INVESTIGATION INTO SELF-ASSEMBLING

MICROCRYSTALS AS AN ALTERNATIVE TO

POLYSACCHARIDE-BASED CONJUGATE VACCINES

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DECLARATION

I, Seen-Wai Lavelle confirm that the work presented in this thesis is my own. Where

information has been derived from other sources, I confirm that this has been

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ABSTRACT

The main virulence factors of encapsulated bacteria such as Haemophilus influenzae type b (Hib) and pneumococcus are their capsular polysaccharides and antibodies against them have been shown to be protective against Hib and pneumococcal invasive diseases. However, polysaccharides are poorly immunogenic and do not induce protective antibody response in children under 2 years when administered on their own. In order to induce a memory B cell response and isotype switching, a protein carrier is usually conjugated to the polysaccharide to stimulate a T cell-dependent response. However, this conjugation process is expensive and the final product is not thermostable.

In this project, self-assembling thermostable microcrystals were used to co-immobilise a model capsular polysaccharide (Polyribosylribitol Phosphate; PRP) from Hib and tetanus toxoid (TT) to create a virtual polysaccharide-protein conjugate to mimic the commercially-available Hib conjugate vaccines.

Self-assembling microcrystals loaded with both PRP and TT were prepared, and their physico-chemical and immunogenic properties were investigated. In vitro, the factors that influence crystal formation were determined, and antigen loading onto and release from the microcrystals was assessed. The dynamics of the interactions between the crystals and some cells of the immune system were also investigated. In vivo, the crystals were tested in combination with varying buffers, administration routes and adjuvants in an attempt to optimise anti-PRP antibody production.

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It was found that the crystal morphology and antigen entrapment could be manipulated by preparation conditions. The microcrystal formulation increases cellular uptake of antigens into bone marrow-derived dendritic cells and neutrophils. Despite the use of microcrystals with low level of free PRP, and combinations of different adjuvants, routes, buffers and doses, the microcrystals were not as immunogenic as a commercially-available Hib conjugate vaccine.

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LIST OF ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
AD	Assay Diluent
BCR	B Cell receptor
BSA	Bovine Serum Albumen
CaP	Calcium Phosphate
CaP-PCMC	Calcium Phosphate (coated) Protein Coated Microcrystal
CpG/CpG-ODN	Oligodeoxynucleotide (OD) Rat CpG-B DNA (Hycult biotech)
DC	Dendritic Cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbant Assay
FACS	Fluorescent Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
G-CSF	Granulocyte Colony Stimulating Factor
HBSS	Hank's Balanced Salt Solution
HBSS	Hanks Balanced Salt Solution
Hib	*Haemophilus Influenzae* type b
MFI	Mean Fluorescence Intensity
MHC	Membrane Histocompatibility Complex
OPD	O-Phenylenediamine Dihydrochloride
OVA	Ovalbumin (Hyglos)
PBST	Phosphate Buffered Saline with Tween 20
PCMC	Protein Coated Microcrystals
PE	Phycoerythrin

PRP	(Haemophilus influenza b polysaccharide) Polyribosyl Ribitol Phosphate
PRP-TT	PRP conjugated to Tetanus toxoid
PS	Polysaccharide
SCS	Subcapsular Sinus
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SHD	Single Human Dose
SR	Solid Recovery
SSC	Side Scatter
TEM	Transmission Electron Microsocpy
TPL	Theoretical Protein Loading
TT	Tetanus toxoid

Chapter 1. Introduction

Infectious diseases are a major cause of premature death and disability worldwide and responsible for approximately one-third of all deaths. Particularly affected are infants and young children in the world's poorest regions. Since the introduction of routine vaccination schedules for infants, there have been significant decreases in the number of cases of childhood associated diseases such as diphtheria, tetanus, pertussis, polio and bacterial meningitis and pneumonia. However, infectious diseases still remain as one of the highest causes of worldwide morbidity and mortality, particularly in developing countries.

There are 5 million deaths per year in children under the age of 5 years from infectious diseases, 2 million of them could be prevented by the use of existing vaccines (1-3). This represents approximately 20% of deaths of children under the age of five years, and 30% of deaths from those between 1-59 months of age (WHO 2011). In these developing countries, the implementation of vaccination programs can be problematic and therefore leaving children vulnerable. The diseases which inflict the highest toll upon young children include acute respiratory infections, diarrhoea and meningitis.

Figure 1.1. Childhood preventable deaths in 2012.

Data from WHO 2011.
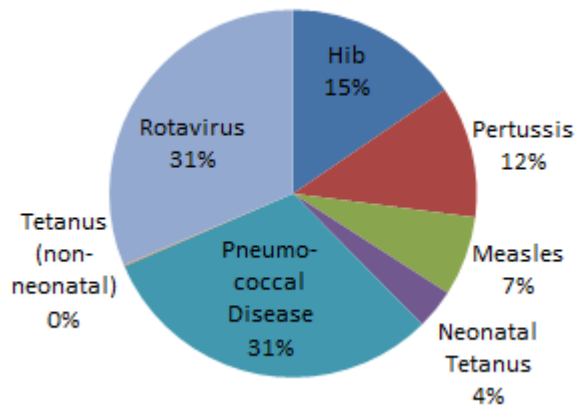
1.1 Encapsulated bacteria

The leading cause of respiratory infections and meningitis are a group of bacteria which have a polysaccharide (PS) capsule, known as encapsulated bacteria. The group includes organisms such as *Neisseria meningititis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, *Group B streptococcus, Klebsiella* pneumoniae and *Salmonella typhi*. The capsule is the most external structure and is made up of long polysaccharide chains which are usually negatively charged. The capsule is the first bacterial structure to be encountered by the immune system upon infection. The capsular polysaccharides can vary in structural features such as repeating unit composition, ring forms, glycosidic linkage position, anomeric-centre configurations and conformations (4). These differences contribute to the different immune responses to the polysaccharides.

The capsule is often the most important virulence determinant on the cell surface of many gram-negative and gram-positive bacteria. The capsule acts to prevent desiccation, aids adherence and resistance to non-specific host immunity. Most capsules are composed of neutral or acidic polysaccharides which are linked to the bacterial surface via covalent attachment to either phospholipids or lipid A molecules. Capsule polysaccharides are composed of monosaccharides joined by a means of a glycosidic linkage.

The capsular polysaccharide can acts to evade the immune response by masking the underlying pathogen-associated molecular patterns (PAMPs) that are otherwise potent activators of the complement system as well as recognised by Toll-like receptors (TLRs). Encapsulated bacteria only weakly activate the complement system and opsonophagocytosis.

The complement system is an important part of the humoral mechanism of defense against bacterial invasion, and is composed of many distinct proteins which react with one another sequentially to induce inflammatory responses. The complement system can be activated through three pathways: the classical pathway, the alternative pathway, and the lectin pathway. Each pathway follows a sequence of reactions to generate a key protease called C3 convertase. The complement system prevents infection by three steps; opsonisation of pathogens so that they are engulfed by phagocytes bearing receptors for complement (C3b, C3bi), recruitment of inflammatory cells (C3a, C5a) or direct killing of pathogens by creating pores at the bacterial membrane (C5b, C6, C7, C8, C9) (5).
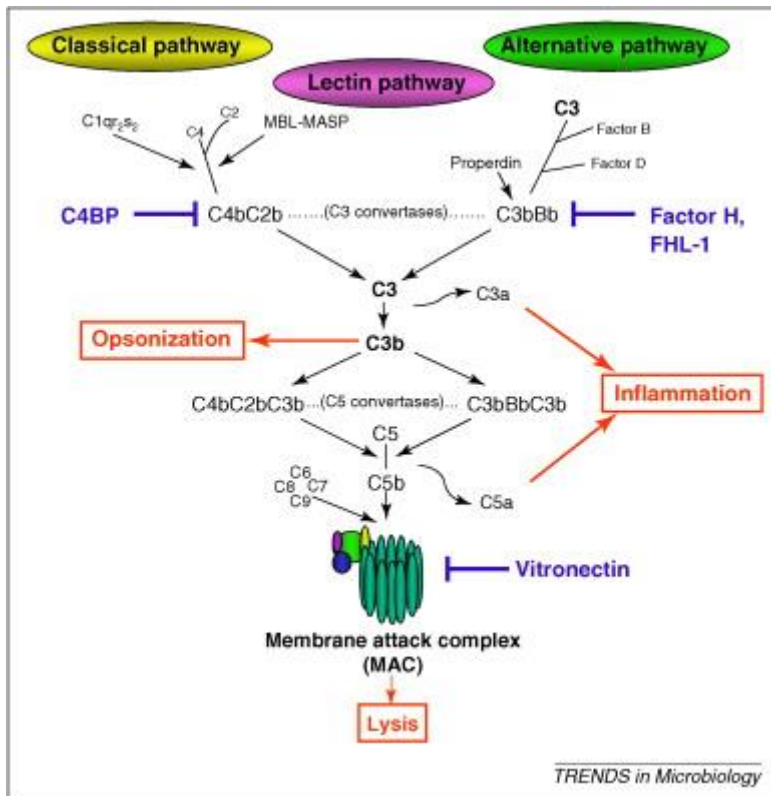
Figure 1.2 Schematic representation of the complement pathways.

Figure from (6)

The clearance of encapsulated bacteria is also dependent on the production of a humoral response, a response which unvaccinated children under 2 years are unable to produce, leaving them very vulnerable to infection. Between 0.8-1 million children under the age of 5 die from pneumoccoal disease annually (7) and Hib and meningococcus are thought to account for approximately 400,000 and 50,000 deaths respectively each year (2, 8). According to recent research (9, 10) these pathogens are responsible for as many child deaths as HIV/AIDS, malaria and tuberculosis combined, despite the development of safe and effective vaccines against common serotypes of all three (11).

S. pneumoniae is an encapsulated diplococcus and is divided into at least 91 serotypes. The classification of the serotypes is based on the chemical structure of the capsular PS. It can cause pneumonia, meningitis and septicaemia, and also cause disease in the form of otitis media, sinusitis and bronchitis (9). Increasing resistance of *S. pneumoniae* to common antibiotics is a growing problem, which increases pressure to prevent disease by immunisation (12-15). Studies of community-based incidence studies of invasive pneumococcal disease (IPD) suggest there are approximately 814,000 pneumococcal deaths in children over the age of five years in developing countries each year (16, 17). 1-4 million cases of pneumococcal pneumonia are occurring in Africa alone. One vaccine targeting 7 pneumococcal serotypes was licensed for young children in 2000. In contrast to the 23-valent polysaccharide vaccine used in adults, the 7-valent conjugate vaccine affects pneumococcal carriage and transmission. Early after its introduction, incidence of invasive pneumococcal disease declined among older adults, a group at high risk for pneumococcal disease (17).

N. meningitidis is also a significant public health problem, particularly in developing countries. It is a leading cause of meningitis and fulminant septicaemia. Five of at least thirteen different PS serotypes cause approximately 90% of infections worldwide. Serogroups B and C are most prevalent in Europe, while serogroups A, C and W135 are responsible for most cases in Asia and Africa and serogroups B, C and Y predominate in the Americas (18). Unlike *S. pneumoniae* and *Haemophilus influenzae* type b (Hib), carriage rates of *N meningitidis* are highest amongst adults and adolescents and rare in those under 10 years of age. Polysaccharide vaccines against meningococcal serogroups A, C, Y and W135 have been available for decades, but have poor

immunogenicity in young children and little effect on nasopharyngeal carriage. However, the introduction of conjugate serogroup C meningococcal vaccine has dramatically decreased incidence of the disease in industrialised nations.

Haemophilus influenzae is a gram-negative rod-shaped bacterium. In 1931, Pittman showed that *H. influenzae* could be divided into two groups; the first encapsulated and able to cause invasive disease, and the other unencapsulated and not isolated from the blood or cerebrospinal fluid of patients with sepsis or meningtitis (19). The encapsulated group can be divided into six serotypes (a-f) based on capsule type and the untypeable isolate have no capsule. Serotype b (Hib) is by far the most common, and causes over 80% of invasive infections (20, 21). Unencapsulated strains are found in 95% of cases of *Haemophilus influenzae* nasopharyngeal carriage, with equal frequencies between the six serotypes in the remaining 5% (22).The type b capsule is composed of a polymer of polyribosyl-ribitol phosphate (23), which acts as the main virulence factor of the bacteria (19, 24), whereas the other capsule types contain a hexose instead of a pentose (25, 26).

Hib infection can cause a variety of diseases, such as osteomyelitis, septicaemia, cellulitis, epiglottitis, and septic arthritis but is particularly well known as a cause of bacterial meningitis and pneumonia. Treatment with antibiotics can cure the infection, but severe neurological sequelae occur in 15% to 30% of those who survive Hib disease.

The bacterial capsule plays a role in helping it evade the immune system by preventing phagocytosis and activation of the alternative complement pathway. Therefore, the encapsulated bacteria to invade such systems as the blood and cerebrospinal fluid and cause severe infections. However, in the presence of anti-capsular antibodies, both phagocytosis and bacteriolysis are induced.

There are many bacterial pathogens which express a PS capsule, and the development of effective PS-based vaccines has had an important role in reducing the prevalence of related bacterial infections in countries where the vaccine has been included into the vaccination schedule. This project focuses on Hib infection as a model of infection by encapsulated bacteria. Although there is an efficacious vaccine currently available, its low uptake into the immunisation schedule in some countries has limited the worldwide prevention of Hib infection.

1.2   Epidemiology of Hib

Hib is a major cause of serious infections in children worldwide, though the rate of invasive Hib disease varies greatly between resource-poor and developed countries as shown in Figure 1 (27-29). 61% of Hib related infant deaths worldwide occur in just ten countries within Africa and Asia, as is shown by Figure 1.1.
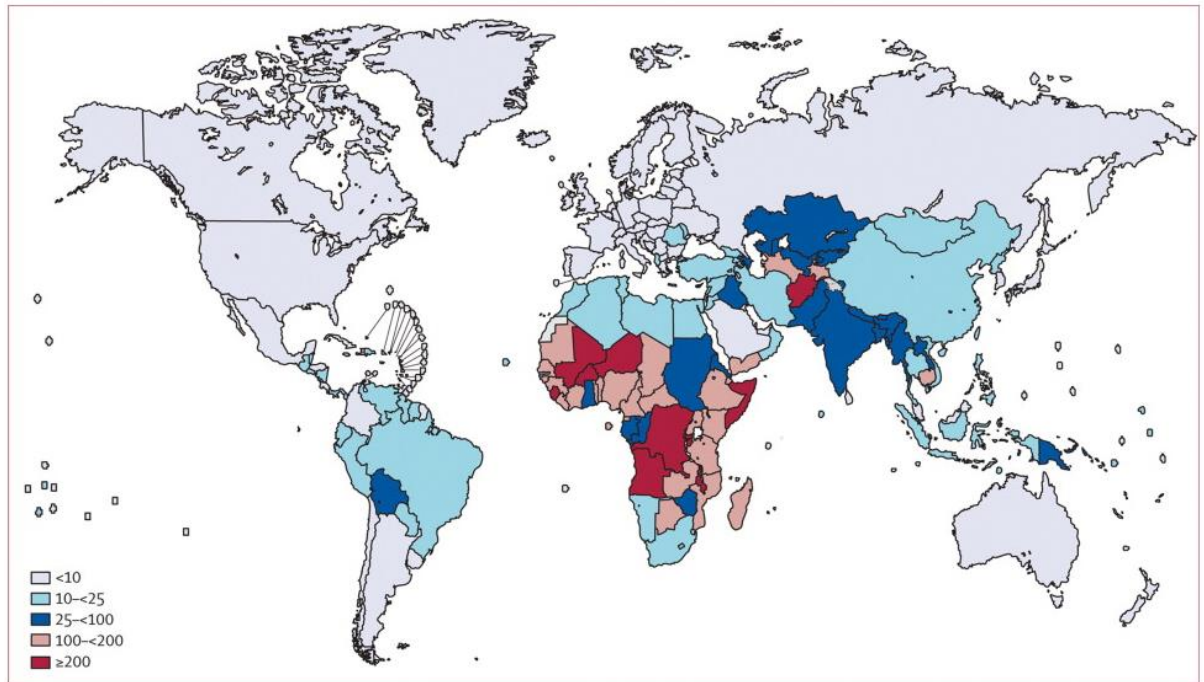
Figure 1. Hib deaths per 100,000 in children aged 1-59 months (HIV-negative Hib deaths only). Map from Watt et al. 2009 (10)

In the year 2000, there were approximately 8.13 million serious Hib disease cases and

371,000 deaths in children under 5 years of age, with HIV infected children under 5

accounting for 2.1% of these deaths (10) . However, the true number of Hib disease

cases are likely to be underestimated as many Hib meningitis cases go undiagnosed

(10) and the incidence of Hib disease which is not meningitis or pneumonia also often

gets underestimated. A high proportion of Hib disease (85%) occurs in young children

under four years of age (30) and one third of the survivors of bacterial meningitis are

left with long term neurologic complications, including sensorineural deafness,

cerebral palsy and epilepsy (31).

As Hib has no non-human hosts, pharyngeal carriage of the bacteria is the main source

of infection (32). The highest rates of asymptomatic carriage are amongst toddlers

(33), and this population acts as a reservoir for infection. Hib was recovered from

nasopharyngeal cultures in 30-50% of infants older than 6 months (34), but this level is markedly increased amongst those living in crowded conditions (35) and with larger numbers of siblings in the family (33). As most bacteria increasingly become antibiotic resistant, the use of vaccine becomes increasingly important to prevent infectious diseases.

1.3   Polysaccharide-based vaccines against encapsulated bacteria

As previously mentioned, PS capsules of *S. pneumoniae*, *H. influenzae* and *N. meningitidis* are virulence determinants that are made up of repeating saccharide units. The bacterial capsule has multiple functions which enable the bacteria to establish and maintain an infection in the human host (4). In naïve individuals, the alternative complement pathway is responsible for protection against invasion by bacteria. This occurs through the binding of the complement component C3b to the microbial cell wall which triggers the formation of the membrane attack complex that leads to swelling and bursting of the microbial cell. However, capsular PS inhibits deposition of complement on the cell membrane. In addition, interaction between the phagocytic cells' complement receptors and the techoic acid-deposited C3b on the cell wall, or the cell wall itself are sterically hindered by the capsule (36, 37).

Since the discovery that the capsule is one of the main virulence factors of these bacteria, polysaccharide vaccines were developed to protect against infection by encapsulated bacteria (38). The first large scale clinical trial in 1945 of a tetravalent pneumococcal polysaccharide vaccine demonstrated that the PS vaccine was effective

in preventing disease in South African gold miners (38). Subsequently, the availability of antibiotics that were able to cure infections in a non-serotype specific manner delayed further research into vaccine design until the 1960s. However, it soon became clear that patients treated with antibiotics might still suffer from the effects of the infection due to the immune reaction to lysed cells and therefore development of a suitable vaccine became the focus of research.

1.4 Hib polysaccharide vaccine

PS antigens are T cell independent antigens (39), they induce short-lived B cell responses by cross-linkage of the B-cell receptor on marginal zone B cells. This cross linking drives the fast development of plasma cells and production of an antibody response, but without the generation of B memory cells, which necessitates vaccination at regular intervals to maintain the protective antibody level.

By the late 1960s, the high fatality rate caused by invasive Hib infection, the high incidence of complications in meningitis survivors and the increasing antibiotic resistance in emerging strains meant that the development of a Hib vaccine was crucial. Human anti-PRP antibodies were shown to confer protection against infection by the bacterium, the purified capsular PS was trialled as a vaccine candidate (40) In a clinical trial in Finland where 100,00 children were immunised with the Hib capsular PS vaccine, interestingly an age-dependent antibody response was found. In children older than 18 months, the vaccination provided good protection against invasive Hib

disease, while the PS vaccine did not protect children under 18 months of age, even with a booster dose (41).

This age-dependent response to Hib polysaccharide is attributed to the nature of the polysaccharide, which is a linear polymer of ribose, ribitol and phosphate; a T cell independent antigen (23). Activation of B cells by PS is mediated by binding of polysaccharide to the B cell receptor (BCR) and of the complement component C3D to its receptor (CD21) which is expressed on marginal zone B cells (42-46). The marginal zone B cell repertoire has low-level of self-reactivity which allows them to remain in a constant pre-activated state. They can therefore respond rapidly to blood-borne pathogens that enter the spleen.

The antibody responses induced by the marginal zone B cells do not need any T cell co-operation and are therefore known as T-cell independent (TI) responses. The reason that infection is so high in infants less than two years of age, is due to the immaturity of their immune system; in particular to the low level of expression of CD21 on their marginal zone B cells and this also makes them unable to mount an antibody response to PS-based vaccine (10, 47).
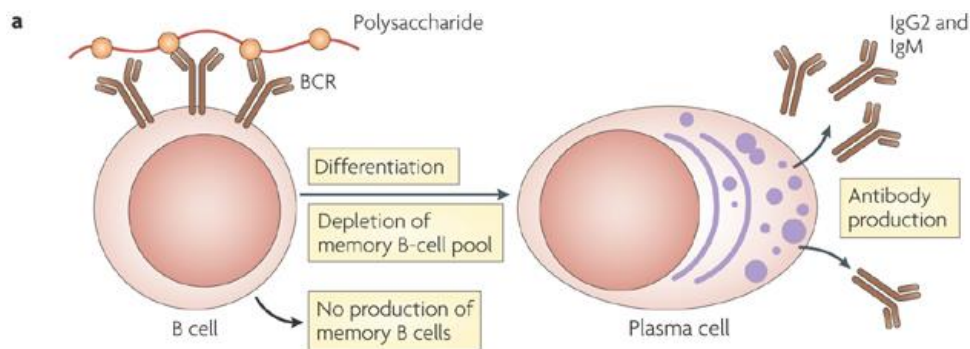
Figure 1.3. Schematic presentation of Immune response to polysaccharide (a T cell independent response)

Adapted from (48)

1.5 Conjugate vaccines

As mentioned earlier, Hib conjugate vaccine followed the use of the polysaccharide vaccine when introduced in 1985, but was withdrawn in 1988 due to limited efficacy in infants under 18 months old. (49). Due to the inability of the polysaccharide vaccine to induce protective immunity in infants younger than 18 months old, polysaccharide has been chemically conjugated to a protein carrier. This converts the polysaccharide to a T cell dependent antigen which is able to induce an antibody response in young infants. The serum antibody to the type b capsule confers protection against invasive Hib disease, and conjugate vaccination primes the follicular B cells for long-term immunologic memory to the Hib polysaccharide, which does not occur after either immunisation with the unconjugated polysaccharide, or even after recovery from invasive Hib infection. Since the licensing of Hib conjugate vaccines in 1987 there has been a huge fall in the number of cases of invasive Hib infections (20, 50).

Effective Hib conjugate vaccines were first licensed for use in US children greater than 18 months old in December 1987 and for infants over 2 months old in October 1990. The success of the Hib conjugate vaccine was seen when the age-specific incidence of Hib disease among children less than 5 years old decreased by 71% from 37 per 100,000 people in 1989 to 11 per 100 000 people in 1991, however *Haemophilus influenzae* meningitis incidence decreased by only 2% between 1951 and 1991 (51). An advantage of the conjugate vaccine is the enhanced IgG booster responses after repeated immunisation, something which does not occur with the unconjugated Hib polysaccharide (52).

Furthermore, the antibodies generated by immunisation with the Hib conjugate undergo affinity maturation, which results in higher avidity antibodies which are more efficient at activating complement-mediated bacteriolysis or opsonisation of Hib. It has also been shown that immunisation with Hib conjugate vaccines lowers the rate of nasopharyngeal colonisation and transmission of Hib, thereby decreasing the incidence of Hib even amongst non-immunised individuals (53, 54).

The success of the Hib conjugate vaccine was soon followed by protein conjugation of polysaccharides from *S. pneumonia* and *N. meningitidis*. These highly effective vaccines induce long-term immune responses as they promote the differentiation of B cells, into long-lived memory B cells in addition to antibody-secreting cells. The existence of memory B cells also results in an increase the magnitude of the antibody response when the vaccine is given as booster doses.

Studies of the incidence of invasive Hib disease within the various age groups in England and Wales was shown to drop soon after the introduction of the first conjugate vaccine, ActHIB in 1992. The rate of incidence went from 22.9 per 100,000 1990 (the pre-vaccine era) to 0.65 per 100,000 in 1998. In the UK, the conjugate vaccine was administered as three doses at 2, 3 and 4 months of age, a booster dose was then introduced at 12-13 months of age (55). This led to a dramatic fall in the number of cases of invasive disease in children under 4 years of age (as shown in Figure 1.4).

Until the year 2000, widespread use of Hib vaccine use was mainly limited to countries within Europe and the Americas. However, by 2006 several other countries had included the Hib vaccine into their vaccination routines (56) and this has led to a massive reduction in Hib disease in those countries. This is due to the protection afforded by the vaccination of the individual as well as the reduction of asymptomatic nasopharyngeal carriage in the population. This herd immunity is vital for the protection of the unvaccinated population, even in areas with low coverage (57-60).
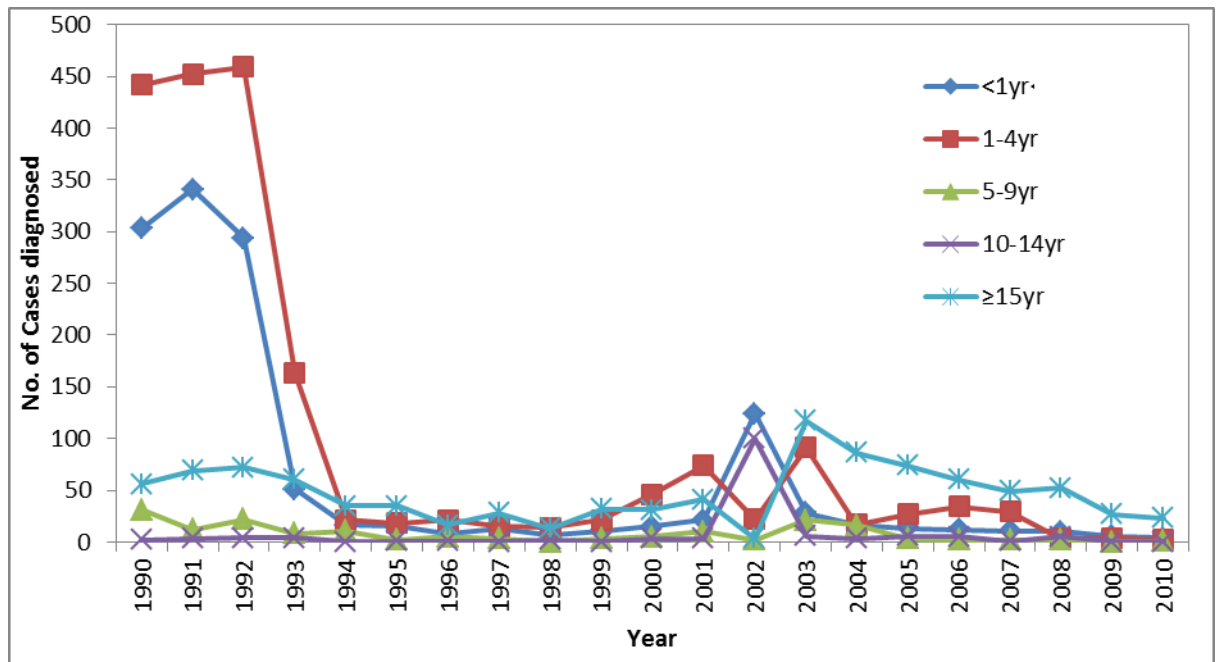
Figure 1.4 Number of diagnosed cases invasive Hib disease in different age groups in England and Wales from 1990-2010 (Data from UK Health Protection Agency)

Hib conjugate vaccines were prepared from either the PRP polysaccharide or oligosaccharide which was conjugated to diphtheria toxoid (PRP-D), CRM$_{197}$ (a modified non-toxic fragment of diphtheria toxin; PRP-HbOC), or to the outer membrane protein of *Neisseria meningitidis* (PRP-OMP) (61). It was subsequently found that conjugation of PRP to tetanus toxoid (TT) was as efficient at generating antibody responses as PRP-OMP, PRP-D and PRP-HbOC. The technology to conjugate PRP to tetanus toxoid was not protected by patent laws, and so TT became the most commonly used (62). It was also found that PRP-D did not produce as good a memory response compared to the other conjugates, and is no longer used (49, 63).

Table 1.1 The effectiveness of various Hib conjugate vaccines with different protein antigens. (64)

Vaccine commercial name	Protein Carrier	Protective efficacy (%) (Ref)
PRP-D	Diptheria toxoid	90 (65) 35 (63) 87 (66)
HbOC	Mutant diphtheria toxin CRM197	100 (67) 95 (66)
PRP-OMP (or –OMPC)	Outer membrane protein complex	95 (68)
PRP-T	Tetanus Toxoid	100 (69)

As can be seen in Figure 1.4, there was a brief resurgence in Hib cases around the years 2000 to 2004. The incidence went from 0.65 per 100,000 in 1998 to 4.6 per 100,000 in 2002 (70). The increase was identified as being caused by the use of of the DTaP-Hib combination vaccine (a 5-in-1 combination vaccine against Diptheria, Tetanus and Pertussis) which contains acellular components of pertussis, instead than the equivalent whole cell pertussis preparation (DTwP) (70). Although the protection

induced by conjugate vaccine is known to rely on the formation of immunological memory and pre-existing antibody, it was identified that there was an increased risk of Hib vaccine failure in fully immunised children who had received two or more doses of the trivalent DTaP vaccine (Infanrix) administered concurrently with the Hib-TT vaccine (71).

It was found that although the DTaP vaccine administered in combination with Hib-TT produced lower antibody titres than when administered in combination with the DTwP, the nature (isotype and IgG subclasses) and function (avidity and opsonic activity) of the antibodies were the same with both the acellular- and the whole cell pertussis-combination vaccine. Hib immunological memory was also induced by both types of combination vaccines and the sera from immune individuals was also able to protect rats against invasive disease after a passive transfer assay (72). These observations imply that the immunological response and formation of memory induced by these vaccines should be sufficient for protection against invasive Hib disease.

There are many proposed mechanisms by which the DTaP vaccine might be interfering with the response to the Hib-TT conjugate. In a study by Dagan et al (73) it was seen that the reduction in the level of anti-PRP antibodies occurred when the PRP-TT conjugate was administered simultaneously with DTaP vaccine and a pneumococcal conjugate vaccine containing the same carrier protein (TT). It has also been noted that the decrease in the anti-PRP antibody response occurred with the highest dose of PncT (Pneumoccal and Tetanus toxoid) conjugate (within the combination). It is possible that the elevated concentration of antibodies to protein carrier could interfere with

conjugate immunogenicity through antigen capture and/or modulation of antigen presentation. When infants immunised with the Hib-TT were studied, it was also noted that the decrease in the level of the anti-PRP antibody response was proportional to the dose of TT injected from both vaccines.

Other previous studies also indicated that the immune response to the carrier protein might interfere with and decrease the antibody response to the polysaccharide. It was found that pre-immunisation with the protein carrier can reduce the antibody responses to both the carrier and to PRP following immunisation with Hib-TT conjugate (74). A similar effect has also been described with other protein epitopes (75, 76). It was also demonstrated that the presence of maternal antibodies to the TT inhibited the response of infants to the PRP when immunised with the Hib conjugate vaccine.

It is also possible that the suppression is mediated by the clonal dominance of B cells specific to the carrier epitopes. As the frequency of polysaccharide-specific B cells clones is much lower than the frequency of B cells specific to the carrier protein (77, 78). The decrease in the response against the polysaccharide is proportional to the intensity of the immune response to the carrier and this effect is increased with polysaccharides with epitope for which a low frequency of B-cell precursors exist.

A study by Fattom et al. (79) used two vaccines, a 12-valent *E. coli* vaccine and an 8-valent *S. aureus* consisting of polysaccharides conjugated to the same carrier protein. It was found that when the two vaccines were administered together at the same site, there was a significant reduction in the antibodies against the polysaccharide compared to when the monovalent conjugates are administered separately.

The rising incidence of vaccine failure cases seen in Hib and Meningococcal serogroup C immunised individuals despite the presence of immunological memory (47) made it clear that the waning antibody titres are responsible for the vaccine failure. It was also demonstrated that even in individuals recently vaccinated with the conjugate vaccine there is a delay in the induction of a detectable increase in antibody concentration-leaving the individual vulnerable to invasive infection over the first few days following immunisation (80).

As a result of the increase in Hib-cases, the DTaP-Hib combination vaccine (Infanrix) was withdrawn in 2003 and use of the DTwP-Hib combination vaccine was resumed. A Hib vaccination booster campaign was implemented from May to September 2003. In 2004, the UK switched to Pediacel- a combination containing DTaP, inactivated polio and Hib (but a different acellular pertussis component than that contained in the earlier vaccine), and was shown to induce a satisfactory immune response to the PRP (81). It was necessary to switch from the whole cell pertussis containing vaccine to one containing the acellular pertussis vaccine because of the reduced reactogenicity of the latter. In 2006, the Department of Health introduced a booster dose of a Hib and MenC combination vaccine (Menitorix) to infants at 12 months.

1.6 Mechanisms of induction of antibody response to foreign antigen

The initial step in a humoral response requires the acquisition of antigens by B cells via surface immunoglobulin. However, between the events of immunisation and production of an antibody response against the antigen, the vaccine or the pathogen will undergo trafficking through the lymphatics. A soluble antigen such as the

conjugate vaccine will passively diffuse through the blood stream or enter straight into the lymphatic vessels until it reaches a secondary or primary lymphoid organ, such as a lymph node or the spleen. The soluble antigens can diffuse directly from the lymph node the subcapsular sinus (SCS) in the lymph node follicle to be acquired by antigen-specific B cells in the follicles (82). In the case of particulate antigens, transport is initially predominantly neutrophil uptake, and from 24 h to 72h by dendritic cells (DCs).

1.7 Neutrophils

Neutrophils are an abundant effector cell in the blood (40 to 75% of the white blood cell content in mammals) and play a vital role in phagocytosing and killing extracellular bacteria. Neutrophils start their existence in the bone marrow and continue circulating in the bloodstream until recruited in large numbers to a site of infection. They are one of the first inflammatory cells to migrate towards the site of injection due to local inflammatory signals. They migrate through blood vessels, then through interstitial tissue following chemical signals such as Interleukin-8, C5a, fMLP and Leukotriene B4 (83).

Phagocytosis by a neutrophil is the most likely outcome of introduction of a small particulate antigen at the site of immunisation. The particle may be recognised by being opsonized by antibodies (84). A neutrophil may phagocytose many particles, each phagocytic event results in the internalisation of the particle into a phagosome. Within the phagosome, the particle is exposed to reactive oxygen species and hydrolytic enzymes in an event known as the 'respiratory burst'.

After the initial influx of neutrophils to the site of immunisation or infection, the neutrophils which have phagocytosed the particles proceed to migrate to draining lymph nodes and shortly undergo apoptosis. The cellular debris is then most likely to be either taken up by resident macrophages, and the antigens recognised and processed by circulating dendritic cells either in the local tissue or in the draining lymphoid organs (85).

1.8 Dendritic Cells

Dendritic cells (DCs) are the most functionally important cells in the trafficking of antigen to the lymph nodes, as well as presenting the antigen to other immune cells such as B cells. DCs can take up a wide range of antigens and present them to T cells in the context of class I or II MHC. They are especially suited to stimulating naïve T cells, and are therefore crucial in the priming of a T cell dependent response against the conjugate vaccine.

They exist in two main states *in vivo*; immature until they capture antigen and go through a maturation process that allows them more capacity for activating other immune cells. Before activation, they exist in many non-lymphoid organs, but then can migrate to the afferent lymph or the T-cell dependent areas of the spleen. Immature DCs are common in the peripheral tissues and are adept at endocytosis and express relatively low levels of surface MHC class I and II molecules. MHC Class II molecules are synthesised by the cell, however it is sequestered intracellularly in endocytic compartments. Immature DCs readily take up antigen, which is then transferred to lysosomes containing MHC Class II, although these are used for use as immunogenic

peptides rather than to form complexes with MHC Class II (86, 87). The immature DCs are therefore less efficient at presenting antigen to T cells.

In the mature state, DCs have much less of a capacity for antigen uptake, but an increased capacity for T cell stimulation. During the maturation process, the MHC CLASS II is redistributed from the intracellular compartments to the plasma membrane. Co-stimulatory molecules such as CD80, CD86, MHC I and T cell adhesion molecules are also upregulated. Any previously or newly internalised antigens are then able to form complexes with MHC Class II (86, 87). The structure of the cell changes, and long membrane folds of "dendritic" extensions act to increase T cell interactions.

Dendritic cells can retain and transport intact antigen and present the antigen to naïve B cells (88). DCs can also give cell-bound signals essential for class switching, and this interaction can occur in a primary antibody response and is independent of T-cell intervention. It has also been suggested that the interaction of CD40L on the DC and CD40 on B cells also increases survival of the B cell (88).

1.9 Macrophages

Macrophages are large phagocytic cells that are found in essentially all tissues (89). Macrophages patrol of potential pathogens and play a critical role as part of the innate immune system. They also play a role in priming the adaptive immune response through recruiting other immune cells such as lymphocytes through inducing inflammation. A monocyte (the macrophage precursor) enters damaged tissue, such as that of an immunisation site, through extravasation through the endothelium of a blood vessel. Unlike neutrophils, macrophages can survive several months in the body.

In one study by Ambrosino et al (1992), the conjugate of the Hib capsular polysaccharide to OMP showed an increase in spleen size and number of macrophages, therefore concluding that the macrophage activation and cytokine release increased the immunogenicity of the Hib polysaccharide (90).

After either endocytosis of foreign pathogen or vaccine antigen, the antigen presenting cell will require presentation in the context of a MHC class II molecule before recognition by the specific T cell receptor.

1.10 Antigen processing and presentation by MHC Class II

The MHC Class II is formed of a heavy and light chain in the endoplasmic reticulum. Initially it is associated with another peptide, the invariant chain, which guides the trafficking of the membrane bound complex from the endoplasmic reticulum into the endocytic pathway within a vesicle. The gradual acidification within the endocytic vesicle leads to activation of proteases, namely cathepsin S, which cleave the invariant chain in two locations. This process leaves the class II invariant associated chain peptide (CLIP) bound to the MHC class II groove, preventing binding by self-peptide fragments during transit.

Foreign peptides such as pathogens or vaccine particles are engulfed by the cell into the endocytic pathway; here they also undergo a reduction in the pH which results in their degradation by acid activated proteases. Fusion of the endocytic vesicles containing the MHC class II and the pathogen or vaccine occurs, but the antigens cannot bind to the MHC class II groove until the HLA-DM molecules catalyses the removal of the CLIP from the groove, and also catalysing the binding of the foreign particles to the cleft. The vesicle is then trafficked to the cell surface where it fuses

with the phospholipid bilayer and the MHC class II associated antigens are exposed to

the extracellular space. A schematic diagram of the intracellular trafficking of MHC is

shown in Figure 1.5.
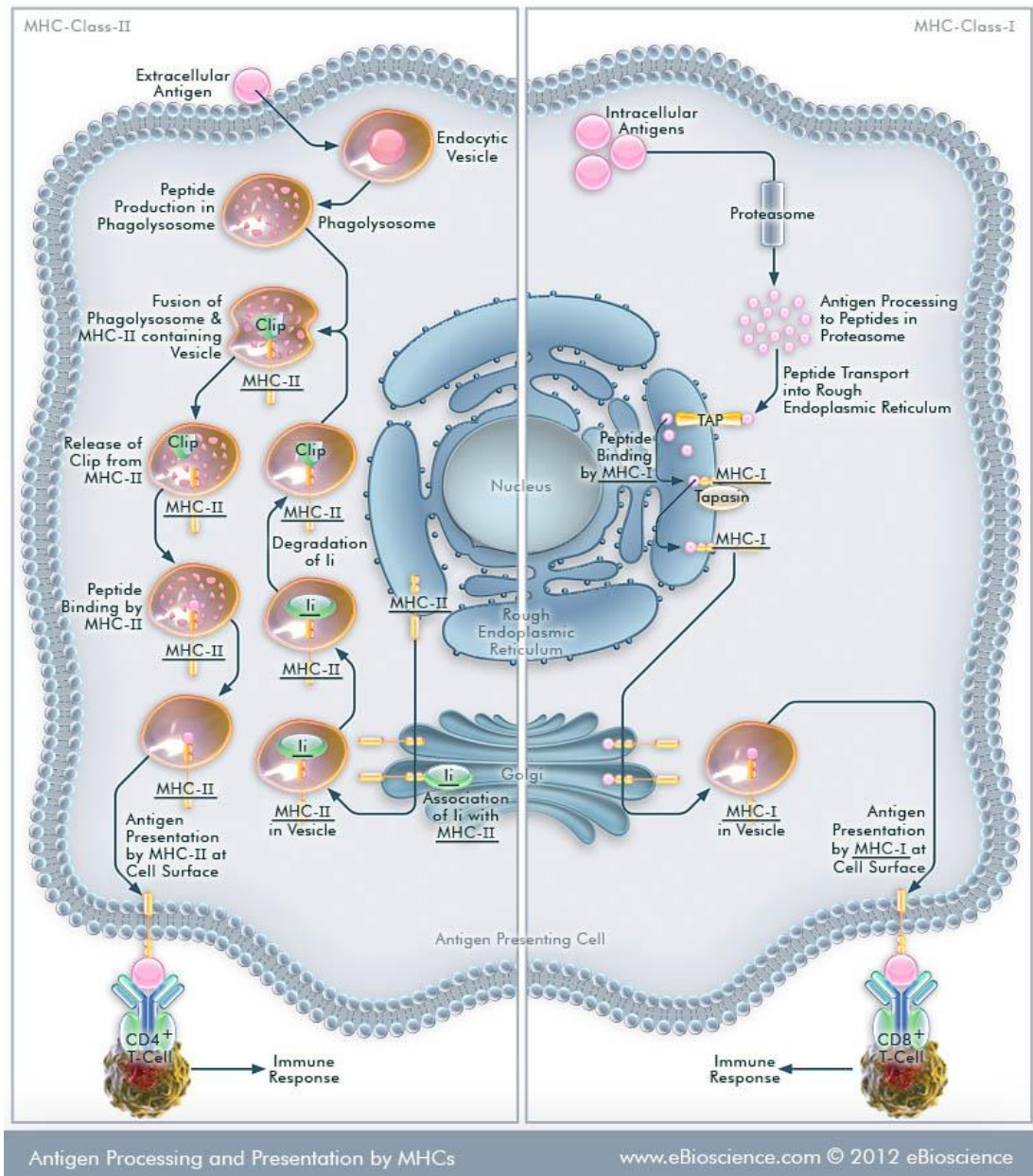
Figure 1.5. Schematic representation of antigen uptake, processing and presentation in the context MHC Class I and MHC Class II.

Image from (91)

1.11 Processing by B cells

B cells have been shown to be able to prime naïve CD4+ T cells in both vitro and *in vivo* (92-94). Although B cells may not be essential for the priming of T cells (95, 96) in order to induce a protective humoral response, uptake of the antigen and involvement of the T cell is essential.

B cells encounter antigen in secondary lymphoid organs after either diffusing there through the lymph or by trafficking by cells of the immune system in the case for soluble and particulate antigen respectively. Antigen-activated B cells and T cells migrate towards the borders of the B cell follicles and the T cell zones of secondary lymphoid organs, respectively. A schematic diagram of the cellular movement is shown in Figure 1.6. B cell–T cell interactions occur and enable B cells to receive helper signals from cognate CD4$^+$ T cells. Activated B cells and T cells then migrate to the outer follicles, where B cells undergo proliferation.

Some of the proliferating B cells differentiate into short-lived plasma cells, which give rise to the extra-follicular foci, and some develop into memory B cells). Alternatively, the activated B cells can return to the follicle and can undergo rapid proliferation to form the germinal centre. In the dark zone of the germinal centre, the clonal expansion of antigen-specific B cells is accompanied by B cell receptor (BCR) diversification through somatic hyper-mutation.

The B cells that exit the cell cycle relocate to the light zone, where affinity selection takes place through interaction with immune complex-coated follicular dendritic cells

(FDCs) and antigen-specific T follicular helper cells. The affinity-matured germinal

centre B cells can re-enter the germinal centre cycle. Alternatively, these germinal

centre B cells exit the germinal centre, either as memory B cells or as long-lived plasma

cells that contribute to serological memory. The strength of signals that B cells receive

is likely to determine their fate; stronger signals (indicated by bold arrows) favour

development into plasma cells or germinal centre B cells, whereas weaker signals

(indicated by narrow arrows) determine memory B cell differentiation.
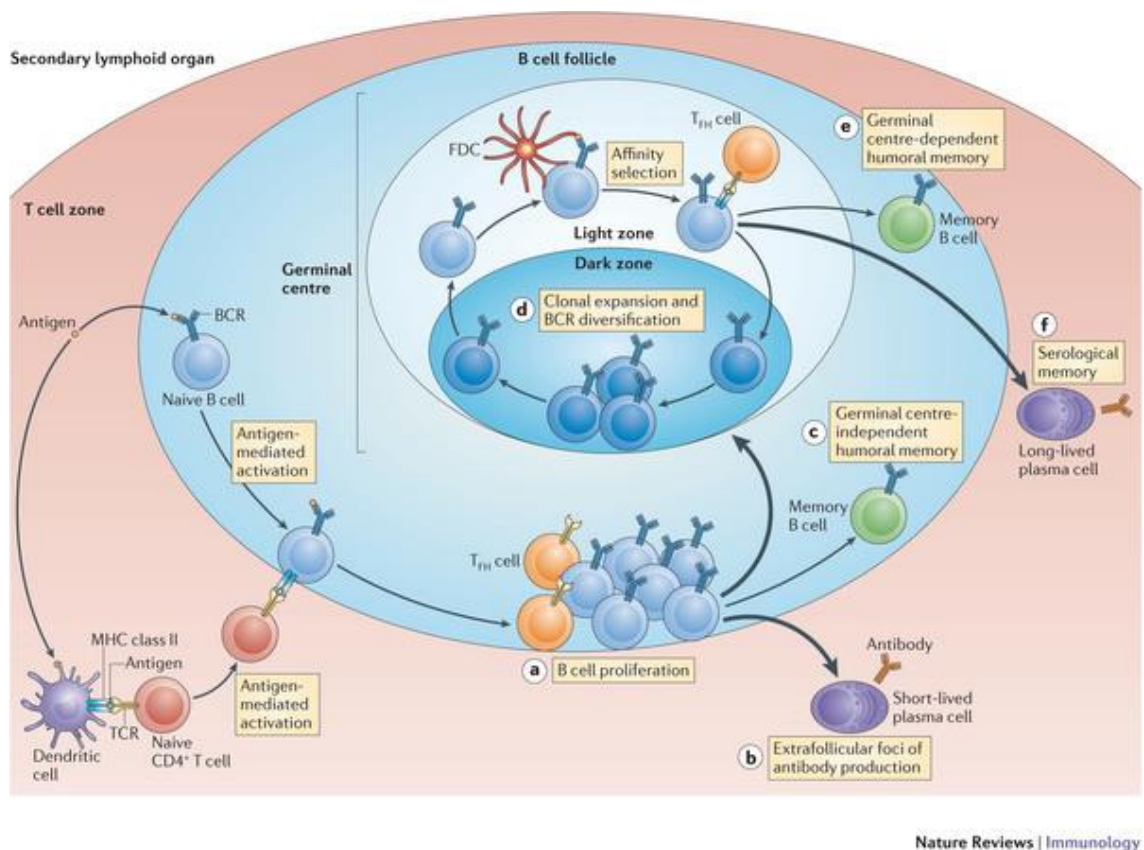
Figure 1.6 Schematic representation of the movement of the B cell within the secondary lymphoid organ

Image taken from review by Kurosaki et al (97)

In the immunologically naïve individual, the polysaccharide chain in a conjugate

preparation binds to polysaccharide-specific membrane immunoglobulin expressed on

B cells (Figure 1.7). The conjugate is then internalized into the cell and the carrier

protein is digested by proteases within an endosome. Resulting peptides are

subsequently transported to the cell surface in association with the type II major

histocompatibility complex (MHC Class II). Peptide specific T cells can then recognise

the peptides presented on the B cells. This leads to activation of T cells and secretion

of cytokines which lead to the maturation and differentiation of the polysaccharide

recognising B-cells into specific plasma and memory B cells. Plasma cells undergo

avidity maturation and isotype switching, producing IgG, IgA or IgE antibodies which

have defined roles in the immune system (3, 48). Only after this process are the B-cells

described as polysaccharide specific.
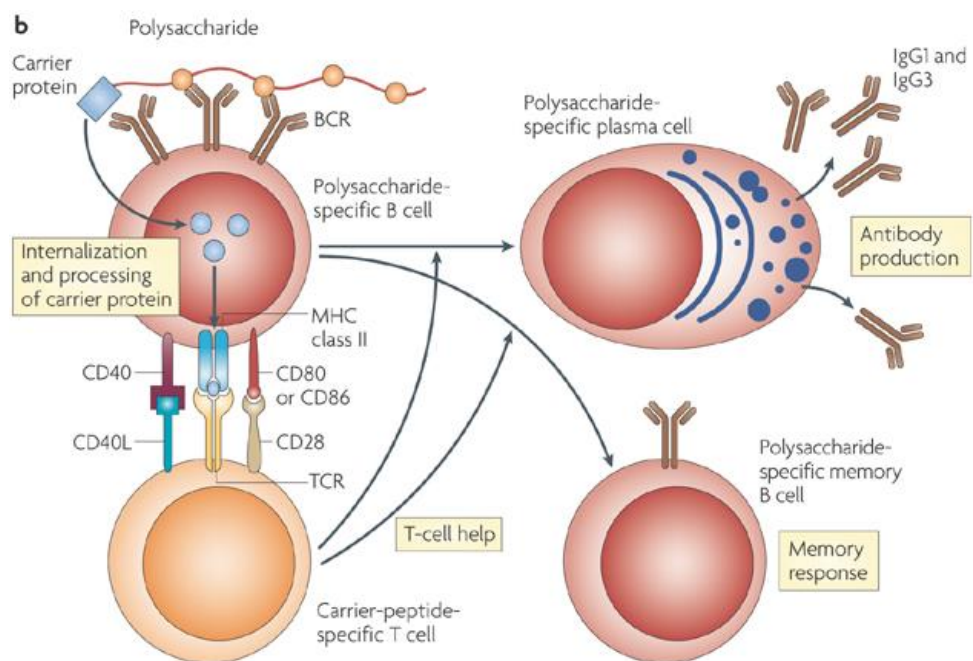
Figure 1.7. Schematic representation of the induction of the anti-polysaccharide response by conjugate vaccines.

Image taken from **(48)**

The two signals which are essential for T cell dependent naïve B cell activation are the BCR cross-linkage by the antigen (signal 1), and the cognate interaction with the helper T cells through the formation of an immunological synapse (signal 2). Upon formation of the immunological synapse the antigen specific T cells become activated and form CD40L-CD40 interactions as well as secrete cytokines to stimulate the B cells. The binding of the antigen to the BCR forms a complex which is internalised and degraded in the endosomal pathway. This process initiates activation of the B cell, which initiates a signalling cascade that leads to the up-regulation of co-stimulatory molecules and also increases the efficiency of antigen internalisation, processing and presentation of the antigenic peptides in the context of MHC class II molecules (98, 99).

Isotype class switching (also referred to immunoglobulin class switching, or class switch recombination) is a mechanism that allows B cells to produce antibodies of a different class, for example IgG, instead of IgM. The differences induced by this process are in the constant portion of the antibody heavy chain, whereas the variable region, and therefore the specificity remains the same. The antibodies of different classes can interact with different effector cells; therefore daughter cells produced from the same activated B cells can produce both different isotypes and subtypes. The process of Class switch recombination (CSR) is responsible for the antibody-heavy chain locus to be removed from the chromosome and the surrounding gene segments to re-join, forming a new antibody gene which expresses a different isotype (48).

There is increasing evidence which indicates that other signals and cell types may play accessory roles in T cell dependent B cell responses. Toll-like-receptor (TLR) signals can enhance the activation of B cells in response to T cell dependent antigens (100).

1.12 Current Limitations for wide use of the Hib conjugate vaccine

In 2003, it was estimated by WHO that 92% of the eligible population in the developed world was vaccinated against Hib, but in developing countries the coverage was 42% and as low as 8% in the poorest countries (101).

Despite the high efficacy attributed to the conjugate vaccine, there are some drawbacks which limit more widespread use, especially in poor countries. The difficult and expensive conjugation chemistry requires a tight process control and further purification steps to remove impurities and unconjugated polysaccharide, leading to high manufacturing costs, which limits its availability in poorer countries.

Another limitation is the additional cost of the cold chain for storing and transporting the vaccine. Globally, 50% of vaccines are wasted due to poor thermo-stability (102, 103). It is also estimated that 80% of the financial cost of vaccination is maintaining the cold chain (104). When combined with the high cost of manufacturing, widespread vaccination with the Hib conjugate vaccine becomes unfeasible in some countries, particularly those with underdeveloped infrastructure.

Despite the high cost, there have been recent efforts to increase the uptake of Hib conjugate vaccines in developing countries though the Global Alliance for Vaccines and Immunization (GAVI) -funded Hib initiative. Whilst this work is of incredible value, finding a replacement for the conjugate vaccine which is cheaper to produce and thermally stable would encourage even more countries to introduce the Hib vaccine into their national immunisation programmes.

Thus, in this project our aim is to investigate the possibility of a replacement Hib vaccine candidate which would remove the need for expensive chemical conjugation of the polysaccharide to the protein carrier by co-adsorbing the polysaccharide and the carrier protein onto a protein-based microcrystal, producing a virtual conjugation.

A pilot study using Protein Coated Microcrystals (PCMCs) loaded with PRP, TT and CpG-ODN (as an adjuvant) produced an anti-PRP IgG response in 3/8 animals following 3 immunisations (Figure 1.8). This preliminary data formed the basis of this project.
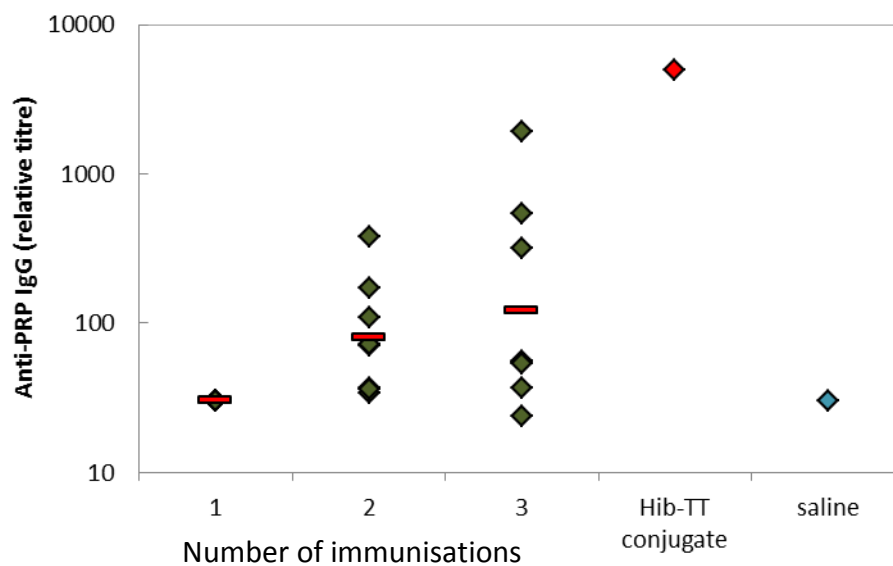
Figure 1.8. Anti-PRP response induced by PCMCs loaded with TT, PRP and CpG.

Rats were immunised with a PRP equivalent dose of 6µg in CaP-PCMCs on days 0, and a PRP dose of 1.2µg of CaP-PCMCs on days 28 and 56. Test bleeds were taken on days 28, 42 and 56. Serum anti-PRP IgG response was measured by ELISA. Levels of anti-PRP responses were determined by parallel line analysis relative to an immune serum from animals immunised with a Hib-TT conjugate vaccine with an assigned titre of 5000.

Response from individual animals is shown as green diamonds and the geometric mean titre of the group is shown as a red horizontal bar.

Thermostable vaccine delivery approaches

As discussed earlier, the removal of the requirement for the cold chain can improve the cost and ability of the vaccine to be delivered to areas with restricted infrastructure. Much research has therefore been focussed on developing formulations for stabilising biological molecules such as vaccines and antibiotics. Such methods include the use of nanoparticles, micro-encapsulation, and liposomes (104) and are discussed below.

Micro-encapsulation

Tiny particles or droplets are surrounded by a coating to give small capsules of useful properties- a process which allows the stabilisation of the particle contained within the capsule. This process has been used to produce formulations which can decrease dosing frequency and prevent the degradation of the pharmaceutical. Most microcapsules have cores with diameters between a few micrometres and millimetres. Biodegradable polymer microspheres have been developed for the controlled release of antigens. This reduces the number of doses needed for primary immunization a single dose. A variety of vaccine antigens have been formulated into microspheres usually composed of PLGA. Depending on the size of the microspheres, molecular weight of polymer and ratio of lactic to glycolic acid in the polymer, the antigen can be targeted to various cells of the immune system or act as a depot a the injection site. However, problems in developing controlled-release vaccines have included instability of vaccine antigens during micro-encapsulation, storage and hydration pre-injection (105).

One material used for microencapsulation is silk fibroin. Silk is a biologically derived protein polymer purified from domesticated silkworm (*Bombyx mori*). The advantages of this material for use within pharmaceutical applications are that it is biocompatible, it has robust mechanical strength and it has a slow, controlled degradation (106, 107). The silk is processed entirely in an aqueous environment, using mild ambient conditions in terms of temperature and pressure. These conditions allow the use of labile biomolecules without compromising their bioactivity (108, 109).

Silk has been used to stabilise the live measles, mumps and rubella vaccine (MMR). The commercial MMR contains protein and salt stabilisers, but still remains susceptible to degradation when exposed to variations in temperature (110). However upon microencapsulation within silk microspheres the vaccine remained viable for up to six weeks at 60° Celsius.

A study by Gupta et al (105) used tetanus toxoid (TT) and PRP conjugated to TT (Hib-T) inside PLGA microspheres as a vaccine. The antibody levels in mice were evaluated and it was found that a single injection of these micro-encapsulated vaccines induced increased antibody levels which persisted for several months, antibody levels were the same or high than those induced by immunisation of conventional formulations of AlPO4-adsorbed TT or soluble Hib-T conjugate vaccine.

Nanoparticles

Nanoparticles have an advantage of being a similar size to cellular components, allowing them to enter living cells using cellular endocytosis mechanism (111). Nanoparticles have qualities that lend themselves to vaccine formulation. They increase antigen stability, can improve immunogenicity and can be used for targeted

delivery and slow release (112, 113). In particular, the use of biodegradable polymeric

nanoparticles with entrapped antigens such as proteins, peptides, or DNA are being

investigated as a method for controlling the release of antigens, as well as controlling

the type of immune response by targeting antigen-presenting cells (APCs). The delivery

of the antigen to the APCs, such as dendritic cells (DCs) is vital for the production of an

effective immune response to the vaccine.

Nanoparticles have been increasingly used for vaccine formulations (Figure 1.9).

Nanoparticles work well as a delivery system, to enhance processing and also can be

used as an immune-stimulant to enhance immunity. Nanoparticles have been used for

both therapeutic (114, 115) and prophylactic vaccines (116-118).
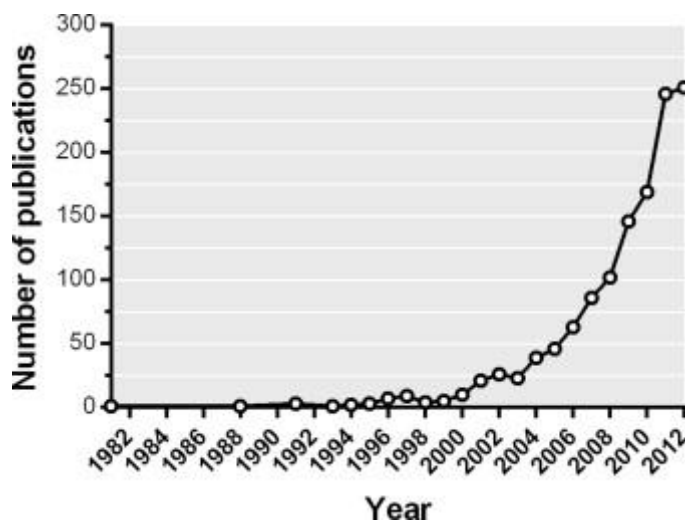
Figure 1.9. Number of publications returned using the search terms "nanoparticle* and vaccin*" from Web of Science (http://apps.webofknowledge.com/; results for a search conducted on 29 July 2013). Taken from (112)

Nanoparticles can be made from synthetic polymers which give slow degradation rates such as:

- Poly (D L-lactide-co-glycolide) (PLG)

- Poly (D L-lactic-coglycolic acid) (PLGA)

- Poly (g-glutamic acid) (g-PGA)

- Poly(ethylene glycol) (PEG)

- Polystyrene

These polymers have also been used to synthesize hydrogel nanoparticles; a type of nano-sized hydrophilic three-dimensional polymer network. Nano-gels have the advantage of flexible mesh size, large surface area for multivalent conjugation, high water content, and potential for high antigen-loading (119).

Natural polymers based on polysaccharides have been used. Examples include:

- Pullulan (120, 121)

- Alginate (122)

- Inulin (123, 124)

- Chitosan (125, 126)

As well as vaccines, natural polymers such as chitosan are used for prolonged controlled release drug delivery systems, wound dressing, blood anticoagulants and bone tissue engineering scaffolds. Chitosan-based nanoparticles have been a focus of recent studies due to good biocompatibility, biodegradability, nontoxicity and ability to control morphology (127-129). Chitosan has been used in the preparation of various nanoparticle vaccines including HBV vaccines (130), Newcastle disease vaccines (131),

and DNA vaccines (128, 132-134). Inulin acts through the activation of complement via the alternative pathway (135), and therefore has the advantage of being an adjuvant. Chitosan nanogels have also been used as a vaccine against Clostridium botulinum type-A neurotoxin subunit antigen Hc for an adjuvant-free intranasal vaccine (136) and recombinant NcPDI antigen for Neospora caninum vaccination (137). There are also adjuvants derived from inulin, such as Advax™, a nanoparticle suspension. Use of Advax™ has shown enhancement of immune response in vaccines against various viruses including influenza (123) and hepatitis B (124).

As well as natural polymers, synthetic substances for nanoparticle vaccines have also been used for vaccination. Although non-biodegradable, synthetic materials have an advantage in that they have a rigid structure and highly controllable synthesis (138). One of the most researched inorganic materials for nanoparticle vaccines is Silica. Silica-based nanoparticles (SiNPs) are biocompatible and act as excellent nanocarriers for applications such as selective tumor targeting (139), real-time multimodal imaging (140), and vaccine delivery. The SiNPs can be prepared with tunable structural parameters. The particle size and shape of SiNPs can also be controlled to change the manner in which they interact with cells (141), an important advantage in vaccine design. The abundant surface silanol groups are also able to be modified to increase functions such as cell recognition, absorption of specific biomolecules, improved interaction with cells, and enhancement of cellular uptake (142-145).

One form of nanoparticle formulation that is used for stabilisation of vaccines is the liposome. The liposome has been identified as a potential adjuvant since 1974 (146). Liposomes and liposome-derived nano-vesicles such as archaeosomes and virosomes

are increasingly investigated as potential carriers of antigen in vaccine development. Liposome based vaccine delivery systems are highly versatile, as their preparation and composition can be adapted to produce the desired properties. Hydrophilic components can be entrapped within the liposome, whereas the lipid-soluble components can be integrated into the lipid bilayer or linked to the surface of the liposome by chemical linkages.

The liposome approach was also used to replace the need for conjugation of oligosaccharide to a carrier protein as shown in a study by Deng et al (147). A self-assembling liposome containing pneumococcal oligosaccharides (serotpye 14) and NKT cell adjuvant was used to successfully induce a natural killer T cell response that mediated the production of high titres of polysaccharide-specific IgG response, comparable to level of response induced in animals immunised with the pneumococcal conjugate vaccine (Prevnar) in a non-liposomal form.

1.13 Our approach to producing a more economic thermo-stable vaccine against Hib

1.13.3 Protein coated microcrystals (PCMCs)

Microcrystals can be engineered to stabilise a wide range of biomolecules including proteins, peptides, DNA/RNA and polysaccharides onto a core crystalline material.

Small microcrystals made up of protein, sugar or salt can be produced by the rapid dehydration of an aqueous solution to produce self-assembled micro-particles, which can be coated with other particles such as antigens. The inert crystalline water-soluble

core is around 3-5μms in diameter and can be coated with one or a combination of proteins. These are therefore known as protein coated microcrystals. These crystals can also be coated with a thin layer of Calcium Phosphate (CaP), the thickness of which can be varied by the preparation process. In an aqueous environment, this CaP layer dissolves slowly and therefore allows a suspension of the PCMCs to be made until the CaP layer gradually erodes and leaves the crystals to dissolve. Representative images of the PCMC forms are displayed in Figure 1.10.

The PCMCs are prepared by dissolution of the excipient, along with the biomolecule of interest into an aqueous solution. This aqueous solution is rapidly dehydrated when added to a water miscible organic solvent. The PCMCs are immediately formed with the biomolecule immobilised on the surface of the excipient and can be dried to form a free-flowing powder. The final qualities of the PCMCs such as morphology, size and biomolecule loading can be adjusted by altering the dehydration conditions.
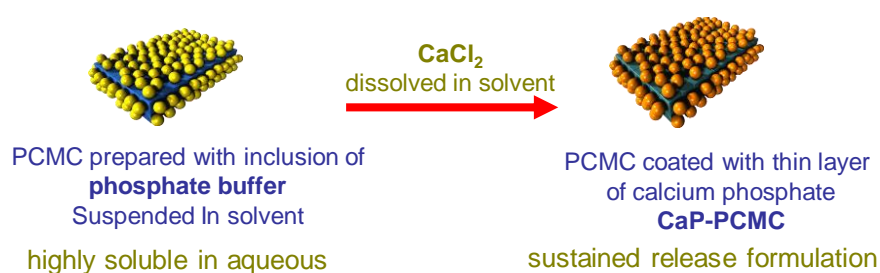
Figure 1.10 Preparation of PCMC and CaP-PCMCs.

Yellow spheres represent the protein adsorbed to the surface of the (blue) microcrystal. Orange spheres represent CaP coated biological molecule adsorbed to the surface of the microcrystal.

The advantages of PCMCs are summarized in Table 1.2.

Table 1.2 The advantages of the PCMC technology.

• High dosage delivery	Can remain as a stable solid in suspension at high concentration. The viscosity still allows delivery through a 27 gauge needle Osmolarity applicable for subcutaneous administration
• Sustained-release of biologics	The release of the active pharmaceutical ingredient from the crystal can be controlled by the dissolution of calcium phosphate. This is easy to include in the normal production process.
• Co-delivery of antigens and immune-stimulants to cells	Immobilisation of the adjuvant onto the same crystal as the antigen allows simultaneous uptake and processing. The similarity of the particle size to a bacterial pathogen may also act with the adjuvant to imitate a bacterial pathogen.
• Elimination of the cold-chain requirement	Active ingredients can be stabilised onto the crystal and the bioactivity is retained.
• Possible for pulmonary delivery of biologics	Can be handled as a powder to deliver multiple active ingredients simultaneously
• Can use continuous manufacture	The manufacturing method of the PCMC can be adapted for larger continuous production of the formulation.

(From(148))

Work using the PCMC technology has resulted in products for the pulmonary administration of medicines which can contain more than one active agent, allowing the simultaneous administration of drug and a secondary agent such as an adjuvant.

The ratio of the multiple active agents adsorbed on the PCMCs can be specified, which may be crucial to the action of the components. PCMCs also have high thermostability which allows easy storage without the necessity for the cold-chain (149).

Adenylate cyclase (Cya) toxin from *Bordatella pertussis* immobilised onto PCMCs in combination with calmodulin (CaM) or bovine serum albumin (BSA) was formed to retain its enzymatic activity, even when kept as a dry powder at 37°C for 2 weeks (150). Diptheria toxoid was immobilised onto PCMCs and the immunogenic activity was found to be retained in immunogenicity studies in mice, although it was reduced when the PCMCs were stored at 45°C for 4 weeks (149).

In this project, new formulations of PCMC that contain L-glutamine microcrystals coated with the Hib polysaccharide PRP and TT antigen will be prepared and evaluated both *in vivo* and *in vitro*. Characteristics of the formulated PCMCs will need to be considered and the manufacturing processes that affect these characteristics will need to be understood. Properties of the existing conjugate vaccine may give a guide as to suitable characteristics that are required in the PCMC vaccine.

The immune response required to produce adequate protection and memory will need to be characterised and considered in order to formulate an effective vaccine. Previous studies have showed that anti-PRP antibody titres could be used as a correlate of immunity for the Haemophilus influenzae type b (Hib) polysaccharide (21). A study in unvaccinated population in Finland showed a decrease in age-specific incidence of Hib disease when the majority of the population had antibody concentrations of 0.15 μg or more; and in the efficacy study of PRP vaccine, (151) age-specific vaccine efficacy correlated with the induction of 1.0 μg of antibody post-vaccination, therefore an

antibody level of 0.15 µg is probably protective against bacteraemia. In this study, the antibody titres will be measured and compared to existing Hib-TT conjugate immunised groups.

It has been shown that the rat model would be the most appropriate model to use to study antibody response to Hib conjugate vaccines. When immunised with Hib polysaccharide alone, both rats and guinea pigs fail to induce a memory response compared to when immunised with the Hib-TT conjugate. Mice have been tried as a model, however they mounted similar antibody responses to Hib when immunised with PRP alone or with the Hib-TT conjugate (152). Therefore, a rat model will be used in the study to assess antibody responses to immunisation with the formulation.

Chapter 2. Aim and Objectives

Induction of an anti-PRP IgG response has been demonstrated after immunisation with a PRP-TT-CpG-CaP-PCMC formulation. This demonstrates the ability of the formulation to act as a virtual conjugate of both the PRP and the protein antigen. However, only three out of eight animals responded to three doses of the crystal formulation. The aim of this project is therefore to investigate the qualities of the CaP-PCMC to enhance the ability of the CaP-PCMCs to induce an anti-PRP response to a level similar to that induced by the conjugate vaccine.

Specifically, the objectives were to:

- Characterise the crystals and determine how the changes to the preparation procedure affects the morphology of the crystals.

- Evaluate the immunogenicity of selected CaP-PCMC formulations in terms of anti-PRP, anti-TT responses, as well as splenocyte proliferation in response to TT and bactericidal qualities of the serum from immunised animals.

- Understand the interaction of the CaP-PCMCs with cells of the immune system by co-incubation of cell cultures of neutrophils, dendritic cells and B cells with fluorescently labelled CaP-PCMCs.

Chapter 3. Investigation into Preparation Parameters on Physico-chemical properties of CaP-PCMCs

3.1. Introduction

To create a virtual conjugate of the polysaccharide PRP and the protein TT antigens using the Protein Coated Microcrystal (PCMC) technology, it is necessary to understand the characteristics of the PCMCs prepared under various experimental conditions as they are likely to affect the immunogenicity of the crystals.

The advantage of using a particle vaccine compared to soluble vaccineis a much higher rate of antigen presenting cell (APC) uptake (153, 154). Once taken up by an APC, the antigen can be trafficked to the draining lymph nodes, as well as being presented within the major histocompatibility complex (MHC) class I or II for activation of T cells.

Uptake of the particulate formulation into APCs is dependent on the physico-chemical characteristics of the particle. These can include size, surface charge, as well as hydrophobicity. Uptake into the APCs might also be through different mechanisms such as receptor mediated endocytosis, micropinocytosis and phagocytosis, which depends on the nature of the antigen(154-157).

At this point, it is not fully understood how the changes in shape or size of the crystals may affect the immunogenicity of a given formulation. However, it is important to consider that the PCMCs need to be delivered to the draining lymph nodes for

recognition by B cells. Trafficking of the PCMCs may occur by circulating dendritic cells or other cell types such as neutrophils. Using polystyrene particles of various sizes and shapes, Champion et al. (2006) reported that particle shape, and not size, plays a dominant role in particle uptake and phagocytosis by alveolar macrophages (158). In particular, the local particle shape at the point of initial contact dictates whether macrophages initiate phagocytosis or simply physically spread onto the particles.

The aim of the work in this chapter is to understand the formation of the CaP-PCMCs and optimise the preparation process to control the size and morphology of the crystals. PCMCs were prepared under varying conditions to determine how these influence the characteristics of the crystals formed. These were analysed for physical properties, such as shape and size. Antigen loading and immunological characteristics are discussed in later chapters.

PCMCs are produced by firstly dissolving the L-glutamine, the PRP, TT and the adjuvant CpG in an aqueous solution and then precipitating these together by addition into a large volume of Isopropanal (IPA) in which these are not soluble. Water-insoluble PCMCs where the antigens, adjuvant and L-glutamine, are prepared with a Calcium Phosphate layer (CaP) is formed by reacting the Sodium Phosphate (dissolved in the aqueous solution) and Calcium Chloride which is added one min after the aqueous solution has been added to the IPA (for two step preparation, Figure 3.2) or in the Isopropanal on the magnetic stirrer (for the one step preparation, Figure 3.1). The quantity of Calcium Chloride needed is calculated from the reaction:

$$CaCl_2 + 2\ NaH_2PO_4 = Ca(H_2PO_4)_2 + 2\ NaCl$$
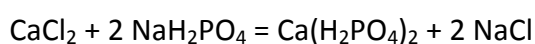
It can be seen from the equation that half a mole of calcium chloride was needed for each mole of sodium phosphate. In our experiments the Calcium Chloride was added at a molar ratio of 1:1 or 5:2 with the Sodium Phosphate in order to achieve a 2x or 5x excess respectively, to investigate the influence of the extent of calcium chloride excess.

Small volume of aqueous solution containing the antigen, L-glutamine and NaH_2PO_4

Large volume of IPA containing $CaCl_2$
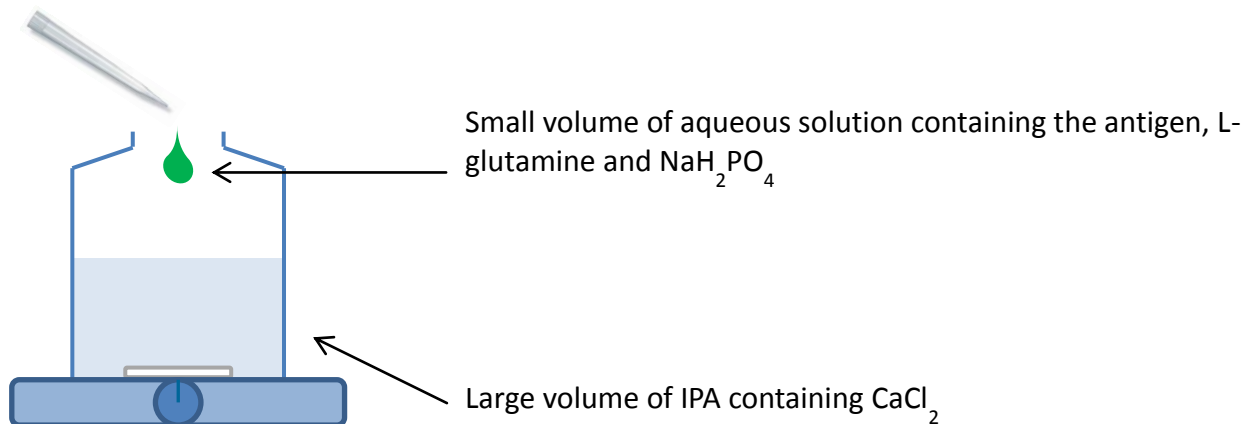
Figure 3.1. Schematic representation of the CaP-PCMC production

Step 1

Small volume of aqueous solution containing the antigen, L-glutamine and NaH_2PO_4

Large volume of IPA

Step 2

Small volume of $CaCl_2$ in IPA added after 1 min of adding aqueous components
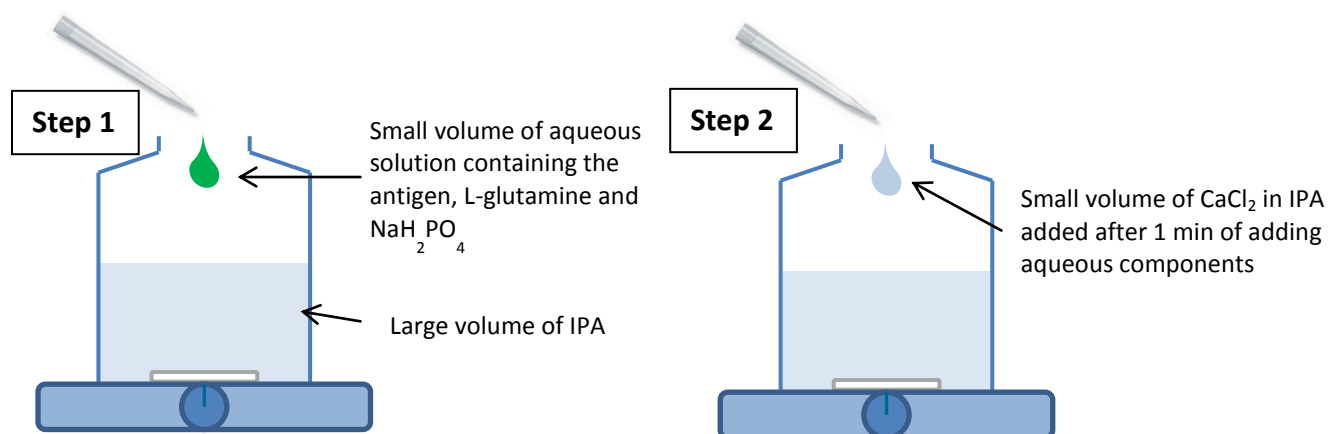
Figure 3.2 Schematic presentation of the PCMC preparation in 2-steps.

3.2. Aims and objectives

The preparation protocol can be varied to alter the formation of the crystals. It is known that crystal size would increase given factors that would slow crystal precipitation. This would include water content of the IPA/water mixture or the temperature of the solvent when the L-glutamine and antigen solution is added. The factors that were chosen to be investigated are listed in Table 3.1.

To thoroughly investigate the effect of every preparation parameter, a systematic multi-factorial investigation would have to be carried out, which would not be practical due to the time and cost required to produce every sample and carry out full characterisation of each combination of parameters. Instead, this investigation relies on the experience and advice of XstalBio who provided a focussed range of factors that were most likely to change the morphology of the crystals.

Table 3.1 Parameters that were varied during the preparation of PCMCs

Parameters of CaP-PCMC preparation		Expected effect on crystal formation
Solvent Temperature (°C)	4, 20, 50	Increased temperature might increase the speed of crystal formation and result in smaller crystals or a difference in CaP layer
Water content (aqueous volume as percentage of total mixture volume, v/v %)	3, 5, 7	Decreasing water content may increase the dehydration of the aqeous components and speed of precipitation and therefore smaller crystals
Number of steps to form CaP layer	1, 2	Adding the $CaCl_2$ in a second step allows the L-glutamine and antigens to precipitate before the CaP and therefore may result in a different CaP structure
Mixing Time (min)	1, 16	A shorter mixing time may prevent further deposition of the CaP, or antigens on the surface of the forming crystal shape and result in smaller crystals but higher loss of material during the process
NaH_2PO_4:$CaCl_2$ ratio ($CaCl_2$ excess)	1:1, 5:2 (2x, 5x)	Increasing $CaCl_2$ excess may increase the speed of its reaction with NaH_2PO_4 and increase the speed of formation of the CaP layer which might result in smaller crystals or a less formed CaP layer
CaP Loading (%)	10, 15	Increasing CaP loading maay decrease the solubility of the CaP-PCMC
PRP Loading (%)	0, 0.25, 0.5	PRP and TT loading and ratio may alter the immunogenicity of the PCMCs
TT Loading (%)	0, 0.05, 0.5	
CpG Loading (%)	0, 0.5	CpG presence increases the immunogenicity of the CaP-PCMCs

3.3. Materials and Methods

3.3.1. Materials

The following materials were used in the study:

CaCl$_2$ (Sigma, Dorset, UK)
L-Glutamine (Sigma, Dorset, UK)
Isopropanal (IPA, VWR, UK)
NaH$_2$PO$_4$ (Sigma, Dorset, UK)
CpG (Cambridge Biosciences, UK)
PRP (NIBSC code 02/208, Potters Bar, UK)
TT (NIBSC code 04/150, Potters Bar, UK)

3.3.2. Preparation of the stock solutions for PCMC preparation

A stock solution of CaCl$_2$ was prepared at 20mg/ml in IPA and placed on a rotator overnight at room temperature to ensure full dissolution. Subsequently this stock solution is diluted with IPA to achieve the required CaCl$_2$ concentrations.

The following day, a stock solution of L-glutamine and NaH$_2$PO$_4$ is made with distilled water at the desired concentration. This was mixed for approximately 30 min on a rotator at room temperature. This stock was then used to prepare the PRP and TT solution with or without adjuvant such as CpG at the concentration required for the formulation.

To prepare the PCMCs, 2-3mL of an aqueous solution of L-glutamine, antigens, CpG and Sodium Phosphate were added into 40-60mL of IPA and the mixture is stirred for 16 min (unless stated otherwise). The resulting suspension of PCMCs was filtered through a 45µm nylon filter fitted in a glass scinter vial (both Millipore). The filter onto

which the PCMC were collected was left to dry at room temperature overnight. The

filter and the dried residue were weighed before the PCMCs were removed from the

filter and placed into a storage container. The solid recovery (%) was calculated from

this weight. The PCMCs might be milled down into a finer powder if required for

analysis or if being mixed with another formulation.

To prepare the CaP-PCMCs with a 2-step method, the same procedure was followed as

for production of the 1-step precipitation with the exception of the $CaCl_2$ which was

made up in a separate more concentrated aqueous solution and was added 1 min after

the antigen and L-glutamine whilst stirring.

3.3.3. Influence of PCMC preparation conditions on PCMC morphology, solid recovery and crystallinity

Formulations with varying preparation parameters were prepared at the School of

Pharmacy. The preparation conditions chosen are shown in Table 3.2.

Table 3.2. Preparation parameters of CaP-PCMC formulations prepared at XstalBio

Sample I.D.	TT load (%)	PRP load (%)	CaP load (%)	Process	Water Content (%)	Mixing time (min)	Temperature (°C)	CaCl$_2$ excess
1	0.5	0	9.4	2-step	4.8	16	20	2
2				1-step				
3	0.5	0	9.4	1-step	3	1	20	2
4					7	1		
5					3	16		
6					7	16		
7	0.5	0	9.4	1-step	5	1	20	2
8						16		2
9						1		5
10						16		5
11	0.5	0	9.4	1-step	5	1	4	2
12						16		
13						1	50	
14						16		
15	0.05	0.5	9.4	1-step	5	16	20	2
16	0.5							

A second batch of samples were prepared to study the effect of antigen/adjuvant load on the morphology of the CaP-PCMCs. Antigen-free and CaP-free samples were also produced to determine the effects of antigen and CaP layer inclusion on PCMC shape. The details of this second batch of crystals are shown in Table 3.3.

Table 3.3 Formulation details CaP-PCMC formulations prepared with varying process steps and antigen loadings

Sample I.D.	TT load (%w/w)	PRP (%w/w)	CpG load (%w/w)	CaP (%)	Process	Water Content (%v/v)
17	0	0	0	0	1-step	5
18				9.4		
19	0.05	0.5	0		1-step	
20	0.5					
21	0.05	0.5	0.5		2-step	
22					1-step	
23	0.025	0.25	0		2-step	
24					1-step	

A third batch of samples was prepared at the School of Pharmacy with varying TT, CpG and PRP, the details of which are shown in Table 3.4. These were prepared with the intention of checking the solid recovery and for antigen quantification in Chapter4.

Table 3.4. Details of CaP-PCMC formulations which vary in antigen loading

Sample I.D.	TT load (%w/w)	PRP (%w/w)	CpG load (%w/w)	CaP (%w/w)	Process	Water Content (%v/v)
25	0	0	0	9.4	1-step	5
26	0.05	0.5				
27	0.5					
28	0.05	0.5	0.5			
29			0.05			
30		0	0.5			
31	0.5	0.5	0.05			
32	0.05	0.25				

3.3.4. Morphology of the PCMCs

The shape of the PCMCs were determined by using Scanning Electron Microscopy (SEM) and light microscopy.

SEM: PCMCs were sputter-coated with 10nm gold using a Quorum Q150 Sputter coater, and analysed with a Quanta 200F scanning electron microscope (FEI, UK).

Light microscopy: A small amount of PCMCs were placed sparingly on a standard glass microscope slide using a small paintbrush. Images were then taken at 10x magnification. To image the change in crystal shape whilst in suspension, a droplet of water was added to the PCMCs, a coverslip was then placed on top. Images were taken at various time points without moving the slide.

3.3.1.3 Xray Diffraction Analysis

Powder X-ray diffraction patterns of the particles were collected using an X-ray diffractometer (Philips PW3170 Scanning X-Ray Diffractometer, Philips, Cambridge, UK) equipped with a Cu K target. The X-rays were generated at 45kV and 30mA. The sample was gently compressed into the sample holder and the powder surface was smoothed with a flat Perspex block. The samples were loaded onto the diffractometer and scanned over a range of 2Θ values, from 5° to 50° at a scan rate of 1.0° 2Θ/s.

3.3.1.3 Micro Flow Imagingto measure particle size

A CaP-PCMC suspension was prepared at 10μg/mL in PBS immediately before its placement into the entry portal on the Micro-flow imaging unit (Protein Simple™, California, US). The images were collected at a flow rate of 0.1mL/min.

3.4. Results and Discussion

To investigate the effect of preparation parameters on CaP-PCMC structure, formulations with varying preparation parameters were prepared and are shown in Table 3.2.

3.4.1. Solid Recoveries

Solid recovery, calculated from the mass of the crystals recovered on the filter, was analysed to determine if the formulations are feasible to prepare. In these formulations, the solid recovery levels of the CaP-PCMC samples were between 71% and 87% (Figure 3.3), these values are sufficiently high to make preparation feasible.
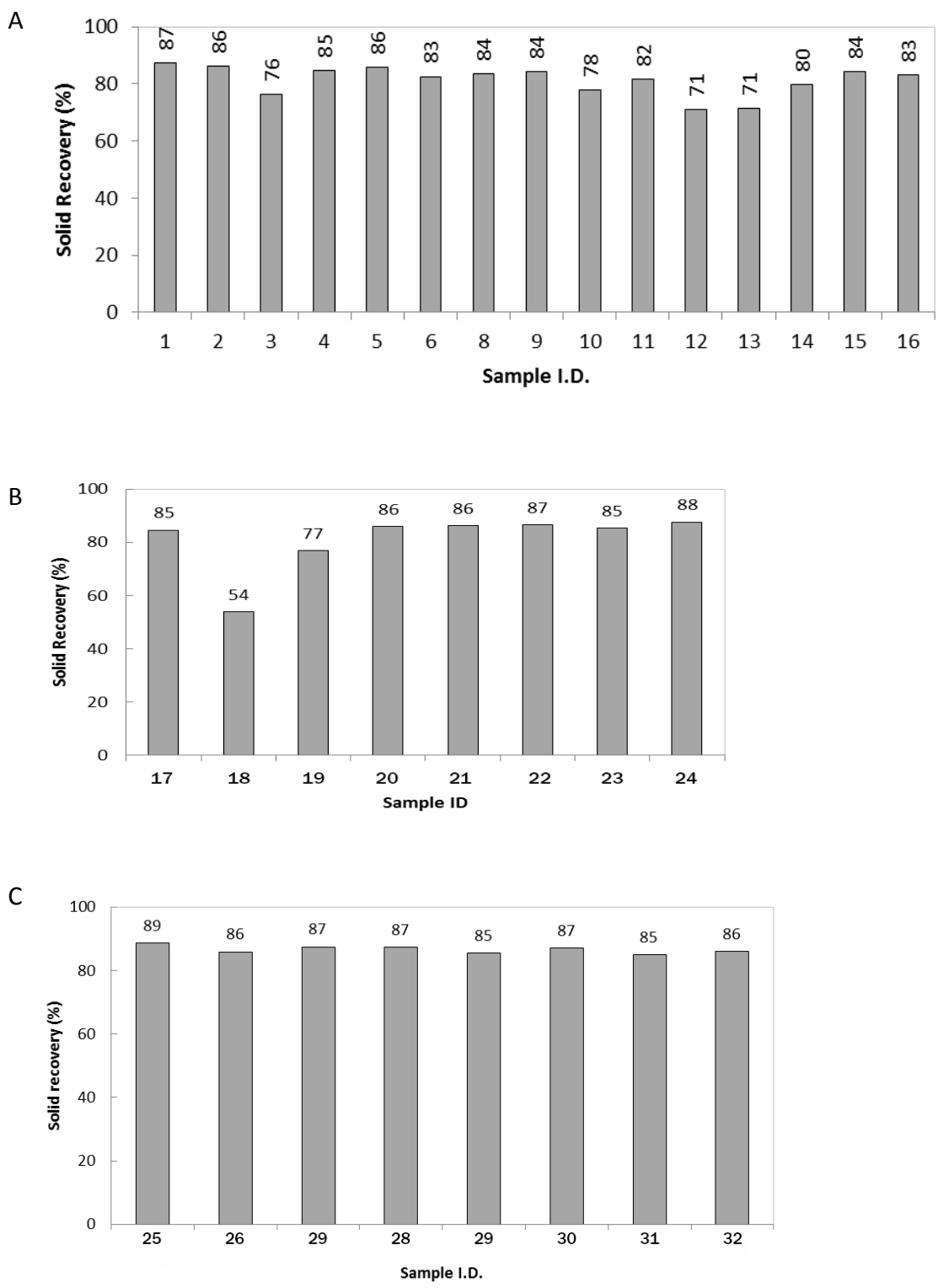
Figure 3.3. Solid recovery of material during the production of the first series of CaP-PCMCs.

CaP-PCMCs were prepared at XstalBio with differing preparation parameters (A), different antigen loadings (B and C). The details of the formulations can be seen in Table 3.2, Table 3.3 and Table 3.4.

Values shown for solid recovery were never 100%, because of the nature of the preparation process. Some material was lost in the soluble form and was not harvested on the filter. Most of the loss occurred when the precipitated crystals remained stuck to the glassware after the preparation and could not be collected.

As the investigations later on in this project are going to be focussed on formulations which contain antigen. The solid recoveries of the formulations prepared with various antigen loadings can be seen in part B of Figure 3.3.

Solid recoveries were found to range from 77% to 88%, similar to recoveries shown in Figure 3.3. This indicates that antigen loading did not influence solid recovery, this could be due to the very low proportion of antigen compared to the other PCMC components. The large quantity of material lost in sample 18, was due to material being stuck on the glassware and not being collected on the filter.

Solid recovery in the third series (shown in part C of Figure 3.3) was similar to the previous series, with all samples recovering between 85% and 89% of the theoretical mass, with a mean of 87% which shows a consistently satisfactory level of recovery.

3.4.2. Influence of formulation parameters on the shape of the PCMCs

Representative SEM images from the first batch of samples are shown in Figure 3.4.

Overall, the SEM appeared to confirm that factors which would increase the rate of

precipitation of the PCMCs decreased the size and smoothness of the crystal shape.
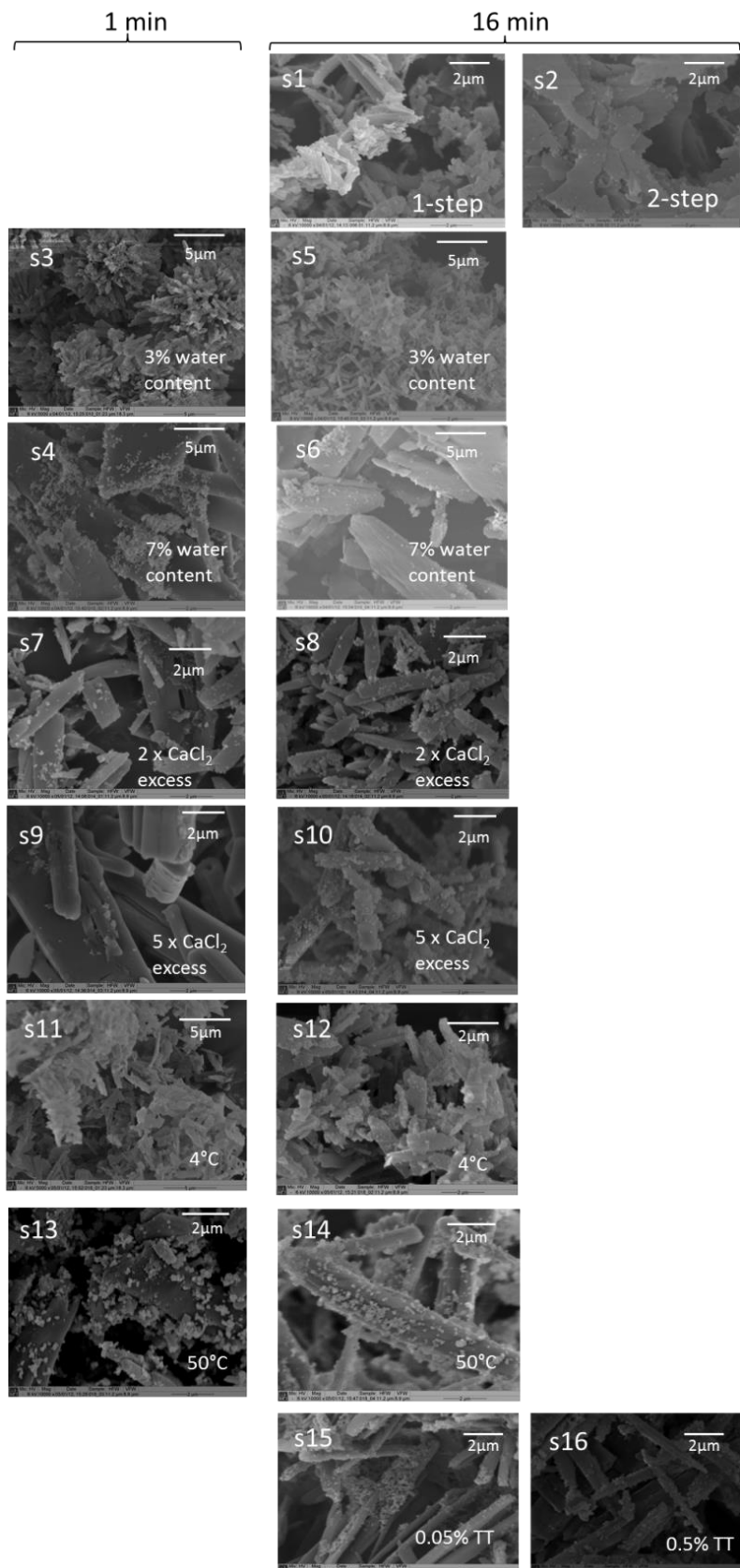
Figure 3.4. SEM images of the samples of CaP-PCMCs prepared with varying preparation parameters. Sample numbers that refer to details shown in Table 3.1

Although many of the formulations have been screened by SEM, the limitations of imaging by SEM is that the formulations were imaged as a dry powder. As a vaccine candidate, these formulations will be reconstituted in a buffer and administered as a suspension, making dry imaging of limited relevance. It is possible that upon particle suspension in an aqueous medium, gradual dissolution of the PCMCs might occur resulting in the release of the smaller particles that are adhered to the larger plate like crystals, and that these particles are taken up by cells and serve as an antigen depot for gradual release. This slow and extended release may improve the quality of the immune response (159). Nevertheless, the SEM is the most effective way, that is available, of determining the morphology of the crystals produced with the different formulations.

3.4.3. Influence of process step

The size and shape of CaP-PCMC samples 1 and 2 are shown in Figure 3.5, both methods produce PCMCs that are quite large in the micrometer size range. The PCMCs formed by the 1 step method appear to be more rod shaped, irregular, and tend to be aggregated, whereas the crystals produced by the 2-step method appear much flatter, and more plate shaped.

Addition of the calcium chloride in a second step, after the L-glutamine, antigens and sodium phosphate have precipitated (for 1 min) may result in a CaP layer that forms more on the surface of the crystal. However if the sodium phosphate precipitates within the L-glutamine, it might not come into contact with the calcium chloride and therefore might not have the opportunity to react and form the CaP layer. From

85

experience, however, we think that the sodium phosphate does not precipitate out in the IPA and that it does not react with the calcium chloride to form the CaP layer.

1-step   2-step

Figure 3.5. SEM images of formulations made using 1 (left) or 2- (right) process steps

3.4.4. Influence of water content

The shapes and sizes of four samples prepared with either 3% or 7% water content and either mixed for 1 min or 16 min are shown in Figure 3.6. The crystals formed with only 3% water content appear much smaller and irregular, and also formed tighter clusters. In contrast, the crystals formed with 7% water content appear much larger in size. In addition, the presence of small surface particles on the surface of the plate-like particles can be seen, which could be adsorbed antigen or CaP particles. The surfaces of the PCMCs are smoother and flatter, and they are not flake-like.

1 min mixing   **16 min mixing**

3%

water

content

7%

water

content

Figure 3.6. SEM images of CaP-PCMCs prepared at different water contents and mixing times.

Samples 3-6 shown, prepared with a 1-step process.

This result was as expected, as larger crystals are often formed from slower precipitations. At a higher aqueous content during the precipitation process, the increased solubility of the crystal components in the mixture of IPA and water will slow the crystal formation and produce crystals with the larger, planar morphologies seen in Figure 3.6.

The One or 16 min mixing times did not seem to influence particles morphology. This shows that PCMC formation occurs fairly rapidly, once the aqeous phase is added to the organic solvent.

3.4.5. Influence of $CaCl_2$:NaH_2PO_4 ratio

To form the CaP on the PCMCs, the $CaCl_2$ was added in excess to make sure that all of the NaH_2PO_4 was reacted. The shape and size of four samples produced with either 2x or 5x $CaCl_2$ excess (molar rations of 1:1 and 5:2 $CaCl_2$:NaH_2PO_4 respectively) and mixed for either 1 min or 16 min are shown in Figure 3.7. The crystals that were produced in a 5x excess of $CaCl_2$ for 1 min appear to be much larger than the crystals produced under the other three conditions. There also seems to be fewer of the small surface particles. Apart from this, the crystals produced in a 5x $CaCl_2$ excess for 16 min mixing duration do not appear to differ greatly from the PCMCs prepared in 2x excess of $CaCl_2$ for 16 min. Although crystals prepared from the 1min mix in 2x $CaCl_2$ excess appear similar in morphology to the 16 min in 2x $CaCl_2$ excess, the former seem to show a slightly flatter and thinner morphology and have fewer small surface particles like the PCMCs produced under the other conditions. In this case, there seems to be a combined influence of mixing time and amount of calcium chloride excess on crystal shape.
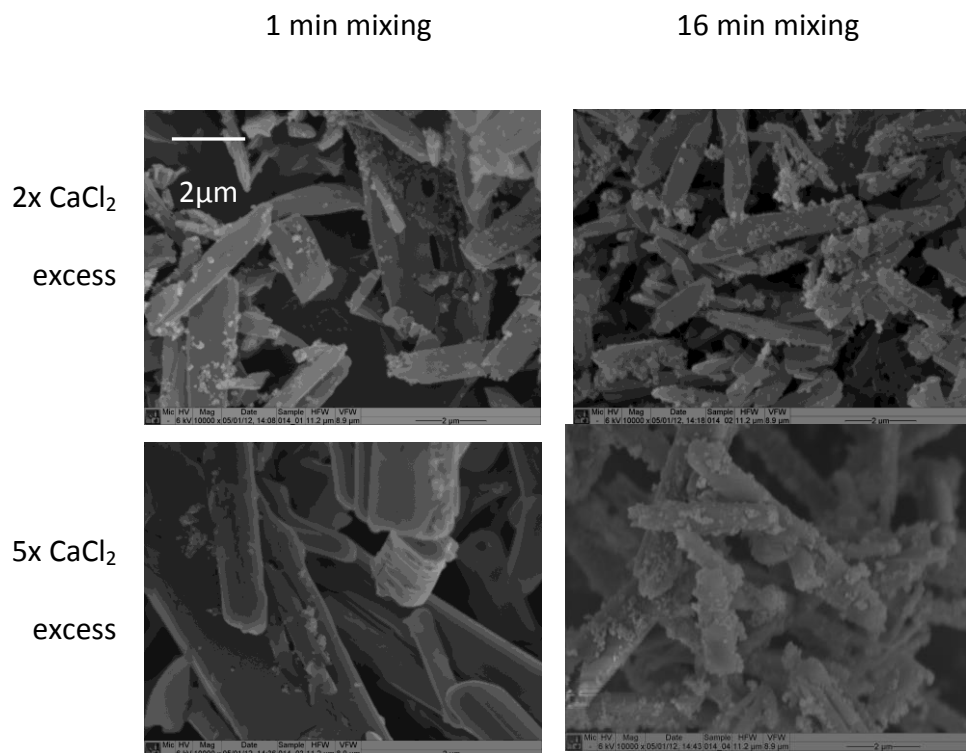
Figure 3.7. SEM images of formulations made at varying CaCl₂ excesses and mixing time.

Samples 7-10 shown, prepared with a 1-step process. Scale bar applies to all four images.

3.4.6. Influence of Temperature

The particles that were produced in IPA heated or cooled to 50°C and 4°C are shown in Figure 3.8. Temperature has a significant influence on the size of the crystals, as they appear to increase at the higher temperature, which was expected as this would increase the solubility of the PCMC components and thereby slow down their precipitation, which results in the growth of larger crystals. In addition the crystals prepared in IPA at 50°C appear to have more of the small particles adhering to their surface. In this case, there does not appear to be an effect of mixing time.
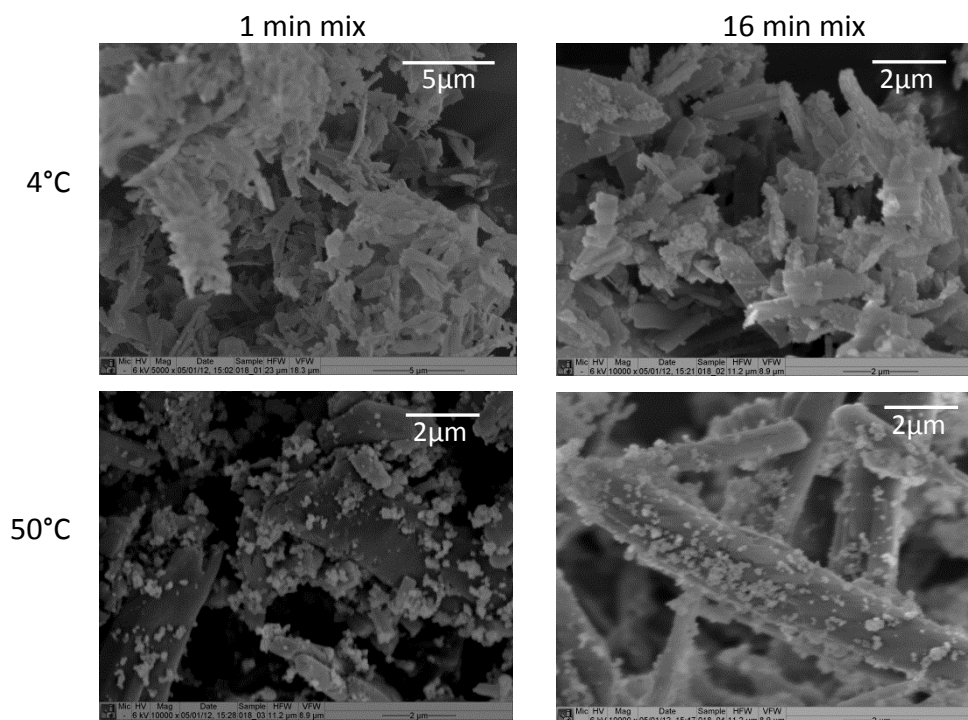
Figure 3.8. SEM images of formulations made at varying solvent temperatures and mixing times.

Samples 11-14 shown, prepared with a 1-step process.

3.4.7. Influence of TT loading in the presence of PRP

Scanning Electron Micrographs of particles prepared with 0.05 or 0.5% TT are shown in Figure 3.9. The crystals seem to be relatively similar in shape and size to each other. This is most probably due to the small proportions of TT in the PCMCs. Thus, a change from 0.05% to 0.5% does not cause any significant change in the PCMC morphology when the majority of the PCMC components did not change.

0.5% PRP loading
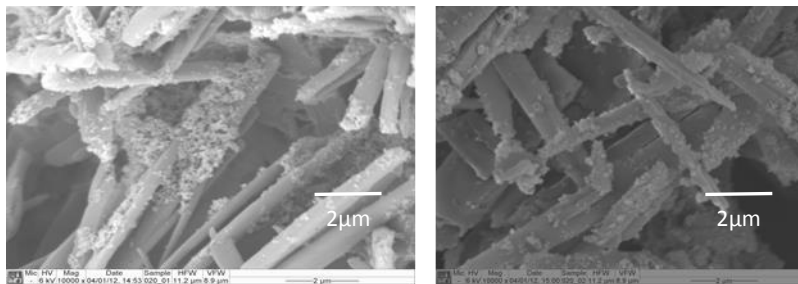
0.05% TT loading   0.5% TT loading

Figure 3.9.SEM images of formulations prepared two loadings of TT

Ssamples 15 and 16 shown, prepared with a 1-step process

3.4.8. The effect of CaP loading on the CaP-PCMC morphology

PCMC formulations prepared with and without a CaP layer were analysed by SEM, the resulting images are shown in Figure 3.10. It can be seen that the two micrographs in Figure 3.10 look very different to each other. Images of PCMCs formulated with CaP show small particles on the PCMC surface, similar to those on the other CaP-PCMC samples, shown in Figure 3.4 to Figure 3.9. PCMCs prepared without appear to have a smooth surface and smooth edges, this suggests that some CaP remained in the

suspension after the PCMCs are formed and then adsorbed to the surface, forming the

small particles. These may be formed from excess CaP in suspension after the PCMCs

are formed and adsorb to the already CaP-coated surface of the crystal.
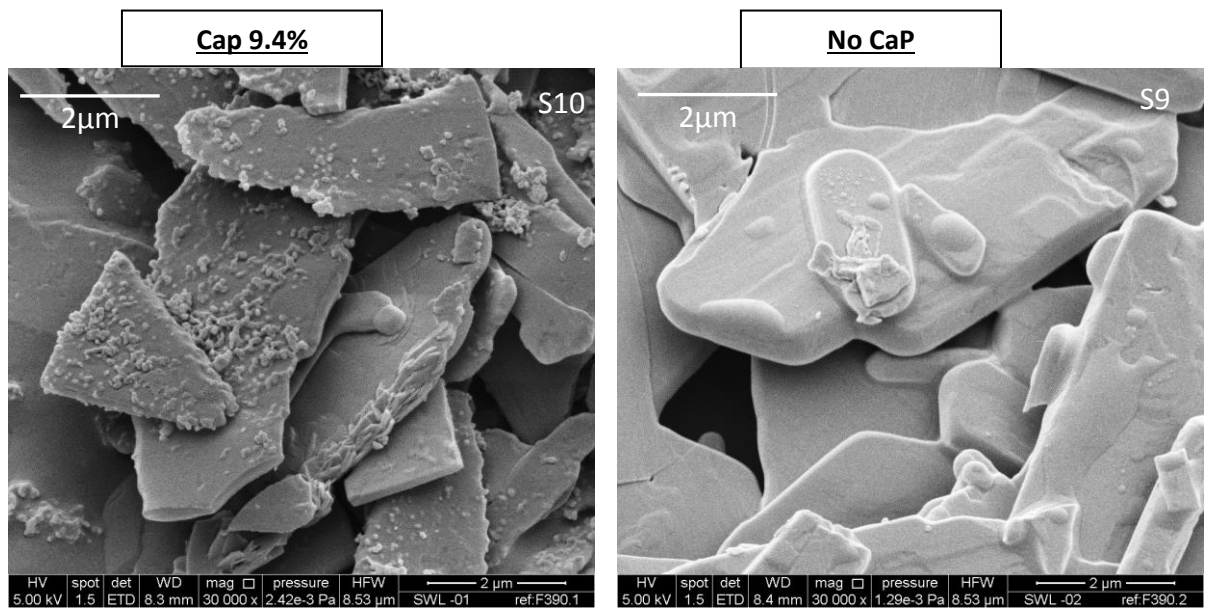
Figure 3.10. SEM images showing the effect of CaP on the shape and structure of CaP-PCMCs

Samples 17 and 18 shown.

3.4.9. Fate of CaP-PCMCs when made to contact an aqueous medium

The effect of suspending PCMCs in water on their shape was observed using light microscopy (Figure 3.11). After 18 hours in suspension at room temperature, the crystals were visible in the same viewing field and displayed no signs of fragmentation, but some aggregation seems to have occurred possibly due to water evaporation, shown by the red arrows. In these images, the CaP-PCMCs seem to be stable in an aqueous medium.
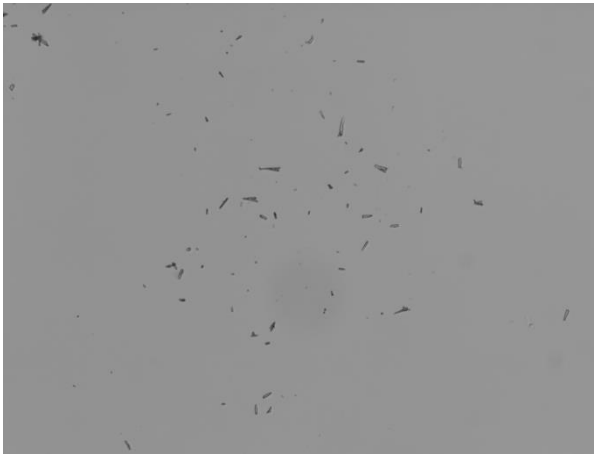
10 min in suspension   >18 hr in suspension

Figure 3.11. Effect of time in suspension on the morphology of the PCMCs (100x magnification)

3.4.10.   PCMC particle size, evaluated by MFI

Micro-flow imaging can be used to determine the particle size in a suspension. The distribution of particle sizes measured on a CaP-PCMC formulation prepared with 0.5% PRP, 0.05% TT, 0.5% CpG and 9.4% CaP (with 5 x $CaCl_2$ excess) is shown Figure 3.12. The minimum size this technique is able to measure is 1μm diameter. This analysis indicated that a majority of the particle sizes are below 1μm in equivalent circular diameter. However, it must be remembered that this technique assumes a spherical shape, and our PCMCs are obviously not spherical, as shown in the scanning electron micrographs.
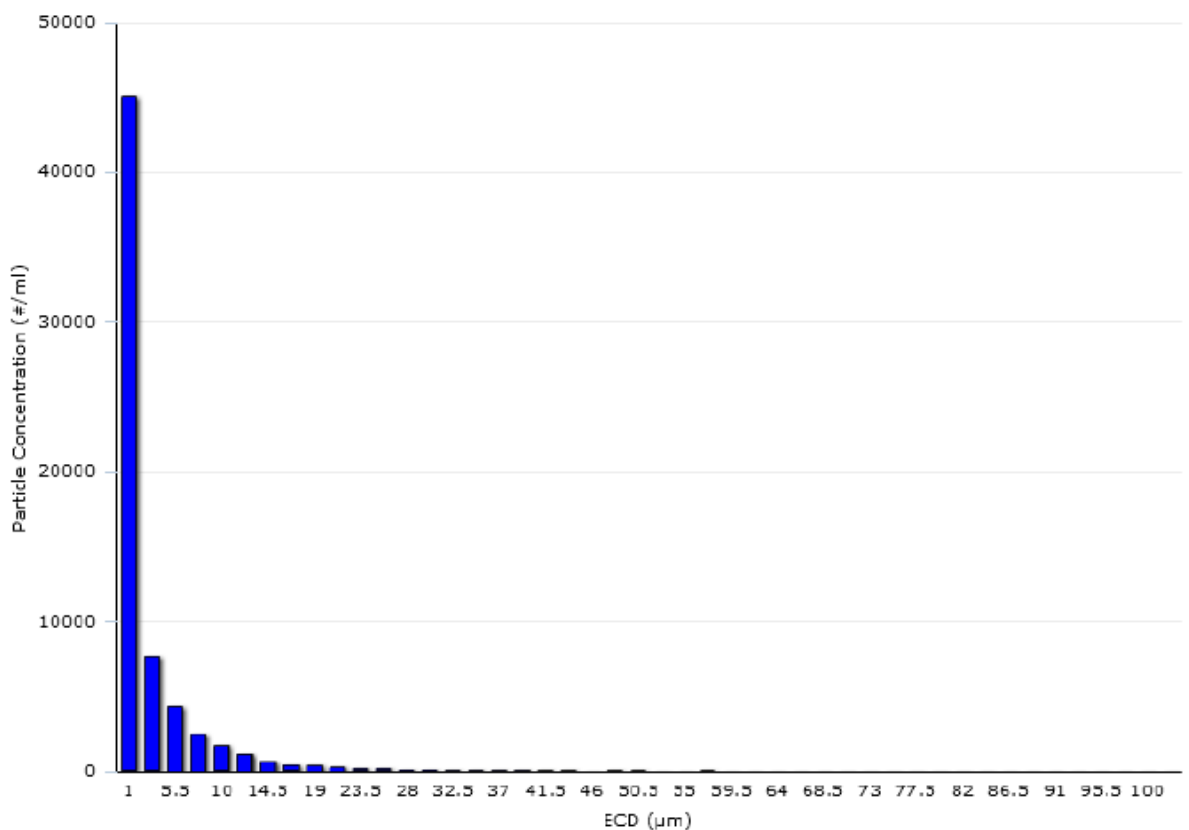
Figure 3.12. MFI analysis of particle size in a suspension.

Concentration of particles for each Equivalent Circular Diameter is shown.

It was also seen in the previous SEM images that the sizes of the crystals was consistently around 2-5µm in contrast to the 1µm or less being given by the MFI.. This may be either because the 'diameter' being measured is narrow due to the rod-like structure of the crystals which is not taken into account by the software. Another explanation could be that the crystal is broken into smaller pieces by the physical stresses when made into a suspension and forced to flow through a narrow tube during MFI measurement.

3.4.11. Comparison of shape of the two samples produced at different locations

Two formulations were prepared both at XstalBio and at the UCL School of Pharmacy to determine if there are differences in the set-ups at the two locations that could affect the morphology of the crystals. These formulations had TT loadings of 0.5% and 0.05%, and a PRP loading of 0.5% and are displayed in Figure 14.

In setting up the crystal preparation in multiple labs, it was a risk that the formulations produced would be different to those previously prepared at XstalBio. Figure 3.13 shows that the PCMCs produced at XstalBio had more of a rod like morphology, compared to the thinner plate-like structure seen in the two samples produced at the School of Pharmacy. These differences in morphology occurred despite the fact that the same preparation parameters had been used at the two sites and indicate that the preparation set up has an effect on the morphology of the crystals. This to be taken into consideration during formulation production.
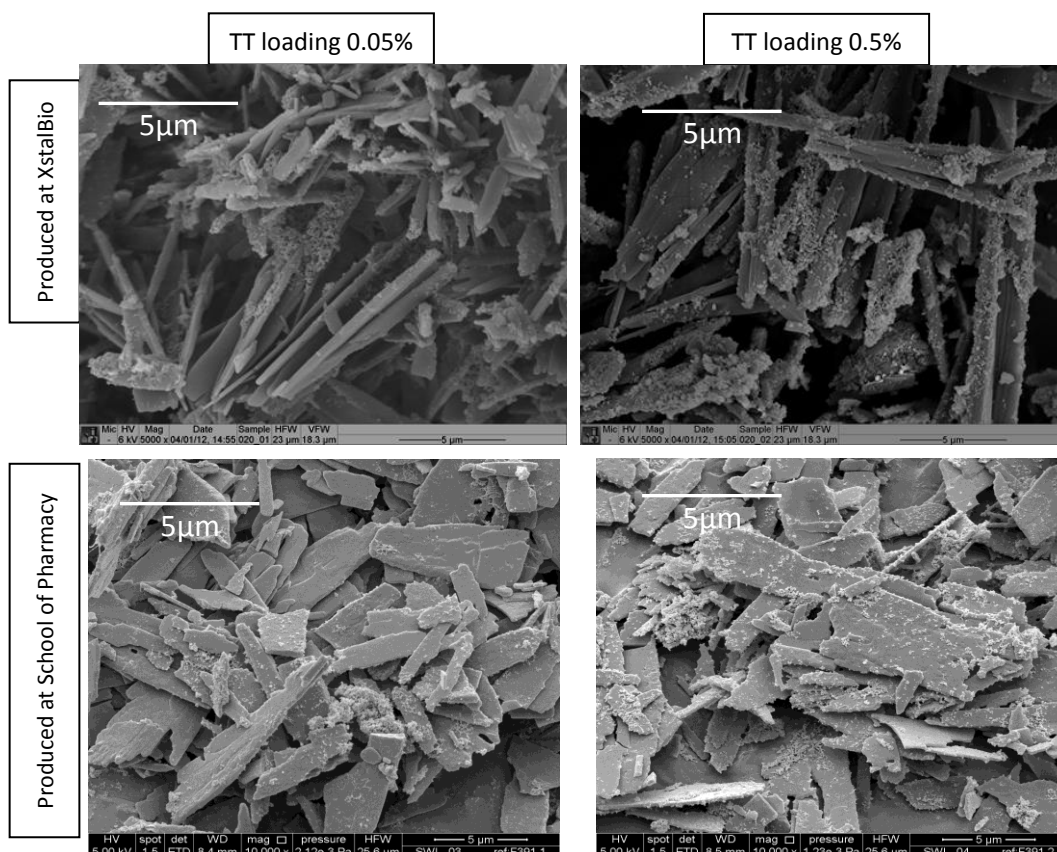
Figure 3.13. SEM images of samples prepared at XstalBio and the School of Pharmacy

Samples 26 and 27 shown.

3.4.12. Determinationation of crystallinity/amorphpous nature of CaP-PCMC particles by X-Ray Diffraction (XRD) analysis

To confirm that the PCMCs formed by the precipitation process have a crystalline nature, a formulation was prepared with 0.5% PRP, 0.05% TT, 0.5% CpG and 9.4% CaP with a 5:2 NaH_2PO_4:$CaCl_2$ ratio and was analysed by XRD.

The spectra (Figure 3.14) produced by the Xray diffraction has clear sharp peaks, which indicates a crystalline material. Therefore, it can be confirmed that the PCMCs prepared in our experiments are crystalline in nature.
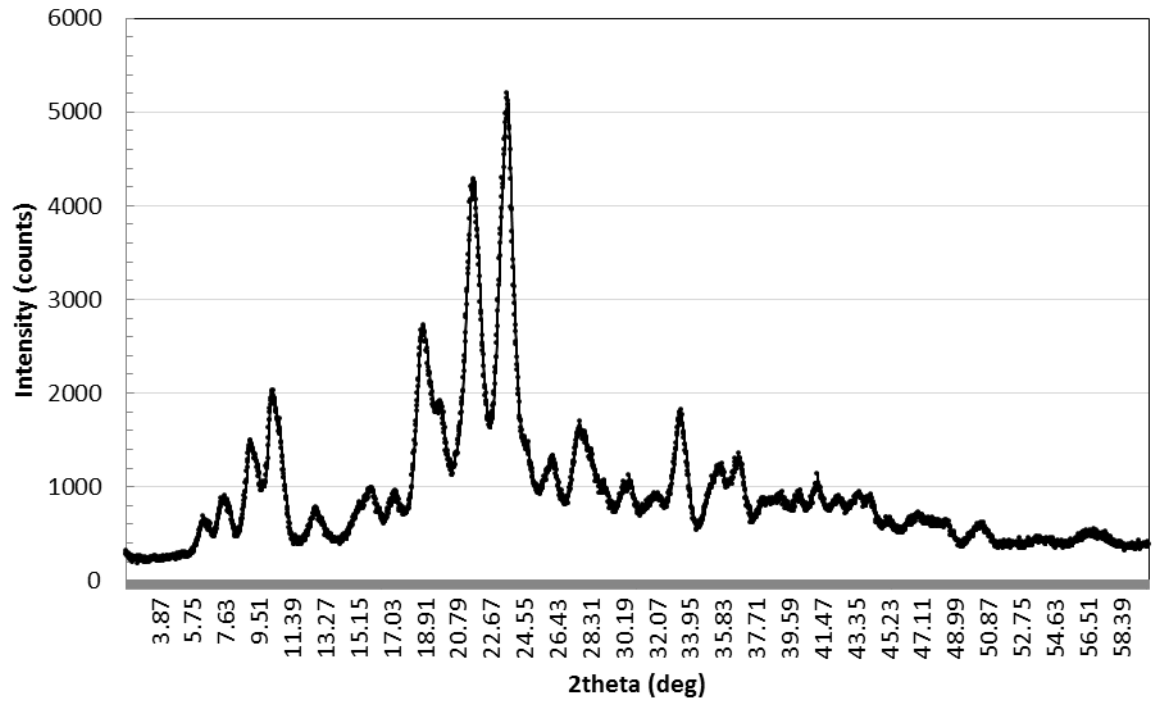
Figure 3.14. Intensity counts from Xray Diffraction analysis.

3.5. Conclusions

First, PCMCs were successfully prepared at all three locations; XstalBio, School of Pharmacy and NIBSC. They are in the micrometer size range, are crystalline in nature and rod-like or plate-like shapes. SEM images of the dry formulations (Figure 3.4 - Figure 3.8) suggest that the preparation coniditons had some influence on particle morphology. In particular, the water content seems to have the biggest effect on the CaP-PCMC shape and size. Other factors that may affect the formuation of the CaP-PCMCs are the temperature and the process step, although not to such an obvious degree.

It is not fully understood how the changes in shape or size of the CaP-PCMCs might affect the immunogenicity of each formulations. However, it is important to consider that the CaP-PCMCs need to be delivered to the draining lymph nodes for recognition by B cells. Previous studies have shown that particle shape, and not size, plays a dominant role in particle uptake and phagocytosis by alveolar macrophages (157, 158), in particular the local particle shape at the point of initial contact seems to dictate whether macrophages initiate phagocytosis or simply spread onto the particles. However, there is also evidence that size, as well as a positive surface charge can affect uptake of particles by circulating dendritic cells (160). Surface charge and hydrophobicity are also factors which can affect the magnitude of the immune response to an antigen. It has been demonstrated that hydrophobic surfaces, such as those exposed on mis-folded protein molecules, can improve antigen immunogenicity (161). Future investigation of the electric charge on the PCMC by zetasizer should be performed to help understand factors that could also increase CaP-PCMC

immunogenicity by changing charge, however there is no evidence if uptake by the dendritic cells might actually increase the magnitude of the antibody response.

In order to create a virtual conjugate vaccine using the PCMC technology, it is very important that the PCMCs are able to hold the two antigens together for processing by the B cell. The stability of the crystals in suspension is expected to be increased by the CaP loading. To determine the stability of the crystals in suspension, the antigen release profile can be studied.

Chapter 4. Quantification and immune characterisation of the antigens adsorbed to the CaP-PCMC

4.1. Introduction

The immunological properties of a vaccine rely on the quality and quantity of the antigens themselves. In the case of conjugate vaccines, where an immune response to the polysaccharide antigen is desired, the characteristics of the polysaccharide antigen are critical. The antigen has to reach the specific B cells whilst in virtual or real conjugation to the protein antigen. The presence of 'free' un-conjugated polysaccharide can be detrimental to the efficacy of a conjugate vaccine. If free polysaccharide was to reach the B cell before the protein conjugated gets access to the B Cell Receptor (BCR), this may induce anergy (52). The production of memory B cells and antibody producing plasma cells relies on full activation of the B cell through co-stimulation by a T cell dependent response (48, 162). Early vaccines consisting of pure PRP failed to stimulate any response in infants under two years of age.

There has been considerable research on reliable and sensitive techniques for the determination of conjugated and unconjugated PRP in protein-polysaccharide conjugate vaccines. The World Health Organisation has published details on the testing of Hib, meningococcal and pneumococcal conjugate vaccines in a Technical Report Series (TRS). The currently manufactured conjugate vaccines are assayed for levels of 'free' soluble polysaccharide that are not conjugated to the protein. The accurate determination of unconjugated polysaccharides has improved greatly with modern

physicochemical methods, based on the structure of the active ingredient. These techniques include optical spectroscopy (circular dichroism and fluorescence spectroscopy), size exclusion, and mass spectrometry. The saccharide component of a glycoconjugate can also be analysed through high performance anion exchange chromatography (163, 164).

The antigenic quality of the polysaccharide antigen needs to be assessed during and after the conjugate vaccine preparation process because activation of the polysaccharide prior to conjugation can shorten or degrade the saccharide chain (165). The structure of the polysaccharide antigen has to be maintained, as the epitope exposed to be recognised by specific B cells has to correspond to the epitope that would be found in the bacteria for anti-polysaccharide antibodies to be protective *in vivo*.

In the development of CaP-PCMC formulations, the assessment of the immunological characteristics of the polysaccharide antigen is extremely important, as preparation techniques may alter the epitopes of the polysaccharide in unknown ways. For these assessments ELISA is a useful tool. It is also possible that during the PCMC preparation process, not all the available antigen adsorbs onto the PCMC before formation of the CaP component. Some antigen may remain dissolved in the solvent and some antigen may adsorb after the CaP formation, the latter would become immediately soluble upon re-suspension of the PCMCs resulting in 'free' antigen. In this chapter, the quality of the PRP epitopes on the CaP-PCMC formulations were assessed, partly, by the detection of the antigens on the crystals through ELISAs, as well as through chemical assays which were also able to measure the soluble polysaccharide.

The presence of the protein antigen must be analysed as it is essential in recruiting T cells for the full activation of the specific B cell. The protein antigen should also ideally be immunogenic enough to stimulate protection, although this function is secondary. In this investigation, the highly immunogenic Tetanus Toxoid along with an adjuvant, CpG, were used for the virtual conjugation. It is hypothesised that the polysaccharide, TT and CpG combination would ensure an adequate immune response if all three are co-immobilised onto PCMCs, such that they are all 'virtually' conjugated.

4.2. Aims and Objectives

The aim of the work discussed in this chapter was to quantify the PRP, TT and CpG loading of the CaP-PCMCs. The PRP content of the crystals were determined using Orcinol assays and ELISA. Orcinol was essential for quantifying the chemical presence of PRP, whereas ELISAs and NMR were conducted to confirm/test that the antigenicity of PRP is retained after formulation. ELISA results show a low PRP concentration compared to Orcinol assay. The lower PRP concentrations measured by ELISA were thought to be caused by a change in the PRP conformation during the CaP-PCMC formulation. In order to investigate this, a series of investigations were conducted to determine the possible formulation parameters which could have caused a conformation change.

4.3. Materials and Methods

4.3.1.   Materials

The following materials were used in the study:

Anti-TT rat monoclonal antibody (NIBSC 10/134, Potters Bar, UK)
Guinea pig anti-TT detection Antibody (NIBSC 10/132, Potters Bar, UK)
Anti-Guinea Pig IgG-horseradish peroxidase conjugate (detection antibody, Sigma)
Rabbit polyclonal IgG anti-PRP-HRP(detection antibody, NIBSC, Potters Bar, UK)

Hib oligosaccharide conjugated to human serum albumin (HBO-HA, NIBSC, Potters Bar, UK)
TT standard (NIBSC code 04/150)

Carbonate Buffer (0.159% Sodium carbonate w/v, 0.293% Sodium hydrogen carbonate w/v pH 9.5-9.7)
Assay Diluent for TT ELISA (20% w/v Marvel Milk powder in PBS, UK)
Assay Diluent for PRP ELISA and anti-PRP antibody ELISAs (1% BSA w/v, 0.5% Tween20 w/v in PBS)
Tris/50mM Citrate/1mM EDTA buffer (pH7.4, all from Sigma, UK)
Sigmafast o-Phenylenediamene dihydrochloride (OPD) substrate (Sigma, UK)
2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt substrate (ABTS, Sigma, UK)
Citric Acid Substrate Buffer (10.51% w/v Citrate monohydrate in water)
H_2O_2 (CalbioChem, UK)
Iron Chloride in Hydrochloric acid ($FeCl_3$-HCl) solution (1.8mM $FeCl_3$ in 36% HCl)
Orcinol-ethanol solution (0.7M Orcinol in absolute ethanol, Sigma, UK)
1st International PRP standard in Sodium Chloride (NIBSC code 02/208, Potters Bar, UK)
SilverXpress silver staining kit (Invitrogen, UK)
Micro BCA protein assay kit (Life Sciences, UK)

4.3.2.   Formulations prepared

Three CaP-PCMC formulations were prepared as before, and production parameters of

the formulations are detailed in Table 4.1. The antigen loadings were selected using

fixed values from a previous sample used by XstalBio in the pilot study which was

previously shown to be partially immunogenic. The $CaCl_2$:NaH_2PO_4 ratio was varied in

these formulations

Table 4.1. Details of the CaP-PCMC formulations produced.

Formulation	PRP loading (%, w/w)	TT loading (%, w/w)	CpG loading (%, w/w)	CaP loading (%, w/w)	CaCl2:NaH2PO4 (CaCl$_2$ excess)
1	0	0	0	9.4	5:2 (5)
2	0.5	0.05	0.5	9.4	1:1 (2)
3	0.5	0.05	0	9.4	5:2 (5)

4.3.3. Preparation of the CaP-PCMC formulations for measurement of total and free PRP CaP-PCMC

To confirm the PRP retained its antigenicity after formulation, the PRP content was assessed using 2 immunoassays. A Sandwich ELISA using rabbit polyclonal antibody (raised in rabbits with PRP-KLH conjugate) was used for coating and for detection and an inhibition ELISA where anti-PRP immune serum (generated from rats immunised with Hib-TT conjugate vaccine) as follows:

- Sandwich ELISA on page 105
- Inhibition ELISA on page 106

To measure the **total** PRP in the CaP-PCMC formulation, CaP-PCMCs were dissolved in the Tris/citrate/EDTA buffer at a concentration of 5-10mg/mL, unless otherwise specified.

To measure the '**free**' or soluble PRP, CaP-PCMCs were suspended at a concentration of 10mg/mL (unless otherwise specified) in PBS (pH 7.5). The suspension was left to stand for 15 min on the bench at room temperature before centrifugation at 12000 rpm for 2 min in a micro-centrifuge. 1mL of supernatant was collected for use in the assays for PRP quantification described below. In initial assays, the pellet was also collected for analysis.

The total PRP content was calculated using a standard curve of various dilutions of the PRP standard. The percentage of free PRP was calculated relative to the total PRP content.

4.3.4. PRP Quantification by Orcinol Assay

To determine the total and the amount of PRP in the formulation, CaP-PCMCs containing PRP were prepared as in Chapter 3 in triplicate, with the exception that the 1mL supernatant was collected and dilution 1:1 in PBS to provide adequate volume for the assay. 500µL of this solution was mixed with 500µL of $FeCl_3$-HCl solution. 50µL of Orcinol-ethanol solution was added to the mixture and incubated for 20 min at 100°C in an oil bath. The glass vials were then added to ice and the optical density was measured at 670nm using a UV-vis spectrophotometer (Perkin Elmer Lambda 800, Waltham, Massachusetts, USA). Optical density was converted to PRP concentration using a standard curve that was prepared with PRP standards in triplicate.

4.3.5. Immune assays to check antigenicity of the formulation

- *Sandwich ELISA*

A sandwich ELISA was used to measure the total and free of PRP based on interaction between antigen and antibodies generated from rabbits immunised with PRP-KLH conjugate.

96-well flat bottom plates were coated with 100µL/well of rabbit polyclonal anti-PRP antibody diluted to 1/200 in carbonate buffer and incubated for 90 min at 37°C. Non-specific binding sites were blocked with 100µL/well assay diluent and incubated for 1hr at 37°C after which they were washed three times. The first well of each row was filled with 200µL/well of the CaP-PCMC samples dissolved in Tris/Citrate/EDTA at 5mg/mL

crystal concentration. The samples were diluted across the 96-well plates in serial two-fold dilutions, and the plates incubated again for 90 min at 37°C. After washing (3x) the plates, a HRP-conjugate rabbit polyclonal anti-PRP detection antibody was added at 100µL/well and the plate incubated for 2 hr at room temperature. The plates were washed and then 100µL of the OPD substrate was added to each well in the dark. The OPD substrate was prepared according to manufacturer's instructions. The reaction was stopped with 50µL/well of 3M HCl. Optical densities were read at 492nm and the concentration of the PRP in the PCMC samples was determined by comparison with a standard curve generated by serial two-fold dilutions of PRP standard on the same plate.

- *Inhibition ELISA*

An inhibition ELISA was used to measure the specificity of binding between the PRP and antibodies from rats immunised with Hib-TT conjugate.

PRP was diluted in assay diluent to a concentration of 4-40µg/mL of PRP (to give a final concentration of 2-20µg/mL). CaP-PCMCs were dissolved in Tris/Citrate/EDTA at a concentration of 10mg/mL (an equivalent of 5µg/mL of PRP) and then diluted in AD to 0.4mg/mL (a PRP equivalent of 2µg/mL). To each tube, 0.5mL of immune serum from rats immunised with Hib-conjugate vaccine in assay diluent was added and these were incubated at 4°C overnight.

The following day, ELISA plates pre-coated with 50µL/well of 1µg/mL of HbO-HA in PBS were washed and blocked with 100µL/well of Assay Diluent for 30 min at 37°C. 100µLs of each sample was added to duplicate wells of the ELISA plate and incubated for 90 min at room temperature. Plates were washed and 100µL/well of anti-rat IgG:HRP

conjugate (at 1/2000 dilution) in Assay Diluent was incubated for 90 min at 37°C.

Plates were washed and 100µL/well OPD substrate was added for 15 min at room

temperature in the dark. The reaction was stopped with 50µL/well 0.1M HCl and the

colour change was measured at 492nm.

4.3.6.   Quantification of TT content in CaP-PCMCs

The loading of TT on the formulations needed to be determined to be sure that the

CaP-PCMCs would be delivering the protein antigen alongside the polysaccharide

antigen. The amount of TT in the formulation was quantified using different ELISA

assays, SDS-Page or a BCA protein assay.

4.3.6.1 ELISA

Tetanus toxoid content of the PCMCs was quantified using a sandwich ELISA. Plates

were coated with 100µl/well of an anti-tetanus toxoid monoclonal antibody diluted to

1:200 in carbonate buffer and incubated at 4°C overnight. Non-specific binding sites

were blocked with 150µL/well Assay Diluent and incubated for 1 h at 37°C. After

washing, 100µL volumes of CaP-PCMC samples (initially dissolved in Tris/Citrate/EDTA

buffer) were added to the plate in serial two-fold dilutions and incubated for 90 min at

37°C. After washing (3x), a guinea pig polyclonal anti-TT antibody was added at

100µL/well and the plate incubated for 2 hr at 37°C. To detect levels of binding, the

plates were washed and then 100µL/well of anti-guinea pig IgG-HRP (1:2000) was

incubated for 1hr at 37°C. The substrate ABTS was prepared according to

manufacturer's instructions in citric acid substrate buffer and added to the plate at

100µL/well. ODs at 405nm was measured after 30 min at room temperature.

Concentration of the TT on the PCMC samples was determined by comparison to a standard curve generated by TT standard in the same ELISA.

4.3.6.2 SDS-Page

To quantify the total TT on the CaP-PCMC formulation SDS-PAGE analysis was carried out. A 10mg/mL CaP-PCMC solution in Tris/Citrate/EDTA buffer was prepared, and 65µL of CaP-PCMC sample was added to 25µL of LDS sample buffer and 10µL of DL-dithiothreitol solution (DTT). The resulting mixture was shaken, before heating at 70°C for 10 min. Ten 10µL volumes were loaded into wells on a 4-12% Bis-Tris gel with a MES running buffer. After running, the gel was then stained using silver staining staining kit according to the manufacturer's instructions. The gel was analysed using a Bio-Rad GS-800 densitometer and Quantity One software.

4.3.6.3 Micro BCA protein assay for quantification of TT content in CaP-PCMCs

To measure the total TT on the CaP-PCMC samples, the crystals were dissolved at 10mg/mL in Tris/Citrate/EDTA buffer. To measure the soluble TT on the CaP-PCMC sample, the suspension was prepared as before in section 4.3.3, but at a crystal concentration of 100mg/mL.

In eppendorfs, 1mL of CaP-PCMC sample was added in duplicate to 1mL of the BCA protein assay kit working reagent prepared according to the manufacturer's instructions. The mixture was then incubated at 60°C for 1 h in a heating block before being left to cool to room temperature. The ODs were then read at 562nm. The

concentration was determined by comparison to a standard curve generated by

varying concentrations of the TT standard.

4.3.7. Quantification of CpG by UV spectroscopy

To determine the quantity of CpG in the CaP-PCMCs, the microcrystals were prepared

as in section 4.3.3 and optical density was measured at 280nm. The optical density of

the samples and standards were also measured at 330nm to take into account light

scattering by particulates in suspension. CpG concentration was calculated by

comparison against a standard curve generated from various concentrations of CpG.

The standard curve can be seen in Figure 4.15. The absorption was corrected using the

equation $ABS_{protein} = ABS_{280} - (1.929*ABS_{330})$

4.4. Results and Discussion

4.4.1.   Quantification and characterisation of PRP in CaP-PCMC formulation

Initial formulations were tested for total and free PRP at XstalBio in a water suspension, rather than PBS. The release of soluble PRP from the CaP-PCMCs was found to be low in water, and so the first formulations were tested in immunogenicity studies. Once new formulations were prepared at NIBSC it became a priority to test the soluble PRP content prior to use in the immunogenicity studies.

The PRP content in the formulation as well as the soluble PRP was measured by both a chemical Orcinol assay and by ELISA. It was essential to set up assays with which to accurately measure the levels of antigen recovery, as potential loss of antigen during the formulation process would make the CaP-PCMC formulation both inefficient to produce and non-immunogenic.

The antigenicity of the PRP when it is a component of the CaP-PCMC formulation is also important to measure in this context. It is for this reason that we cannot solely rely on Orcinol results providing evidence of the chemical presence of PRP. The ELISA to quantify PRP was used in these experiments to investigate if the PRP retained its antigenic form post-formulation. The detection of the PRP in this assay depends on antibodies, which are specific for PRP in the Hib-TT conjugate and known to be effective at killing bacterium [ref], to bind to the same epitope on the CaP-PCMC.

Without this antigenicity, it would be impossible for the CaP-PCMC formulation to induce a protective anti-PRP response.

4.4.2. Orcinol assay for quantification of total and soluble PRP in CaP-PCMC formulation

To determine if the Orcinol assay could be used to accurately quantify PRP content in the presence of CaP-PCMC formulation, an assay was carried out with the standard PRP in the presence and absence of blank (i.e. without PRP) CaP-PCMC.

As seen in Figure 4.1, the relationship between the ODs and the PRP concentration is linear. It can also be noted that the ODs measured at the various PRP concentrations are not affected by the presence of CaP-PCMC. This indicates that the CaP-PCMC formulation does not interfere with the quantification of PRP by this method.
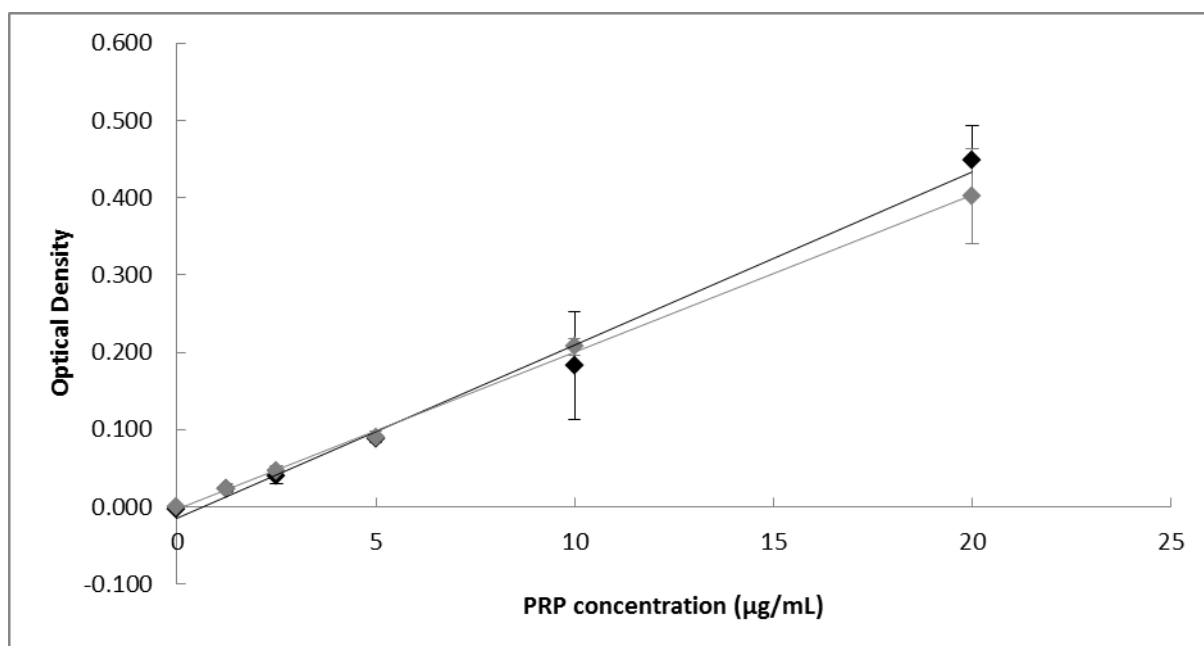
Figure 4.1. Optical density of various concentrations of PRP stock after testing by Orcinol assay

Optical density measurements at multiple concentrations of PRP with and without CaP-PCMC formulation (black and grey lines respectively). Error bars represent standard deviation of the ODs between the three replicates at each concentration.

The Orcinol assay was used to measure the total PRP content in various batches of

CaP-PCMC formulation 3 (Table 4.1) after formulation as well as the soluble PRP.

Examples of total PRP content and soluble values are presented in Table 4.2. The total

recovery was higher than expected, 112% of the expected quantity of PRP was found

in the CaP-PCMC formulation. However, it was found that 69.9% of this was lost as

soluble PRP when in a suspension of PBS.

From these results, we conclude there is a high rate of release of PRP from this

formulation. Positively, the closeness of the calculated percentages from the pellet and

the supernatant to the total measured PRP indicate that this is a reliable assay.

Table 4.2. Total and soluble PRP measurements from CaP-PCMC formulation

	Percentage of total PRP content	Percent of expected
Supernatant	69.9	-
Pellet	27.5	-
Total	-	112

Total and free PRP in PBS was measured CaP-PCMC as measured by Orcinol assay.

This assay was repeated multiple times, measuring the release of soluble PRP from the CaP-PCMC suspension and calculating it against the total PRP content shown in Table 4.2. The values of the soluble PRP release vary from 62.4 to 80.8%. This variation could come from heterogeneity in the crystal suspension, if there was clumping of the crystals or variations in the agitation to form the suspension. However in all the assays, over half the PRP was found to be soluble in PBS. Interestingly, this formulation was found to have a <5% release in water when tested by XstalBio (data not shown). This could be due to the breakdown of the CaP layer in the presence of salt.

Table 4.3. Percentage release of soluble PRP from CaP-PCMC formulation.

Assay no.	Percentage free (%)
1	69.9
2	80.8
3	70.3
4	80.4
5	62.4
Mean	72.8

Multiple suspensions were measured for soluble PRP release by Orcinol assay. The percentage was calculated from the measured total from a previous assay.

Before further testing in immunogenicity studies, a new CaP-PCMC formulation will need to be developedBoth the recovery of PRP on the CaP-PCMC formulation and the low levels of soluble PRP released in suspension are vital for the success of a formulation. Just as the conjugate vaccines are tested for low 'free' polysaccharide, this measurement will need to be taken with every new batch of formulations prepared, particularly those tested in immunogenicity experiments.

4.4.3. Kinetics of soluble polysaccharide release from CaP-PCMC suspension, and effect of $CaCl_2:NaH_2PO_4$ ratio, over time

Two formulations were developed at XstalBio by screening a variety of preparation parameters for low soluble PRP, the parameters can be seen in Table 4.4.

Table 4.4. Formulations developed by XstalBio which have low PRP release.

Formulation	PRP Loading (%, w/w)	TT loading (%, w/w)	CpG loading (%, w/w)	CaP loading (%, w/w)	CaCl$_2$:NaH$_2$PO$_4$ (CaCl$_2$ excess)	Process step
1	0.5	0.05	0.5	10	5:2 (5)	1
2	0.5	0.05	0.5	10	5:2 (5)	2

Multiple formulations with varying preparation parameters were screened for low soluble PRP content in a PBS suspension. The two formulations with the lowest soluble PRP content were both produced with a 5:2 $CaCl_2:NaH_2PO_4$ excess and produced in both 1 and 2 process steps.

To monitor the release of soluble PRP from the CaP-PCMCs while in suspension over time, it was decided to test the soluble PRP at different time intervals. The rate of release of PRP from the formulation was investigated in suspensions in two different ways:

1. CaP-PCMC suspensions (1.5mL) which were replenished with 1mL of PBS after the harvesting of 1mL supernatant.
2. Individual CaP-PCMC suspensions prepared used for each time point

The two methods were used on the two formulations and the suspensions were kept both on the rotator for constant resuspension of the CaP-PCMCs and on the bench where the suspensions were left to settle.

The soluble PRP from both formulations on the rotator are shown in Figure 4.2. It can be seen that the initial release of soluble PRP from the crystals was 5.7% and 32.6% of the total from the formulation prepared with monobasic and dibasic sodium phosphate. The monobasic formulation shows a steady increase of cumulative PRP release to 26% after an hour, 61% after two hours, 89% after three, and up to 109% after four hours when the suspension was centrifuged, the supernatant harvested and the pellet resuspended with PBS. However, the suspensions that were kept for the different time points and had the supernatant harvested once, showed low PRP release, showing a peak of only 14% after 4 hours.

The dibasic formulation showed similar patterns, but with increased PRP release from both suspensions. After only 1 h, there was over a two-fold increase at 79% soluble PRP content, which increased to 88% after 2, 96% after 3 and 99% after 4 h in the replenished suspension. Again with the individual suspensions, the soluble PRP was

found to be greatest at the first time point with 33%, and remains at the same level, with soluble PRP values only dropping to 20%.

The data shows a clear effect of the PBS supernatant replenishment. In both formulations, the individual aliquots showed minimal influences of time on the soluble PRP content. Variations in these results are likely due to the variations in the crystals within each suspension. It is also clear, that the replenishment of the supernatant with PBS drives the release of more soluble PRP from the crystals. These results would indicate that the presence of the Phosphate and Saline may act to dissolve the Calcium Phosphate layer and release more soluble PRP.
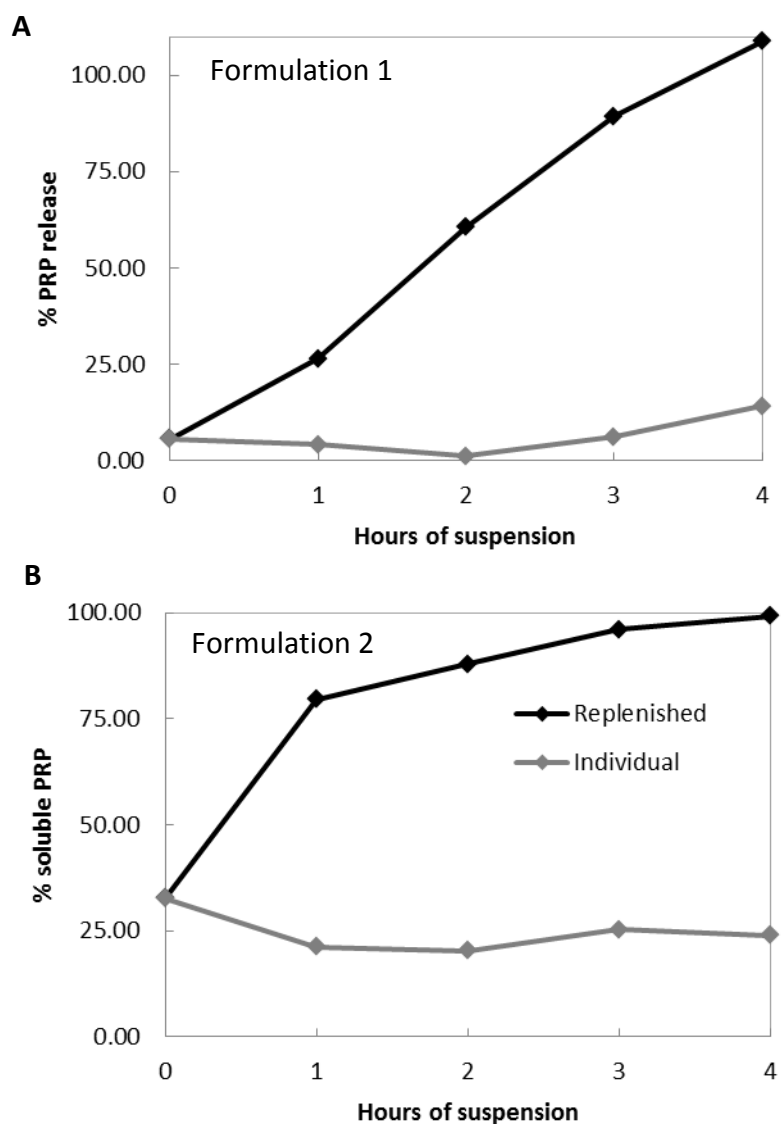
Figure 4.2 Release of soluble PRP from the CaP-PCMC over time from two formulations in a rotating suspension

PRP release from CaP-PCMC formulation prepared with monobasic sodium phosphate (A, formulation 1) and dibasic sodium phosphate (B, formulation 2) and left on a rotator. The release from crystals which had the buffer replenished (black line) is shown against separate samples for each timepoint (grey line).

To investigate the effect of the rotation on the release of PRP from the CaP-PCMCs, the same experiments were carried out but without any movement which acts to continually resuspend the crystals in the PBS. The release of soluble PRP from the stationary CaP-PCMC suspensions over time is shown in Figure 4.3. The initial release

was found to be 12 and 51% in the formulations prepared with the monobasic and dibasic sodium phosphate respectively. Although higher, these results are similar to the previous experiment, and indicate that Formulation 1 which was prepared with the monobasic Sodium Phosphate has a much lower soluble PRP content than the formulation prepared with the dibasic Sodium Phosphate.

The release of soluble PRP from the individual suspensions on the bench showed no effect of time on the release of soluble PRP from the CaP-PCMCs, as seen with the rotated suspensions in Figure 4.2. The formulation with the monobasic Sodium Phosphate (Formulation 1) has a maximum release at 3 h with 23% of the PRP in the formulation released as soluble. The replenished sample, again, showed increasing proportions of the PRP lost from the crystal. The values increase six-fold from 12% to 76% after the first hour. The release continued to increase steadily between 1 and 4 h, reaching 108%.

Formulation 2, which had an initial soluble PRP content of 51%, showed a similarly constant release rate at all time points when measured from separate suspensions, reaching a minimum at 3 h of 43% and a maximum of 59% at 4 h. The same formulation showed an increase of 33% between 1 and 2 h and the cumulative release continues to increase steadily to 101% at 4 h.

The similarity between the release of soluble PRP from samples from both the stationary suspensions and the rotated suspensions indicates that the physical agitation of the crystals is unlikely to be the driving factor in the release, and the presence of the phosphate and saline is increasing the release.
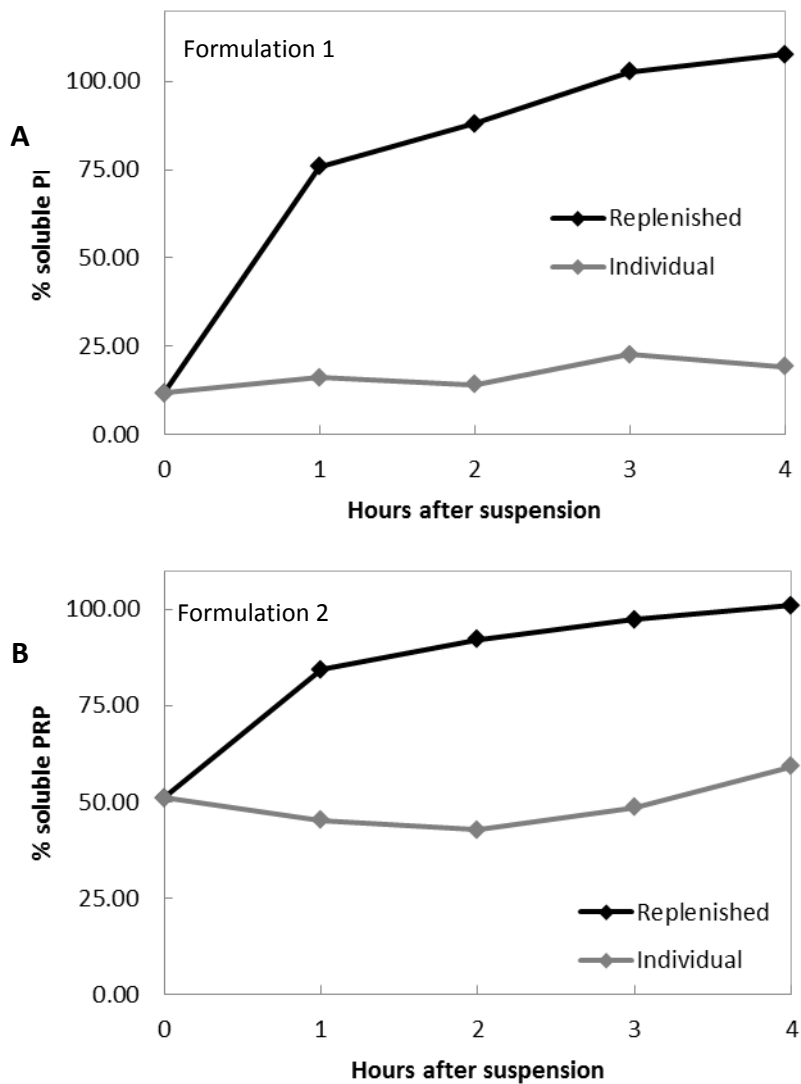
Figure 4.3 Release of soluble PRP from the CaP-PCMC over time from two formulations in a stationary suspension

PRP release from CaP-PCMC formulation prepared with monobasic sodium phosphate (A, formulation 1) and dibasic sodium phosphate (B, formulation 2) and left on a the bench. The release from crystals which had the buffer replenished (black line) is shown against separate samples harvested for each timepoint (grey line).

4.4.4. Quantification and characterisation of antigenicity of PRP in CaP-PCMC formulation by ELISA

The ELISA has the advantage over the Orcinol assay of being more sensitive, being able to quantify PRP concentrations as low as and also being able to measure the antigenically recognisable PRP. A standard curve generated by testing different concentrations of the PRP standard measured by ELISA is presented in Figure 4.4.
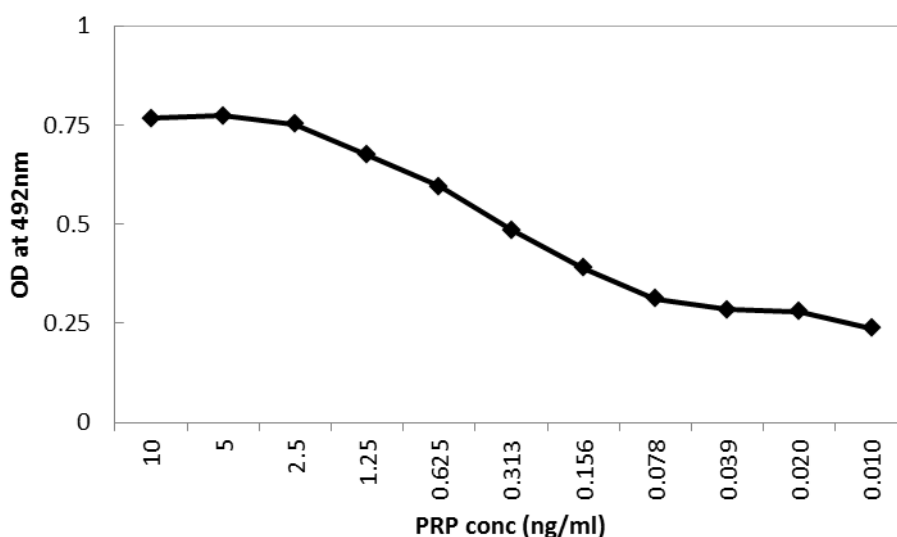
Figure 4.4. Titration curve of PRP standard measured by sandwich ELISA

ODs generated from duplicate wells of an ELISA carried out on serial two-fold dilutions of the PRP standard.

To investigate the potential for CaP-PCMCs to interfere with the PRP quantification by ELISA. A CaP-PCMC formulation with no PRP was added to the PRP standard and added to the plate. As shown in Figure 4.5, there was no significant difference between the titration curves generated in the presence or absence of CaP-PCMC. This result would

indicate that there is no interference with the ELISA from the components or physical presence of the CaP-PCMCs.
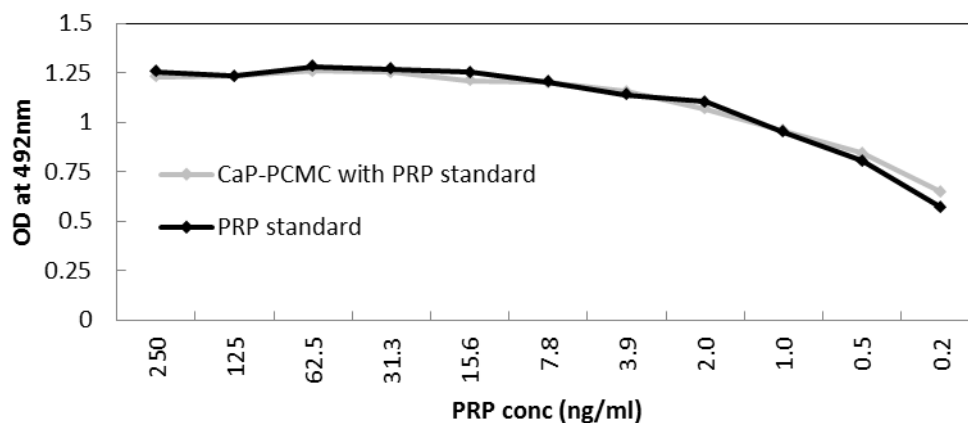
Figure 4.5. Titration curve of PRP standard with and without CaP-PCMCs measured by sandwich ELISA

ODs generated by ELISA to quantify PRP in CaP-PCMC formulation and PRP standard with and without non-PRP CaP-PCMC formulation (grey and black lines, respectively). Points shown are mean values from duplicate wells.

To quantify the PRP on the CaP-PCMC, the CaP-PCMC and PRP standard were titrated together across the ELISA plate and the PRP content of the CaP-PCMC was calculated. The curves are shown in Figure 4.6. The sample, which had previously been shown to have 112% recovery by Orcinol assay (Table 4.2), was shown to have a much lower OD compared to the PRP standard, although not as low as the negative control. The negative control, blank CaP-PCMCs, appear to have very low levels of binding at the highest two PRP concentrations, however these levels are barely above the background level seen at the other concentrations and therefore do not indicate any presence of PRP. The low detection of PRP on the PRP CaP-PCMC sample indicates that

only when the PRP is formulated as part of the CaP-PCMC, the ability to bind to either

the capture or detection antibody is decreased.
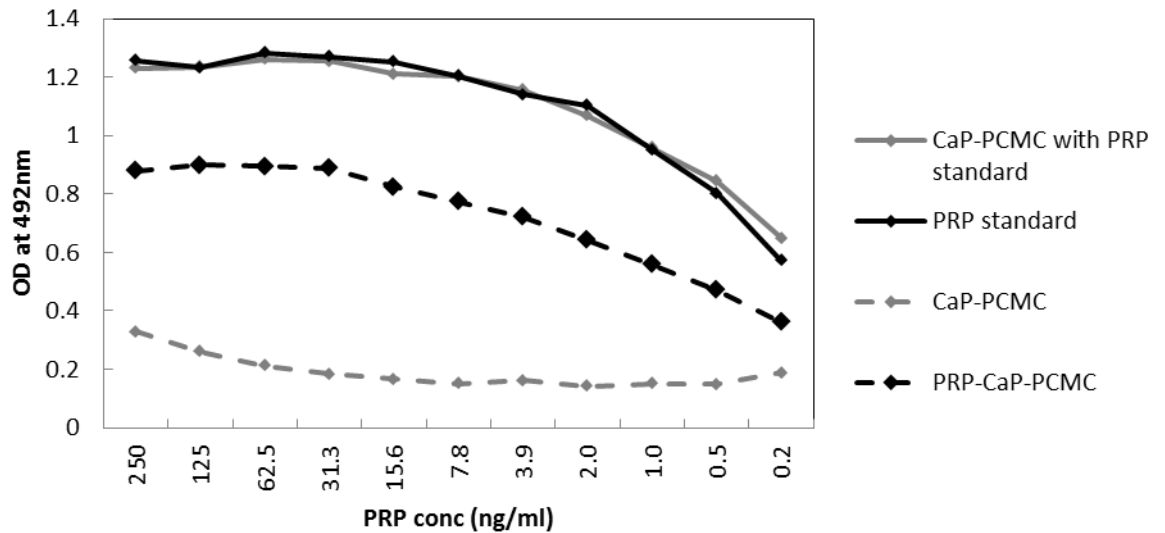
Figure 4.6. Titration curves of PRP-CaP-PCMCs against PRP standard

PRP-CaP-PCMCs were titrated against PRP standard with and without CaP-PCMCs as well as blank CaP-PCMCs. ODs shown are from duplicate wells on PRP ELISA. n=2.

Changes in the antigenicity of PRP leading to failure of recognition by the detection

antibodies used in the assays indicate that we cannot solely rely on Orcinol results

providing evidence of the chemical presence of PRP. There is either a difference in the

interfering capabilities of the CaP-PCMC formulation when formulated with the

antigen, or more likely, that the formulation of the PRP has somehow changed the PRP

so that it is no longer detectable by the ELISA. This could be due to a conformational

change of some of the PRP polysaccharide in a way that does not allow the same level

of binding by the anti-PRP detection antibodies in the rabbit serum from PRP-KLH

immunisation.

The detection of the PRP in this assay depends on antibodies, which are specific for PRP in the PRP-KLH conjugate, to bind to the same epitope on the CaP-PCMC. Without this antigenicity, it would be impossible for the CaP-PCMC formulation to induce a protective anti-PRP response. It is not clear if these changes could affect the immunogenicity of the CaP-PCMC.

The sandwich PRP ELISA was used to measure the total and soluble PRP content of Formulation 1 (refer to Table 4.1), the assay was carried out on two dates and both sets of results are shown in Table 4.5. The pellet was also analysed in these samples. Similar values were obtained when the assays was repeated on two different days.

It was found that the total PRP content was 28% of the expected in the first assay, and only 6% of expected in the second. The PRP measured on the supernatant was 23% in the first assay and 5% in the second, which equates to 82% and 75% of the total PRP measured. The PRP in the pellet was found to be 18% and 2% of the total expected in the two assays, which equates to 64% and 30% of the total measured PRP content.

The low recovery of the PRP measured on this sample which had previously been shown to have 112% of PRP by Orcinol assay (Table 4.2) demonstrates that the ELISA is not able to detect 72-94% of the PRP that is present on the CaP-PCMCs. The measured PRP in the supernatant might indicate that the soluble PRP can be quantified by the sandwich ELISA, but a conclusion is hard to draw, as the soluble PRP was measured between 6-12% as measured by Orcinol assay.

Table 4.5. The concentration of PRP in the CaP-PCMC formulation were analysed twice by ELISA

	First Assay		Second Assay	
	Conc (ng/mL)	Actual recovery (% of theoretical)	Conc (ng/mL)	Actual recovery (% of theoretical)
Total	2.80	28.03	0.67	6.71
Supernatant	2.26	22.55	0.50	5.01
Pellet	1.83	18.28	0.20	2.00

The initial experiment (Table 4.2) appears to demonstrate that the presence of the CaP-PCMC itself is not the cause of the low detection of PRP by ELISA, and rather, it is a change that is occurring as part of the formulation process. Interestingly, when the measurement of the free and CaP-PCMC polysaccharide was carried out by ELISA (Table 4.5), the proportions of the PRP in the supernatant was similar, although the quantities varied considerably. This may indicate that the proportion of epitopes on individual polysaccharide chains might be affected, rather than proportion of the total PRP.

4.4.5. Quantification of PRP mixed with IPA and $CaCl_2$ combinations by ELISA

To understand which component of the CaP-PCMC preparation process could have altered PRP and resulted in reduced binding in the ELISA, the PRP standard was mixed with Isopropanal (IPA), IPA and $CaCl_2$, or $CaCl_2$ in water and mixtures were titrated straight onto the plate coated with rabbit anti-PRP antibodies, and the assay carried on

as before. Results shown in Figure 4.7 indicate that exposure of PRP to IPA did not alter the ability of the PRP to bind to the detection antibody.

These results indicate that there does not seem to be an alteration of the binding epitopes by treatment of PRP with IPA, $CaCl_2$ or the combination of both.
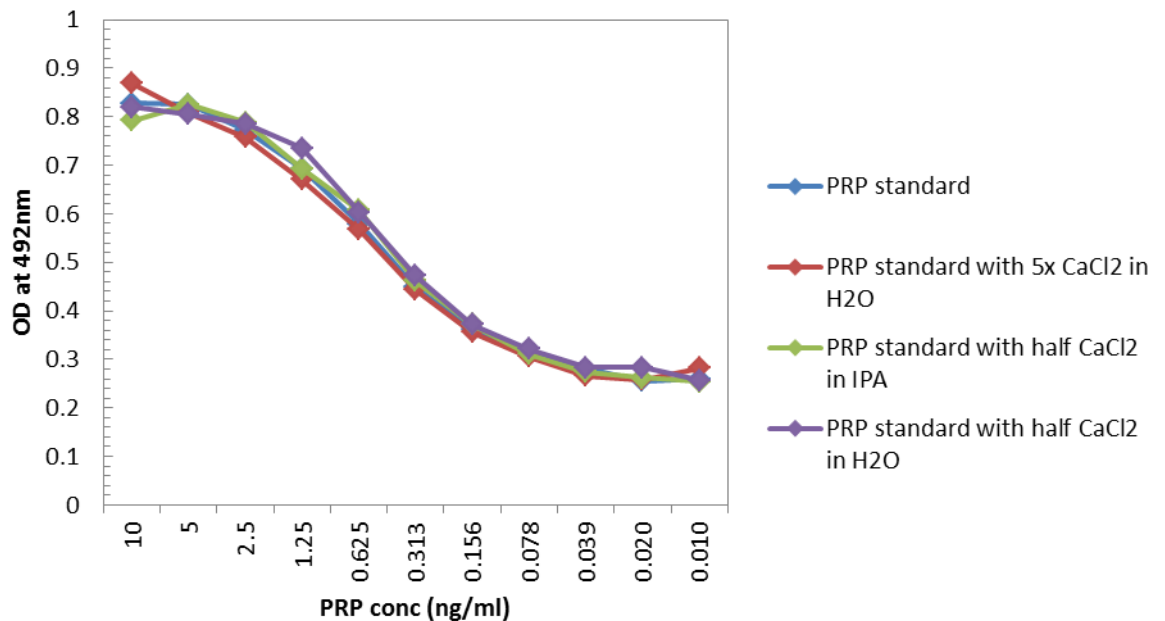
Figure 4.7. Titration curves of PRP treated with IPA and CaCl$_2$ individually and in combination

The PRP standard was mixed with $CaCl_2$ and IPA individually or in combination were titrated against PRP standard. ODs shown are from duplicate wells on PRP ELISA. n=2

Although the PRP standard treatment do not indicate whether the $CaCl_2$ or the IPA are responsible for the decrease in PRP detection when formulated into CaP-PCMCs, the results indicate that the effect is the results of the preparation process. Therefore, it was decided to test the effect of different processes on the antigenicity of PRP by ELISA to determine the factors responsible.

4.4.6. ELISA to quantify PRP on soluble PCMC preparation prepared at XstalBio

To determine if the PRP has endured any changes during the formulation process, a range of formulations were produced by XstalBio. These formulations were designed with the aim of isolating the factor that may be causing the potential epitope change. Due to the effect of the reduced binding seen only in CaP-PCMC samples and not in soluble samples prepared without the CaP layer (data not shown), the factors that these samples focus on are related to the formation of the CaP layer.

As the use of a core protein is necessary for the formation of the crystals, the L-glutamine was replaced with histidine in two samples to investigate the role of the effect of the L-glutamine on the CaP layer. Other factors include the presence of NaH_2PO_4 and $CaCl_2$ in combination with solvent.

The details of the formulations are shown in

Table 4.6. The titration curves from ELISA analysis are shown in Figure 4.8 and Figure

4.9. The ELISA method was carried out at NIBSC and the Orcinol assay was carried out

by staff at XstalBio. A comparison and summary of all the values are shown in Table

4.7.

Table 4.6. PCMC formulations prepared with different core proteins and CaP-PCMC component

Formulation number	Core	CaP component
1	L-glutamine	NaH_2PO_4
2	L-glutamine	none
3	L-glutamine	NaH_2PO_4 and solvent
4	L-glutamine	$CaCl_2$ in solvent
5	Histidine	NaH_2PO_4
6	Histidine	none

Six formulations were prepared with either L-glutamine or Histadine as the main component, and with different combinations of NaH2PO4, or CaCl2 with and without extra solvent. The PRP and TT loadings were kept at 0.5% or 0.05% respectively to reflect previous formulations.

It can be seen that the Orcinol results show close to the expected 100% recovery of PRP in all samples, with the percentages ranging from 74-106. Slightly lower values are expected in soluble (without CaP) PCMCs due to higher loss to glassware. However, it was seen that the percentage of PRP quantified by ELISA was much higher in the two samples which contained NaH_2PO_4 (Formulation 1 and 5). This result was unexpected, as high quantification by ELISA had not previously been seen. This effect might be due to interference by un-reacted NaH_2PO_4 with the binding of the capture antibody, however it is not of direct relevance, as in the CaP-PCMCs, there is an excess of $CaCl_2$ and therefore it is unlikely there is unreacted NaH2PO4 remaining in the formulation.
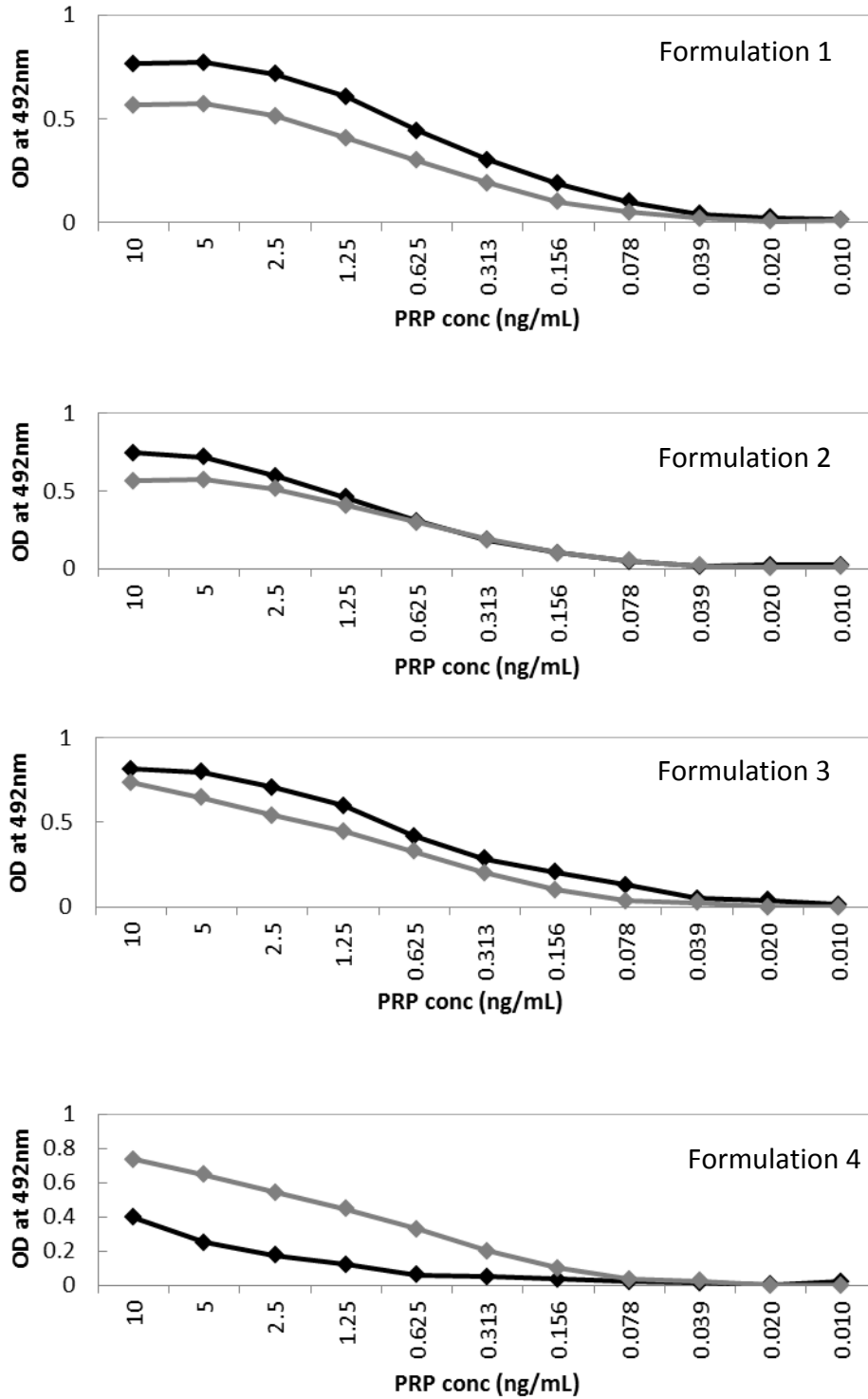
Figure 4.8. Titration curves of PRP-PCMC formulation against PRP standard

PRP-PCMC formulations detailed in

Table 4.6 were titrated against the PRP standard. ODs shown are means of replicate ELISA wells of sample. The PRP content of the PCMC (black line) was calculated against the PRP standard (grey line). n=2 and n=3 for the standard and the sample wells respectively.

It was also seen that only the sample containing the $CaCl_2$ (Formulation 4) was found

to have a much lower PRP quantity by ELISA, with only 9.9% of expected compared to

109.6% in the formulation without the $CaCl_2$ (Formulation 2). It is evident that

inclusion of $CaCl_2$ was responsible for the low PRP detection by ELISA.

The two samples prepared with histidine instead of glutamine did not show a large

difference in PRP quantification by ELISA, with PRP values of 203.1% and 122.1%

compared to 215.8% and 109.6% in the corresponding glutamine samples.
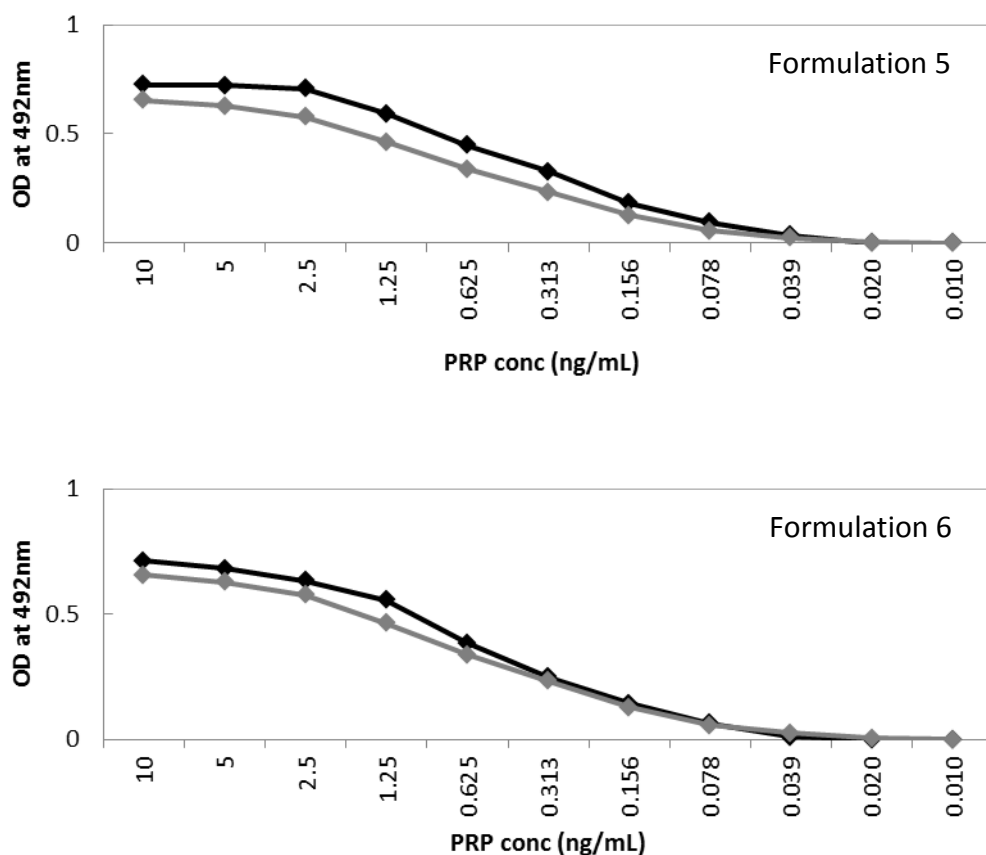
Figure 4.9. Titration curves of PRP-PCMC formulation against PRP standard

PRP-PCMC Formulation 5 and 6 detailed in

Table 4.6 were titrated against the PRP standard. ODs shown are means of replicate ELISA wells of sample. The PRP content of the PCMC (black line) was calculated against the PRP standard (grey line). n=2 and n=3 for the standard and the sample wells respectively.

Table 4.7. The PRP as measured by ELISA compared to the values measured by Orcinol assay at XstalBio. The values of PRP detection by ELISA were calculated by parallel line analysis.

Formulation no.	PCMC components	% of expected by ELISA	% of expected by Orcinol
1	TT, PRP, glutamine, NaH_2PO_4	215.8	74
2	TT, PRP, glutamine	109.6	88
3	TT, PRP, glutamine, NaH_2PO_4 (with solvent shot)	154.4	92
4	TT, PRP, glutamine (with solvent shot containing $CaCl_2$)	9.9	106
5	TT, PRP, histidine, NaH_2PO_4	203.1	90
6	TT, PRP, histidine	122.1	82

The specificity was also investigated by using soluble formulations produced with only one of either $CaCl_2$ or NaH_2PO_4 in combination with either L-glutamine or histidine. The inhibition ELISA titration curves are shown in Figure 4.10.

Table 4.8. PCMC formulations prepared with different core proteins and CaP-PCMC component

Formulation no.	PCMC components
7	TT, PRP, glutamine
8	TT, PRP, glutamine (with solvent shot containing $CaCl_2$)
9	TT, PRP, glutamine (with solvent shot containing NaH_2PO_4)
10	TT, PRP, histidine, solvent shot
11	TT, PRP, histidine (with solvent shot containing $CaCl_2$)
12	TT, PRP, histidine, NaH_2PO_4 (with solvent shot containing $CaCl_2$)

Six formulations were prepared with either L-glutamine or Histadine as the main component, and with different combinations of NaH2PO4, or $CaCl_2$. $CaCl_2$ was added dissolved in a 'shot' of Isopropanal 1 min after the aqueous components were added. The PRP and TT loadings were kept at 0.5% or 0.05% respectively to reflect previous formulations

It can be seen that the glutamine samples with and without NaH_2PO_4 did not show a large difference in binding from that of the PRP standard, indicating a similar antigenicity. However, Formulation 8, which contained $CaCl_2$ showed a decrease in binding. These results confirm the previous finding that the antigenicity of the PRP is affected by the $CaCl_2$ during the preparation process.

However, in Formulations 11 and 12, $CaCl_2$ was included in the formulations and it was seen that there was no decreased specificity of the anti-PRP detection antibodies. These results show that the decrease in antigenicity by $CaCl_2$ in the CaP-PCMC formulations is dependent on the protein core. Unfortunately, histidine is not suitable for use as a protein core in CaP-PCMCs, as previous samples used by XstalBio were found to have high levels of solubility in a water suspension.

A

B
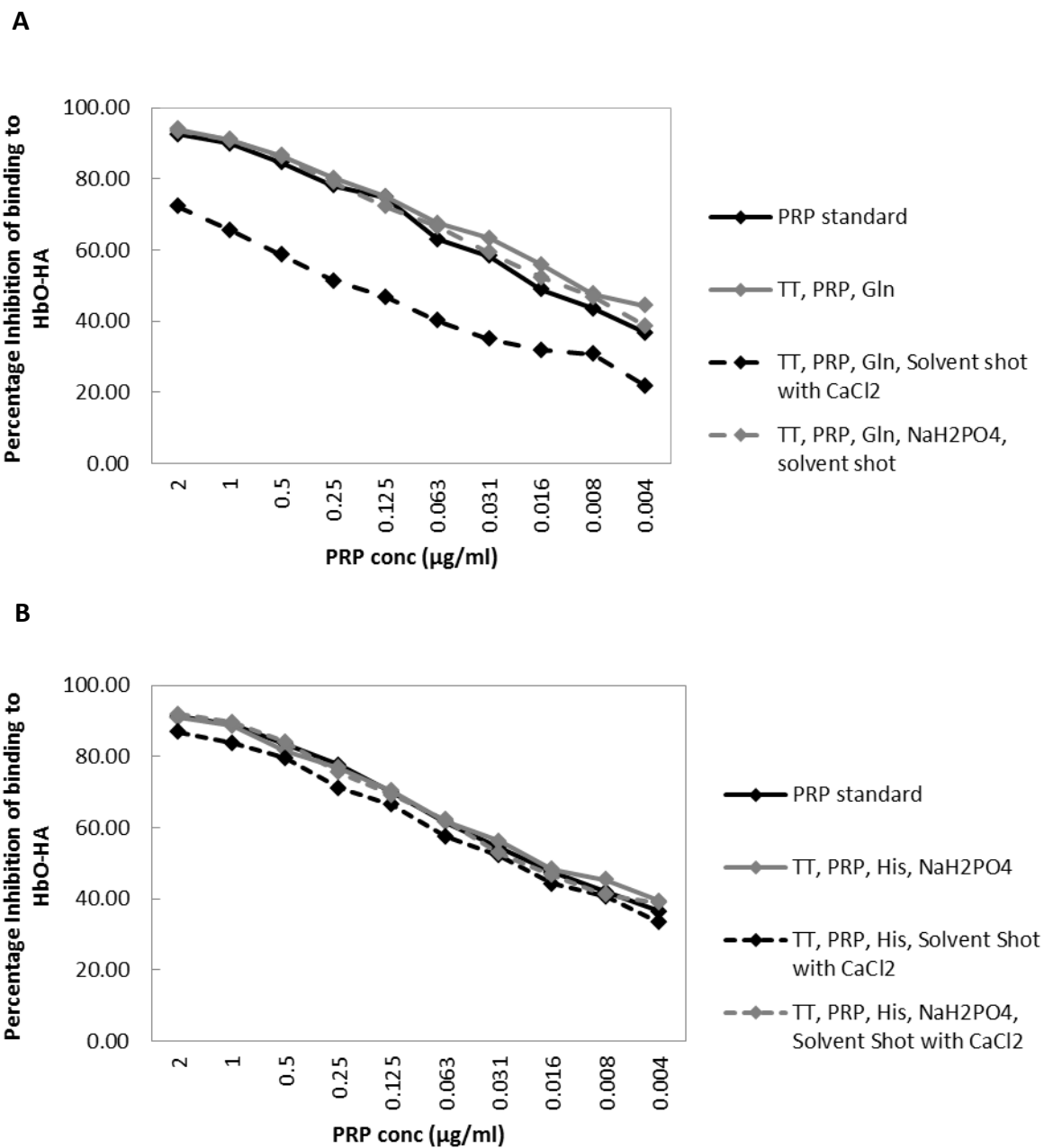
Figure 4.10. The specificity of binding of the anti-PRP antibody from a Hib-TT immunised animal to PRP in different PCMC formulations with NaH$_2$PO$_4$ or CaCl$_2$ in formulations with glutamine or histidine.

The antigenicity of the PRP in formulations with glutamine and histidine (A and B) which were prepared with NaH$_2$PO$_4$ and CaCl2 at doubling dilutions. The points show means from duplicate wells on the ELISA plate.

Once the $CaCl_2$ has been shown to cause a loss of antigenic PRP, a concern is that in immunogenicity investigations, that the CaP-PCMC immunisations might be inducing a specific antibody response against the conformationally altered PRP, which may not be protective.

4.4.7. Investigation into change of PRP conformation of PRP using NMR

Nuclear magnetic resonance (NMR) spectroscopy can be used to detect changes in the PRP structure after formulation. As indicated by results in section 4.4.6, there may be a conformational change induced by a binding of calcium to the PRP.

The main purpose of the NMR experiment was to determine if there was a change in PRP conformation induced by binding of Calcium. After consultation with a polysaccharide NMR expert, the resulting spectra, shown in Figure 4.11, there would be three peaks that are absent, in a location indicated by the green arrows.
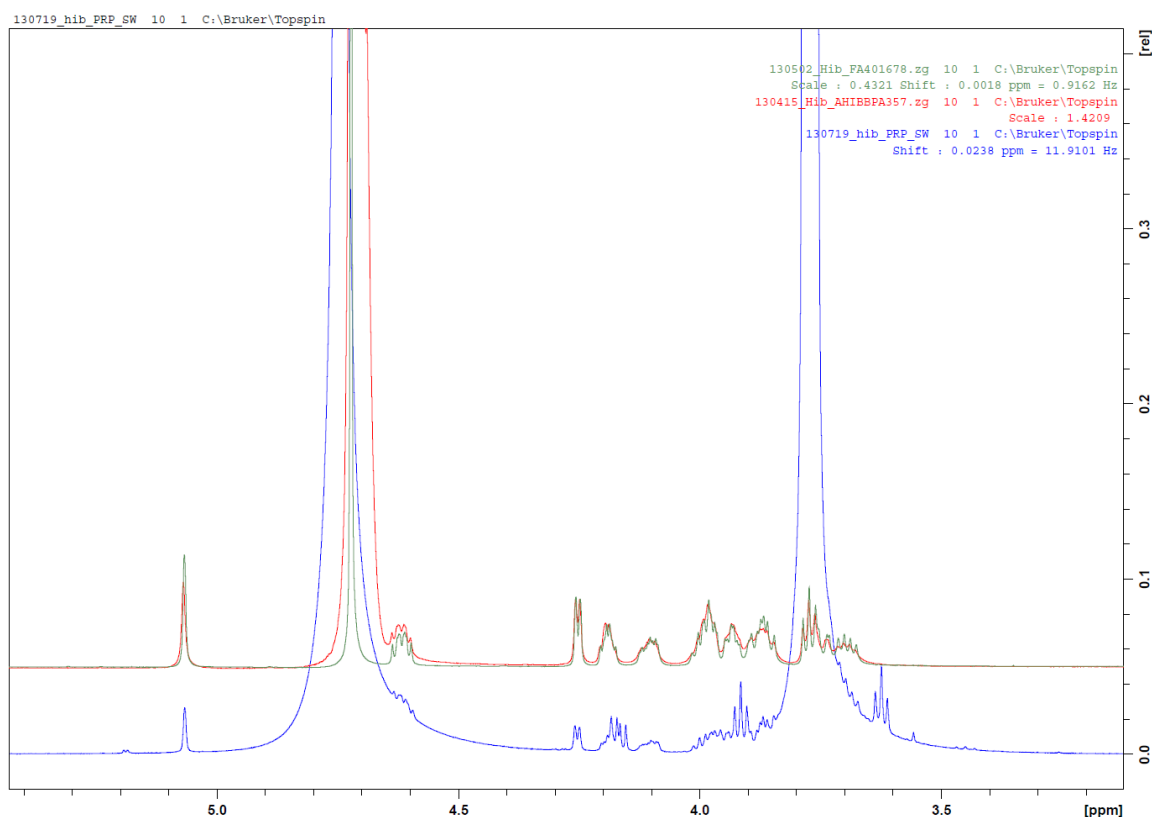
Figure 4.11. NMR of CaP-PCMC sample (blue) overlayed with NMR of two unformulated PRP samples (red and green)

The NMR spectra shown in Figure 4.11 shows that there is a large peak of resonance

present on the CaP-PCMC spectra which can attributed to the presence of a large

quantity of glutamine present in the formulation. Unfortunately, it is a change in the

smaller peaks that a consequently masked by the glutamine peak, that would indicate

changes in the structure of PRP.

There also appears to be a slight shift and sharpness of the spectra from the CaP-PCMC

formulation which is due to the presence of NaCl (from the PRP used in the

formulation).

4.4.8. TT ELISA for quantification of TT on CaP-PCMC formulation

In contrast to PRP, the antigenicity of the TT is of less importance than that of the PRP.

The presence of the TT is very important, as highlighted by the high ratio of TT to PRP

in conjugate vaccines, however, the pure function of the TT in the conjugate is to

recruit T cells to activate the follicular B cells. Therefore as long as there is some

immunogenic protein presence of the TT, the necessary T cell dependent response

could theoretically still be induced.

ELISA was used to measure the antigenically recognisable TT on the four CaP-PCMC

formulations prepared by XstalBio with either 0.05% or 0.5% TT. Determination of TT

loading on PCMCs by ELISA showed very low level of TT recovery for one sample

(0.12%) and none for another sample (Figure 4.12). This suggests that the ELISA

method was not able to detect any TT on sample 15 which had the lower loading of

0.05%. This is possibly due to a loss of TT during formulation or to changes in the

conformation of the TT during the production process, making it unrecognisable during the ELISA.
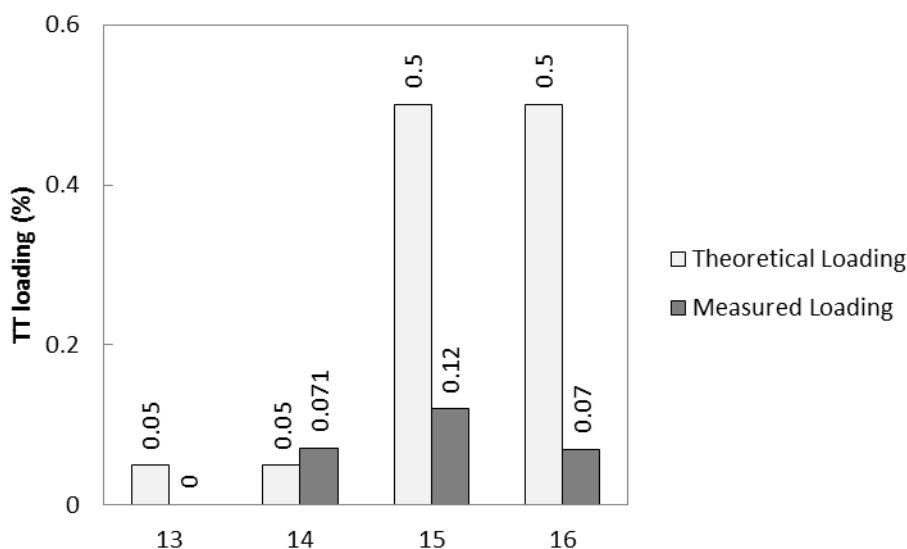
Figure 4.12. TT loadings as measured by ELISA.

Expected loading is shown in pale grey bars, against the loading measured by ELISA in darker grey.

4.4.9. SDS-Page for quantification of TT on CaP-PCMC formulation

SDS PAGE was used to measure the chemical presence of the TT on a range of formulations prepared at XstalBio. The intensity of the bands was used to quantify the TT using a densitometer.

It can be seen in Figure 4.13 that three of the samples with 0.5 or 0.05% loading showed recoveries of 80.1-122%. However, there was one 0.05% loading which only had 11.9% of the expected TT and the two samples with 0.025% TT loading show low recoveries of TT (41.2 and 51.1%) however this can be attributed to the low

concentrations which are at the limit of detection for this assay. The first sample also shows low recovery of TT on the formulation.
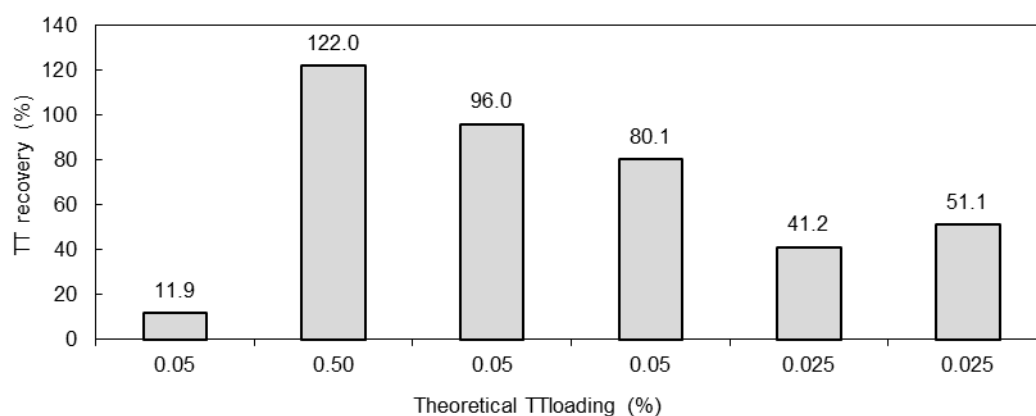
Figure 4.13. Percentage recovery of TT on six CaP-PCMC samples tested by XstalBio.

Six formulations were prepared and tested for TT recovery using SDS-PAGE by XstalBio. The percentage of TT recovery calculated from the intensity of the bars shown on the gel.

It is clear that there was either an issue with the ELISA for TT detection, or there was a problem with low recovery of TT on the CaP-PCMC. The conformation of TT was not investigated in a similar fashion to PRP, as any conformational change would not affect the immunogenicity of the overall formulation.

4.4.10.   BCA protein assay for quantification of TT on CaP-PCMC formulation

The SDS Page, although more specific, is limited by its detection limit. The soluble TT is still important to measure to ensure that delivery of the TT reaches the polysaccharide B cells along with the PRP. To test the release and total recovery of TT in the formulation, a more sensitive BCA protein assay kit was used. A standard curve of TT concentrations were tested, the relationship between the colour change and the TT concentration was found to be linear (Figure 4.14).
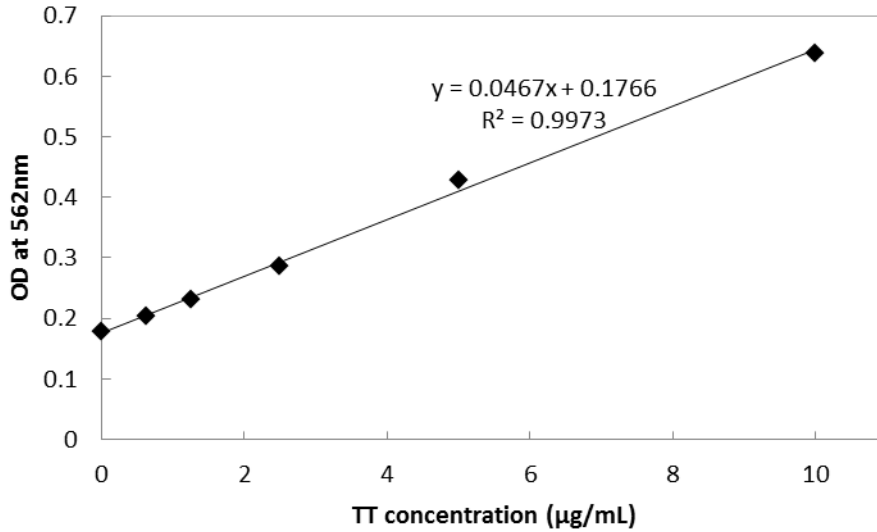
Figure 4.14. The OD generated by the BCA assay carried out on a standard curve of TT

The BCA protein assay was used to quantify TT in different TT concentrations to produce a standard curve. OD points are from duplicate samples.

The equation from the standard curve was used to calculate TT in the CaP-PCMC formulation as well as the TT released into soluble form when the CaP-PCMCs were suspended in PBS. The total recovery of TT was found to be 69.9% of the expected value, and of this, only 2% was found to be soluble.

The BCA assay was more successful in measuring the quantity of TT in the formulation as well as the release of the TT into soluble form when the crystals are suspended in PBS buffer. Although this assay is more sensitive, there is no confirmation of size of the measured protein as in the SDS-Page. The release of TT from the formulation is also very low, which indicates that the TT is stably adsorbed to the crystal surface and will be delivered to the final cellular target of the crystal.

4.4.11. Quantification of CpG by UV spectroscopy

CpG was added to the formulation to stimulate TLR9 which is present intra-cellularly in B Cells. Although CpG is not an essential antigen, the recovery of CpG on the formulation is important to stimulate the B cells. CpG is also costly and so it is of interest that as high a recovery is achieved in the formulation as possible.

The CpG standard was diluted and tested to form a standard curve, shown in Figure 4.15, and this was used to calculate the CpG recovery on two 0.5% CpG loaded CaP-PCMC formulations (Table 4.9). The recovery of CpG was high, with 96.2 and 98.8% of the theoretical CpG measured adsorbed to the crystal.
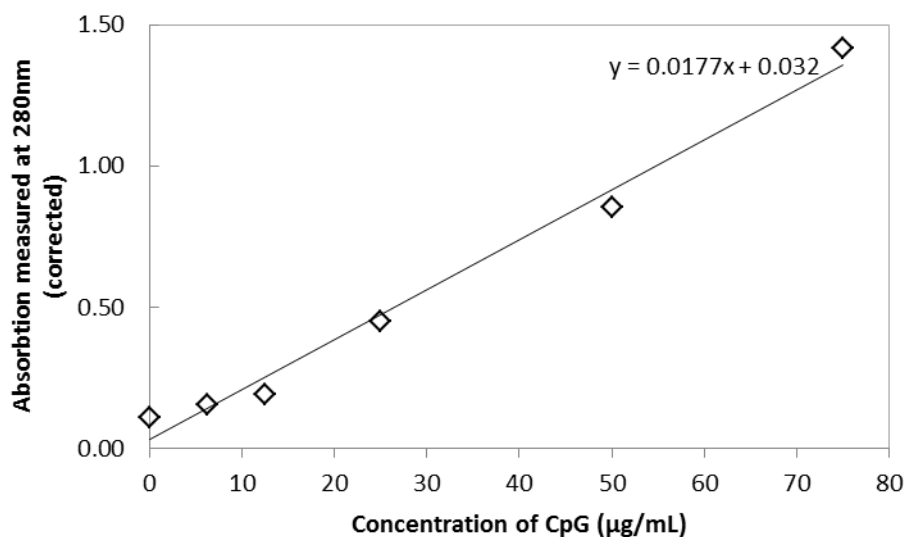
Figure 4.15. Absorbtion at 330nm of different concentrations of CpG corrected for control absorbtion at 330nm

CpG was quantified successfully by using UV spectroscopy (Table 4.9). Unfortunately, this assay required a large quantity of CpG to produce a reference standard for quantification of the sample CpG content. It is for this reason that this assay was not repeated with other formulations.

The high recoveries on formulations from both the 1-step and 2-step protocols indicate that the CpG loss during the production process is not an issue with CaP-PCMC production. Collectively, these results indicate that the preparation of the CaP-PCMCs is successful in adsorbing good amounts of antigen to the crystal upon preparation. This is crucial to ascertain before any investigation moves into the *in vivo* system.

Table 4.9. The CpG measurement and percentage recovery of two formulations as determined by UV spectroscopy

Formulation details	CpG (µg/mL)	Expected	Percentage recovery
0.5% CpG, 2-step	48.1	50.0	96.2
0.5% CpG, 1-step	49.4	50.0	98.8

4.5. Conclusions

These experiments indicate that the PRP, TT and CpG are all chemically present on the crystals. However, the measurement by ELISA of both TT and PRP indicate that there is an issue with changes in the antigenicity of the antigens on the crystals after formulation. This is not a major concern with TT, however for a protective immune response against the Hib capsule to be induced by the CaP-PCMC immunisations, the epitope of the PRP needs to be recognised by the immune response.

The analysis of the different PCMC formulations demonstrated that the inclusion of $CaCl_2$ is changing the PRP on the CaP-PCMCs in a way that it cannot bind the detection antibodies generated against the PRP-KLH vaccine to the same level as PRP in PCMCs formulated without $CaCl_2$. This may cause a decrease in the specificity of anti-PRP antibodies generated in animals immunised with the CaP-PCMC formulation, and this is investigated in the next chapter.

Chapter 5. Immunogenicity of CaP-PCMC and effect of various preparation parameters and adjuvants

5.1. Introduction

The pilot study with CaP-PCMCs demonstrated that the CaP-PCMC formulation was able to induce an anti-PRP immune response (52). The original PRP-TT-CpG CaP-PCMC formulation used had been prepared at XstalBio using a two-step process and the antigen, CpG, CaP loadings, and water content and molar ratio of $CaCl_2$ to NaH_2PO_4 used in the CaP-PCMC preparation are shown in Table 5.1 below.

Table 5.1. The Preparation parameters of the pilot formulation

	Antigen Loading		CpG loading	CaP loading	Water content	$CaCl_2$:NaH_2PO_4 ratio	Number of Process steps
	PRP	TT					
Percentage Loading (%,w/w)	0.5%	0.05%	0.5%	9.4%	5%	1:1	2

The immunogenicity of this formulation had been tested in the rat model at NIBSC, where animals were immunised subcutaneously with three doses of the CaP-PCMC formulation. The immune responses following the primary and two booster doses are shown in Figure 1.8. The CaP-PCMC formulation was shown to be promising as it can be seen that three out of the eight rats given three doses of CaP-PCMC suspension generated substantial levels of anti-PRP antibodies.
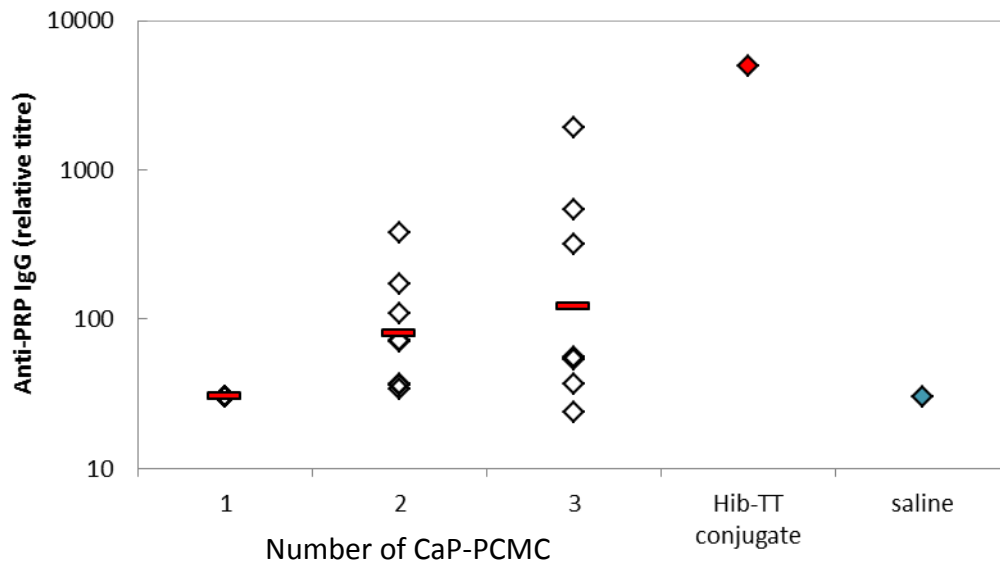
Figure 5.1. Anti-PRP IgG response induced after 1, 2 and 3 doses of CaP-PCMCs loaded with TT, PRP and CpG.

Rats were immunised with CaP-PCMCs with PRP equivalent dose of 6µg in CaP-PCMCs on days 0, and a PRP dose of 1.2µg on days 28 and 56. Test bleeds were taken on days 28, 42 and 56. Serum anti-PRP IgG response was measured by ELISA. Levels of anti-PRP responses were determined by parallel line analysis relative to an immune serum from animals immunised with a Hib-TT conjugate vaccine with an assigned titre of 5000. Responses from individual animals are shown as diamonds and the geometric mean titre of the group is shown as a red horizontal bar.

5.2. Aims and Objectives

The aim of the work discussed in this chapter:

1. To investigate the effect of different preparation parameters on the immunogenicity of the crystals

2. To investigate the effect of dose and dosage regimen on the immunogenicity of the crystals

3. To investigate the effect of soluble adjuvant on enhancing the immunogenicity of the CaP-PCMC formulation

4. To investigate the antigenicity of the PRP after the CaP-PCMC preparation process.

The formulation used in the pilot study between NIBSC and XstalBio was used as a starting point. Thus, PRP, TT and CpG loadings in the CaP-PCMCs were maintained at levels shown in Table 5.1. The preparation conditions shown in Chapter 2 to influence the CaP-PCMC morphology were varied. The CaP loading, number of process steps and water content used during the preparation procedure were therefore varied.

The influence of dosage regimen was tested to determine an optimum dose and dosage regimen using the doses used in the pilot study. As too high, as well as too low a dose can result in sub-optimal antibody responses (52), the original dosage regimen of 6μg followed by two doses of 1μg of PRP (a TT equivalent of 0.6μg, then two doses of 0.1μg) was increased as well as decreased.

The effect of the adjuvants on the immunogenicity of the crystals was studied using Monophosphoryl Lipid A (MPLA), Pam2Csk4, Polyinosinic:polycytidylic acid (Poly(I:C)) and QuilA. MPLA, Pam2Csk4 and Poly(I:C) are Toll-like-receptor (TLR) agonists and can activate immune cells which express the specific TLRs. This action would promote inflammatory responses and recruit higher numbers of immune cells (166, 167) which may act to increase the immune response to the PCMCs and possibly enhance their uptake or stimulation of the B cells.

MPLA is a derivative of Lipid A from Salmonella and was chosen as an adjuvant as it binds to Toll-like receptor 4 (TLR4). It is a detoxified version of Lipopolysaccharide (LPS) and acts as an efficient immune-stimulant (168, 169).

Pam2Csk4 which is a synthetic diacylated lipopeptide that is thought to signal through a TLR2 and TLR6 heterodimer, as it has both a lipid and peptide part (170). However, it

has also been reported to that Pam2Csk4 can also signal through a TLR6 independent manner (171). It has been shown that stimulation of TLR2 by Pam2Csk4 enhances the efficacy of vaccination against other bacterial infections (172). Furthermore, when combined with a TLR 9 agonist, present as CpG-ODN in the CaP-PCMC formulations, the Pam2Csk4 was found to promote resistance against bacterial pneumonia via the TLR2-Myd88-dependent pathway (170).

Poly(I:C) is a synthetic analogue of double stranded RNA, which imitates viral infection. Poly(I:C) binds TLR3 and therefore can be used an immune stimulant (173, 174). Once bound, TLR3 activates the transcription factor interferon regulatory factor 3 (IRF3), which leads to the production of type 1 Interferons (IFNs), such as IFN-β. Activation also triggers the production of inflammatory cytokines and chemokines such as TNF-α, IL-6 and CXCL10 (167).

QuilA is a derivative of a saponin from the Quillaja saponaria plant and is not a TLR agonist. QuilA has been used with CpG-ODN, which is present in the CaP-PCMC formulation, as an adjuvant to enhance antibody response against viral proteins (175).

5.3. Materials and Methods

5.3.1. Materials

The following materials were used in the study:

Plastic microtitre 96-well plates (Nunc Maxisorb, UK)
Hib Oligosaccharide conjugated to Human Serum Albumin (HbO-HA, NIBSC, Potters Bar, UK)
Wash Buffer (PBS containing 0.05% Tween 20 v/v)
Assay Diluent (PBS containing 1% Bovine Serum Albumin, 0.05% Tween 20 and 3.8% Ethylenediaminetetraaectic acid, w/v)
Detection Antibody (HRP-conjugated goat anti-rat IgG, 3030-05 Southern Biotechnology, UK)
Sigmafast OPD substrate (Sigma, UK, prepared 30 min before use)
TT In-house standard (NIBSC code 02/126, Potters Bar, UK)
First International PRP standard (NIBSC code 02/208, Potters Bar, UK)

5.3.2. Animals and immunisation

Groups of 5-10 female, Sprague Dawley rats (aged 6-8 weeks) were immunised subcutaneously with 200µl of a CaP-PCMC formulation (equivalent of 6µg of PRP for first, and 1µg of PRP for second and third doses) per animal on days 0, 21 and 42 unless otherwise specified. The equivalent TT doses were 0.6µg for the first dose, and 0.1µg for the second and third dose. PRP & TT doses were varied by adjusting te mass of CaP-PCMCs used to immunise the rats. The positive control group received 1/25th of the Single Human Dose (SHD) of a Hib-TT conjugate vaccine (0.4µg of PRP and 0.8µg of TT per dose in 200µL volume) subcutaneously on the same days as the CaP-PCMCs, as this was previously determined to be the optimum dose for immunogenicity in the rat model (52).

Animals were bled from the tail vein on days -1, 20 and 41 to determine baseline, primary and secondary IgG responses. The experiments were terminated

approximately 2 weeks after the 3rd immunisation. When blood was collected, it was kept overnight at 4°C before centrifugation at 10,000rpm for 10 min. Serum was collected and stored at -20°C in single use aliquots until tested for antibody responses by ELISA.

5.3.3. Quantification of anti-PRP antibody response by ELISA

Plastic microtitre plates were coated with 100μL/well of 1μg/mL of Hib capsular oligosaccharide conjugated to HbO-HA in PBS and incubated for 2 h at 37°C, then overnight at 4°C. The following day, plates were washed three times before being blocked using assay diluent for 45 min at 37°C. Rat sera were diluted 1/25- 1/50 in assay diluent and added at 100μl/well volumes to duplicate wells and incubated for 90 min at room temperature.

After washing the plates, 100μl of 1/1000 dilution of detection antibody in assay diluent was added to each well and the plate was incubated for 90 min at room temperature. After washing, 100μL/well of the Sigmafast OPD substrate, prepared according to manufacturer's instructions, was added and the plates were incubated for 15 min at room temperature in the dark. The colour change reaction was stopped with 50μL/well of 3M HCl and optical density (OD) was read at 492 nm using an automated reader (Labsystems Multiscan, MS, UK) and Genesis software. Data from the ELISA was presented as mean OD values for each serum sample tested.

5.3.4. Quantification of anti-TT antibody response by ELISA

 Plates were coated with a 100μl/well of 0.5Lf/mL TT and incubated overnight at 4°C. The following day, the plates were washed before being blocked for 45 min at 37° with 100μL/well of assay diluent. After washing, rat sera were diluted 1/1000 in assay

diluent and added, in 200µl volumes to duplicate wells in the first column on the plate, then 10 two-fold dilutions were carried out across the plate except for the final column which contained Assay Diluent and served as a blank. The plates were then incubated for 90 min at room temperature.

After washing, 100µl of a 1/1000 dilution of detection antibody in assay diluent was added per well and the plate was incubated for 90 min at room temperature. After washing, 100µL/well of the Sigmafast OPD substrate was added and plate incubated for 15 min at room temperature in the dark. The colour change reaction was stopped with 50µL/well of 3M HCl and optical density measured at 492 nm using an automated reader as in Chapter 4.

The titre of an anti-TT IgG response in the immune sera samples was calculated relative to a reference serum with an assigned titre of 10,000 using in-house parallel line bioassay programme (Combistats) which relates the logarithm of the assay responses (OD) to the logarithm of the dose, using at least 3 points on the linear portion of the dilution curve.

5.3.5. Preparations of CaP-PCMC formulations used in the immunogenicity studies

In this Chapter, three immunogenicity studies were conducted. The details of CaP-PCMC preparations are shown below.

5.3.5.1. *Investigation into the influence of CaP-PCMC preparation parameters*

Seven CaP-PCMC formulations were prepared under aseptic conditions with variations in the preparation parameters. The CaP loading, the water content and the number of steps in which the solutes are added (process steps) were varied, as shown in Table 5.2.

Table 5.2. Details of CaP-PCMC formulations prepared at XstalBio with varying CaP load, Water content and process step.

PCMC Sample ID	TT load (%w/w)	PRP load (%w/w)	CpG load (%w/w)	CaP load (%w/w)	Water Content (%v/v)	Process	Mixing Time (min)	Solid recovery (%)
1	0.05	0.5	0.5	10	5	1-step	16	80
2					7			76
3					10			83
4					5	2-step		88
5				15				81
6				20				74
7				10	3			81

5.3.5.2. *Investigation into the influence of dose and dosage regimen*

Based on results of the immunogenicity of CaP-PCMC prepared with different

preparation parameters, two formulations were chosen, details are shown in Table

5.3. The two formulations were prepared, and the powders mixed in a 1:1 ration on a

rotator for 3 h.

Table 5.3. Two formulations prepared and combined for immunogenicity studies on dose and adjuvant

Sample ID	TT (%w/w)	PRP (%w/w)	CpG (%w/w)	CaP load (%w/w)	Water Content (%v/v)	Process	Mixing Time (min)
CaP-PCMC A	0.05	0.5	0.5	10	7	1-step	16
CaP-PCMC B	0.05	0.5	0.5	10	10	1-step	16

TheCaP-PCMC concentration in the suspension were adjusted to give different dosages

and dosage regimens. The three chosen regimens are shown in Table 5.1.

Table 5.4. Dose and regimen of PRP and TT given in the three different dosage patterns tested

Dosage Regimen	Theoretical PRP content (µg)	Theoretical TT content (µg)
1	1, 1, 1	0.1, 0.1, 0.1
2	6, 1, 1	0.6, 0.1, 0.1
3	6, 6, 6	0.6, 0.6, 0.6

5.3.5.3. Investigation into the influence of soluble adjuvant

To investigate the effect of soluble adjuvant administered at the same time as the CaP-

PCMCs, the suspension for injection was prepared as above, from formulations shown

in Table 5.3. The soluble adjuvant was then added to the suspension prior to injection.

The doses of each adjuvant were chosen based on those used in previous literature.

Details of the adjuvant doses are shown in Table 5.5. The doses were kept the same for

each specific adjuvant whether used singly or in combination.

Table 5.5. Dosage details for each of the four adjuvants administered in soluble form simultaneously with the CaP-PCMC suspension

Adjuvant	Dose/rat (µg)	Agonist	References
MPLA	10	TLR 4	(176)
PAM2CSK4	5	TLRs 2, 6	(172)
Poly(I:C)	25	TLR 3	(174)
QuilA	20	NLRP3	(177)

Soluble PCMCs were prepared (without the addition of $CaCl_2$ and NaH_2PO_4) and mixed with soluble PRP, TT and CpG and used as a negative control for the components of the CaP-PCMCs. Hib-TT conjugate was used as a positive control.

5.3.6. Investigation into the antigenicity of PRP

To investigate if any changes in PRP are caused by conditions it encounters during the CaP-PCMC preparation process, the antigenicity was assessed through its ability to bind anti-PRP antibodies which were generated against the Hib-TT conjugate.

The influence of Isopropanal (IPA) and $CaCl_2$ were investigated as experiments discussed in Chapter 3 indicated that the combination of these during the preparation process as responsible for a change in the PRP which limits its detection by ELISA. The effect of $CaCl_2$ and IPA individually and in combination were investigated by incubation of $CaCl_2$ in assay diluent, $CaCl_2$ in IPA and IPA on antigen coated plates. The assay was carried out as for the anti-PRP ELISA, with the addition of an extra step of plate-treatment before adding immune serum.

HbO-HA was necessary to use as the PRP is a carbohydrate, and therefore lacks the ability to adhere to the plate surface without conjugation to a protein, in this case,

Human Serum Albumin. Although it should be noted that there may be differences

between the Hib Oligosaccharide in HBO-HA that

Duplicate rows on the HbO-HA coated plates were exposed to conditions for 16 min,

similar to the preparation process. The conditions were as follows:

- IPA (5% water content)
- $CaCl_2$ dissolved in H_2O (0.3mg/mL)
- $CaCl_2$ dissolved in IPA(at a water content of 5%, $CaCl_2$ at 0.3mg/mL)

5.3.7. Statistical Analysis

To analyse the influence of treatment group on the immune response generated in the

animal (either anti-PRP response or anti-TT titre), One way Analysis of Variance

(ANOVA) was conducted on the data from the ELISA. To analyse the influence of

number of immunisations among groups, a Repeated Measures ANOVA was

conducted.

SPSS 21 was used for all statistical calculations. The nature of test used in stated in the

text.

5.4. Results and Discussion

5.4.1. Effect of formulation parameters on the immunogenicity of CaP-PCMCs

To study the effect of the preparation parameters on the CaP-PCMCs' immunogenicity, seven different formulations were prepared (Table 5.2) under aseptic conditions, based on the PRP-TT-CpG-CaP-PCMC formulation in the pilot study, but with variations in water content, CaP loading, process step (ie. One or two step process) and CaP loading, as these parameters were seen to have a visible effect on the crystal morphology. A Hib-TT conjugate vaccine was used as a positive control.

The solid recoveries for the different formulations were high (between 74% and 88%), with a mean of 80% which was similar to the recoveries of the formulations prepared under non-aseptic conditions shown in Chapters 2 and 3.

4.3.2.1 The anti-PRP antibody response

Figure 5.2 shows the anti-PRP responses before and after three doses of each formulation tested. An animal was considered a responder if the anti-PRP response was greater than the mean IgG response in pre-immune sera + 3 standard deviations.

It was found that there were no significant differences between the groups prior to immunisation, as expected (ANOVA, $p < 0.05$). The geometric mean pre-immunisation responses of the groups were low, at 0.7-0.8 OD, which confirms that the rats were naïve and had not been exposed to any PRP or polysaccharide that could induce cross-reactive antibodies.

The IgG responses post-immunisation were found to be significantly different to the responses prior to immunisation (repeated measures ANOVA, $p < 0.05$), however the post-immunisation OD means vary between 0.9 and 0.22 in the CaP-PCMC immunised groups which is low, and therefore may indicate that there is a very low response generated against the CaP-PCMCs.

However, there was no significant difference between the groups after immunisation (ANOVA, $p < 0.05$). Although it can be noted that the highest means, both over 0.2 OD, were from the groups administered with Formulations 2 and 3, prepared with 7 and 10% water content, respectively. The highest anti-PRP response (OD of 1.4) was induced in one rat in the group immunised with PCMC Formulation 2. Two other weakly responding animals were from the group immunised with Formulation 3 (ODs of 0.8 and 0.3). In the responses seen in the pilot study, the formulation parameters were altered. However, as can be seen in Figure 5.2, this did not enhance the immunogenicity of the CaP-PCMCs. It seems that the CaP-PCMCs are not acting as a virtual conjugate of PRP with TT.

As seen in the SEM images in Chapter 2, the increase in the water content from 5% to 7% resulted in larger flatter crystal shape. The CaP-PCMCs prepared with a 7 or 10% water content were not imaged. It appears that the increase in the water content may act to increase the immunogenicity of the formulation and this may be due to a slightly more immunogenic shape which allows better targeting of the follicular B cells.

4.3.2.1 Kinetics of the anti-PRP response in three responding animals

In order to understand if the high responders had generated the anti-PRP response in a T cell dependent manner, the sera from the three individual rats (circled in orange in

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Figure 5.2) with the highest anti-PRP responses were further analysed to determine the kinetics of the anti-PRP response after sequential dose of antigen (Figure 5.3). It can be seen that the responding animal immunised with Formulation 2 mounted a clear booster response after the second and third immunisations, indicating the generation a T cell dependent response and the priming of B cells for a memory response.

In contrast, the two animals immunised with Formulation 3 show responses that are less indicative of a memory response. One animal shows an increase in the OD from the first to the second immunisation of 0.13 to 0.34, while the other shows an increase after the second immunisation from 0.08 to 0.38 OD. In the first case, the lower dose of CaP-PCMCs may not have reached the follicular B cells, and in the second case, it may be that the first dose of CaP-PCMCs did not reach the cells. If none of the first dose of CaP-PCMCs were able to reach the follicular B cells to initiate the differentiation into memory B cell, the next dose will not induce a memory, boosted, response, but instead act to prime the B cells.
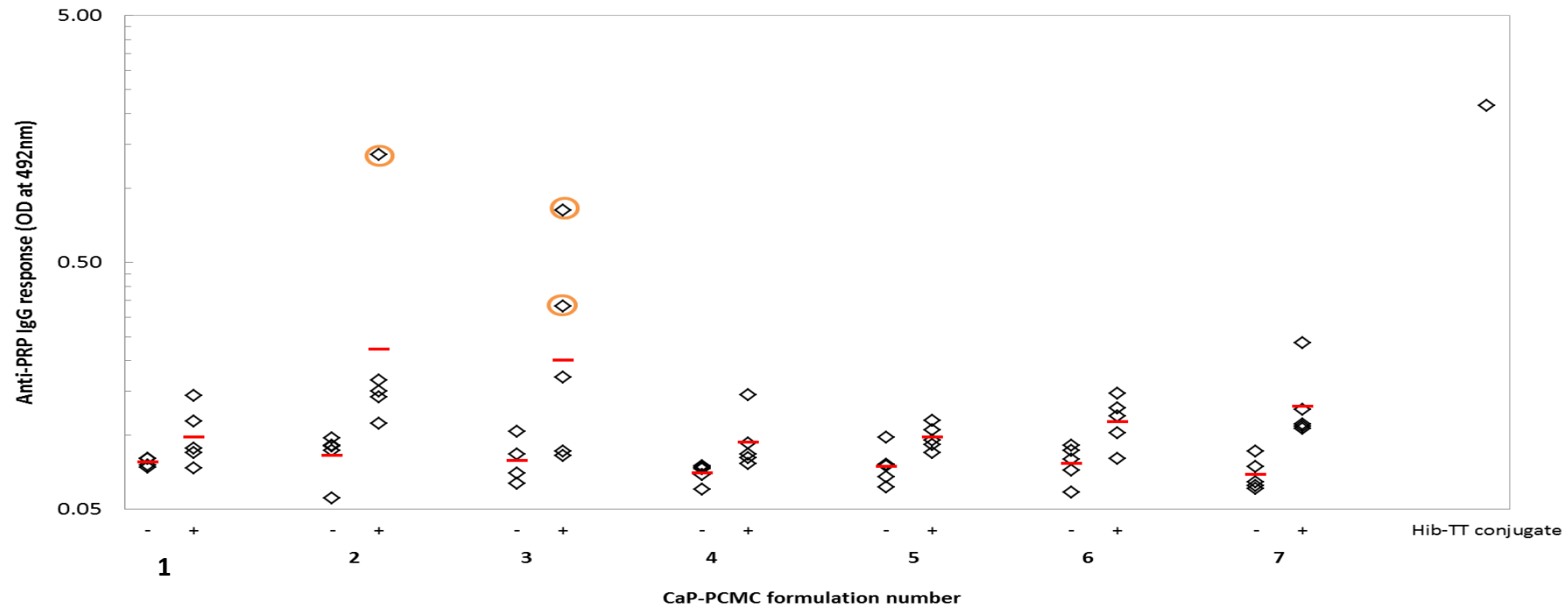
Figure 5.2. Anti-PRP IgG response after 3 doses of different formulations of CaP-PCMCs

Animals were immunised with 3 doses of various CaP-PCMC formulations containing 6μg of PRP/rat for the initial dose (1.2mg of CaP-PCMC) and 1μg/rat for the booster doses (0.2mg of CaP-PCMC). Animals in the control group were immunised with a Hib-TT conjugate containing the equivalent of 0.4μg PRP/dose. Sera obtained before (-) or after 3 immunisations (+) were tested at a dilution of 1/25. The anti-PRP response is presented as OD of individual animal sera (diamonds) and geometric mean of the group (red horizontal bars; n=5). A two-way ANOVA was carried out and there was no significant difference between the CaP-PCMC groups or between immunisations. The orange circles show the IgG titres of the highest responders which were selected for further analysis described in the following section.
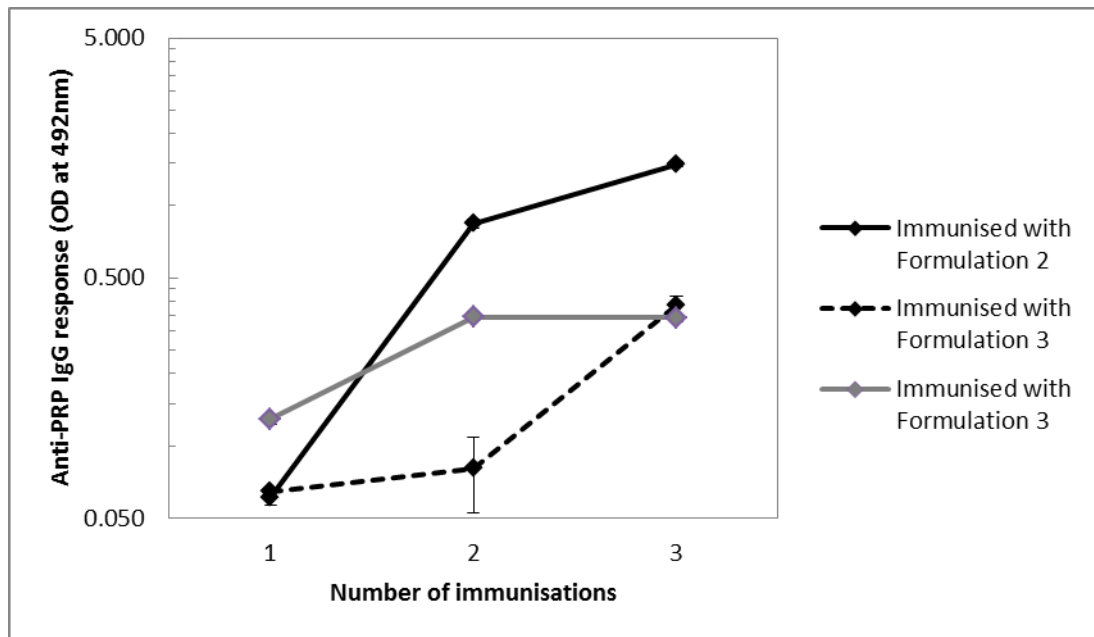
Figure 5.3. Kinetics of anti-PRP IgG responses in responding animals.

Animals were immunised with 3 doses of two different CaP-PCMC formulations containing the equivalent of 6μg of PRP/rat for the initial dose and 1μg/rat for the subsequent two doses. Sera were tested after each immunisation at a dilution of 1/25.Mean OD readings from triplicate wells are shown (error bars indicate 1 standard deviation)

4.3.2.2 The anti-TT antibody response

In contrast to the low anti-PRP responses, substantial primary and memory antibody responses were generated against TT, as shown in Figure 5.4 and Figure 5.5. After each successive immunisation the anti-TT titre increased significantly (repeated measures ANOVA, $p < 0.05$). However, the primary, secondary and tertiary IgG responses did not differ significantly among groups (ANOVA, $p < 0.05$)

In addition, irrespective of the PCMC formulation used, all animals immunised with CaP-PCMCs had anti-TT responses that were similar or higher than the response

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induced by the Hib-TT conjugate, the TT dose in the Hib-TT conjugate (4µg) was higher than in the CaP-PCMC (0.6µg then twice at 0.1µg) and this was most probably due to the inclusion of the adjuvant CpG in the CaP-PCMC formulation.

Interestingly, the three animals which mounted good anti-PRP response, shown in Figure 5.3, also had the highest anti-TT responses compared to other animals in their group (data not shown). This not only indicates that the antibody response to TT does not interfere with the induction of an anti-PRP response, but that there may be a correlation between the responses.

Influence of number of steps in preparation process on anti-TT response

The effect of number of process steps on the immunogenicity of the CaP-PCMCs was investigated, as there is likely to be differences in the nature of the CaP formation on the crystal. The CaP layer may or may not cover the antigens and the core of the microcrystal, and therefore might affect the antigen exposure and recognition by B cells. Data presented in Figure 5.4 showed that the primary response induced by immunisation with Formulation 1, prepared by 1 –step process was much higher than the response induced by the equivalent formulation prepared using a 2-step process (Formulation 4). However, the difference disappeared after subsequent immunisations. The difference may be due to variations in the crystal morphology that affected the ability of the crystal to prime the initial response. Subsequent doses may require lower levels of TT than the priming dose, due to increased pools of anti-TT T cells, which may explain why the final doses even out.

Influence of CaP loading on anti-TT response

The effect of CaP loading on the anti-TT response was investigated in groups immunised with Formulations 4, 5 and 6, where the CaP-PCMCs used were loaded with either 10%, 15% or 20% CaP respectively (Table 5.2). The animals immunised with Formulations 5 and 6 show very similar levels of anti-TT IgG titre at all three doses ($p>0.05$; ANOVA, post hoc Tukey). The animals administered Formulation 4 (the lowest CaP-loading of the three groups) showed a significantly lower anti-TT titre after the first dose but not the second or third doses compared to groups given 15% and 20% CaP-loaded crystals (ANOVA, $p<0.05$) . It is possible that the increased CaP loading in Formulation 5 and 6 would have an effect on the antigen release rate and stability of the microcrystals, and therefore have a possible effect on the immunogenicity of these particles. However this may only have an effect on the priming dose, or at the higher concentration of CaP-PCMCs administered.

Influence of water content on anti-TT response

The effect of water content on the anti-TT response generated by the CaP-PCMCs was investigated with formulations prepared with a 5%, 7% and 10%

The primary anti-TT response in the group immunised with the formulation prepared with a 10% water content (Formulation 3) had similar titres after the first dose as the titre from animals administered CaP-PCMCs prepared with a 5% and 7% water content (Formulations 1 and 2). This result may indicate that the water content does not play a part in altering the immunogenicity of the CaP-PCMCs, at least in terms of the anti-TT responses generated.
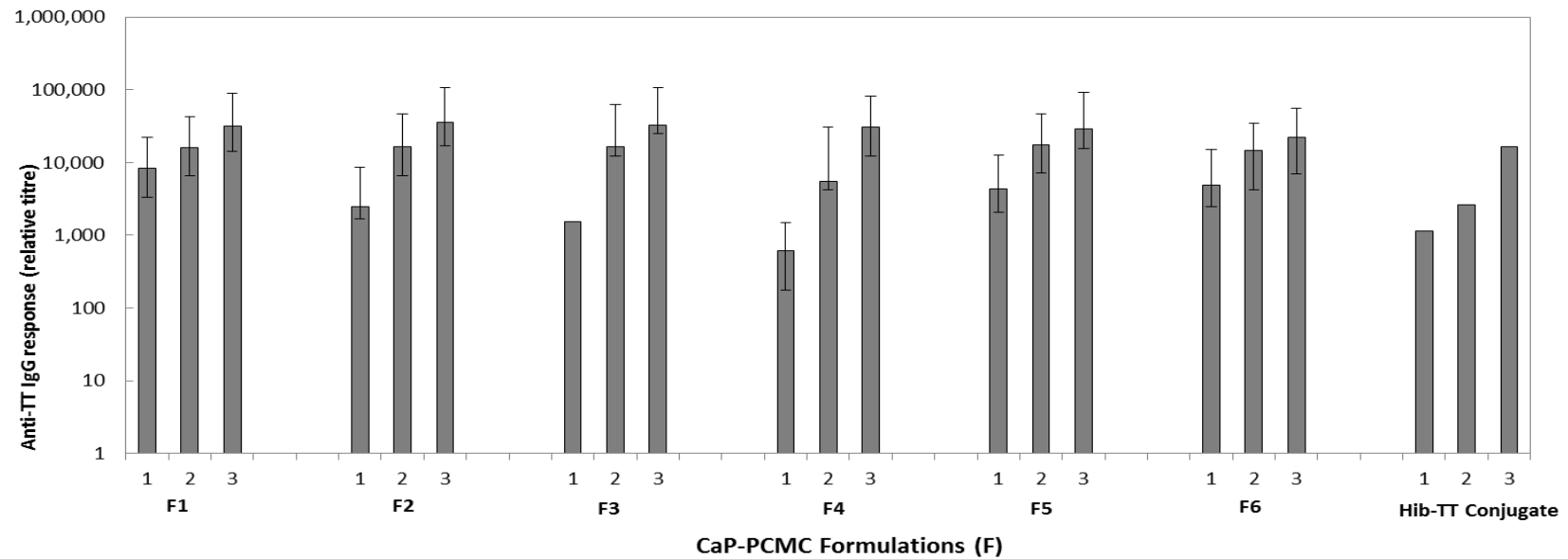
Figure 5.4 Anti-TT IgG antibody responses after the first, second and third (1, 2 ,3 on x axis) doses of different CaP-PCMCs formulations.

Animals were immunised with 3 doses of different CaP-PCMC formulations containing 0.6µg of TT/rat (1.2mg of CaP-PCMCs) for the initial dose and 0.1µg/rat (0.2mg of CaP-PCMCs) for the booster doses. Animals in the control group were immunised with a Hib-TT conjugate containing the equivalent of 4µg TT/dose. Sera were tested after each immunisations at a dilution of 1/1000. The anti-TT response is presented as geometric mean titre (n=5) and error bars indicate the 95% confidence interval.
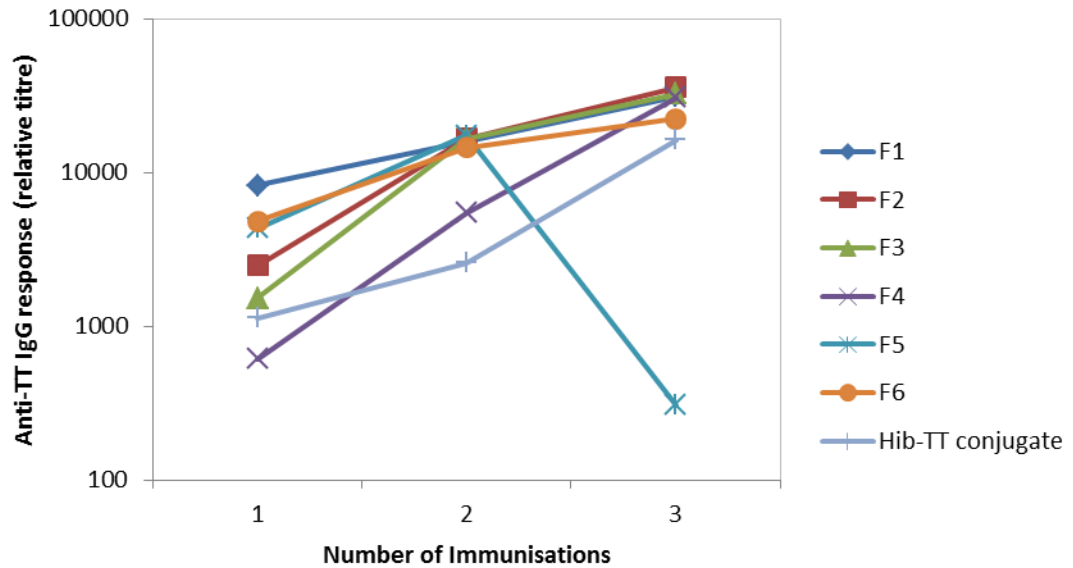
Figure 5.5 Anti-TT IgG antibody responses after the first, second and third (1, 2 ,3 on x axis) doses of different CaP-PCMCs formulations. The data is the same as in Figure 5.4 but presented to visualise the differences among the different groups

Animals were immunised with 3 doses of different CaP-PCMC formulations containing 0.6µg of TT/rat (1.2mg of CaP-PCMCs) for the initial dose and 0.1µg/rat (0.2mg of CaP-PCMCs) for the booster doses. Animals in the control group were immunised with a Hib-TT conjugate containing the equivalent of 4µg TT/dose. Sera were tested after each immunisation at a dilution of 1/1000. The anti-TT response is presented as geometric mean titre (n=5).

5.4.2. The influence of antigen dose and dosage regimen on the immunogenicity of the PCMCs

As the only responding animals were from groups immunised with a formulation prepared with a water content of 7% or 10%, it was decided that two new batches of CaP-PCMCs would be prepared with the parameters shown in Table 5.2, the first with 7% and the second with 10% water content (Table 5.3). The two batches were combined at a mass ratio of 1:1 for further immunogenicity studies in an attempt to increase the immunogenicity of the CaP-PCMCs.

5.4.2.1. The anti-PRP antibody response

To investigate the effect of antigen dose and dosage regimen on the antibody responses, animals were immunised with the doses and regimens seen in Table 5.4. Anti-PRP and anti-TT responses were assessed after the third immunisation and the data is presented in Figure 5.6. It was found that none of the animals in any of the groups had any significant anti-PRP IgG responses that were above the background level (pre-immunisation). Changing the PRP dose and dosage regimen did not increase anti-PRP titre. The low immune responses seen in Figure 5.6 may be due to a too low or too high (ie. Non-optimum) antigen dose, or due to the fact that the CaP-PCMCs are inherently poorly immunogenic.
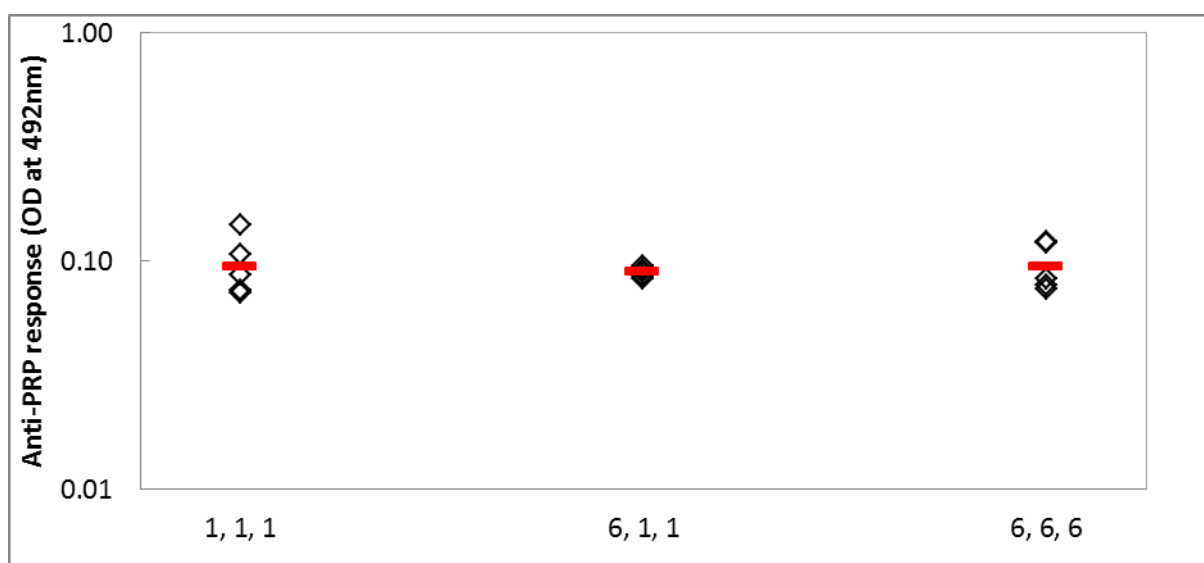
Figure 5.6. Anti-PRP response in terminal sera of groups given different dosage regimens of PRP

Rats were administered with CaP-PCMC dosage regimens shown in **Table 5.2** Diamonds represent the mean of readings from duplicate wells of individual serum samples at 1/25 dilution. Geometric mean of the group shown as red horizontal bars (n=5). Two way ANOVA was carried out and it was found that there was no significant difference between groups.

5.4.2.2. The anti-TT antibody response

The anti-TT response (as shown in Figure 5.7) indicate that an influence of antigen dose was found, with animals receiving the lowest dose (0.1µg TT for all three immunisations) showing the lowest titres which were statistically significant for the primary response (ANOVA, $p < 0.05$).

After the second immunisation a greater than two-fold effect of the dosage difference is seen when boosted with the lower dose (ANOVA, $p < 0.05$). After the third immunisation the group boosted with the 0.6µg of TT dose had a five-times greater response than the group boosted with the 0.1µg of TT, which was significantly different (ANOVA, $p < 0.05$). The group which was given the highest dose of 0.6µg of TT at each immunisation showed higher anti-TT responses to the group given 0.6µg of TT followed by 0.1µg of TT for the third immunisations (ANOVA, $p < 0.05$). These results indicate that the dosage can make a large difference in the magnitude of the anti-TT response induced, especially for the priming dose.

These results correspond to previous findings by Katere et al (178) who found that the increase in antigen load of TT on polymer particles was found to have positive influence on generation of antibody titres. This study showed increasing anti-TT titres after a single immunisation with micro-particles loaded with increasing TT concentrations. However it is not known whether increasing the antigen loading acts differently to adjusting the TT dose through increase particle dose.
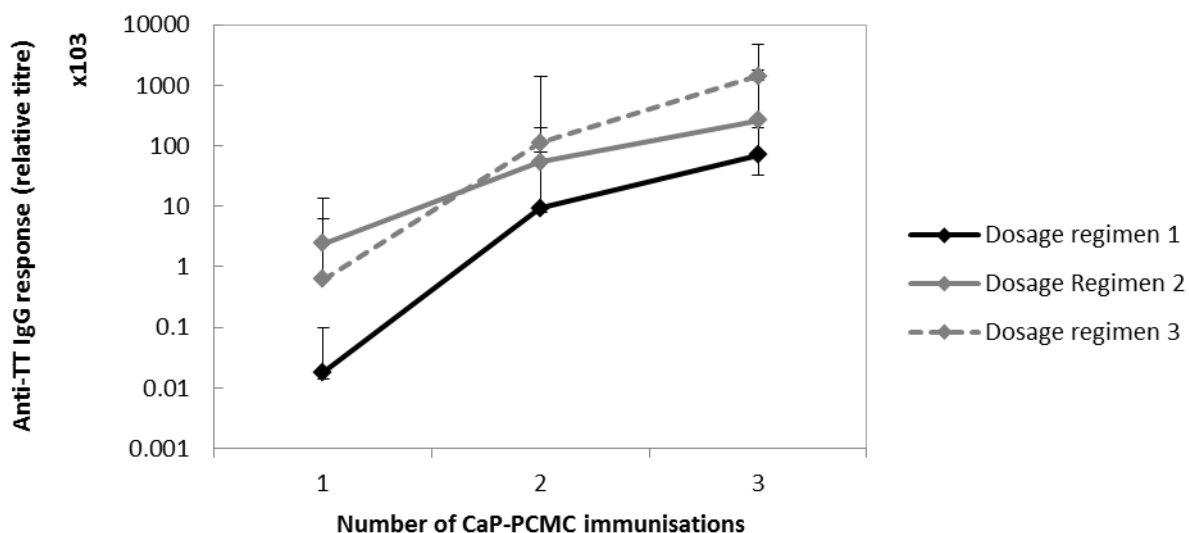
Figure 5.7. Anti-TT responses in groups given different dosages.

Animals were immunised with 3 doses of CaP-PCMC suspension with the dosage regimens shown in**Table 5.4**. Sera were tested after each immunisation at a dilution of 1/1000. The anti-TT response is presented as a geometric mean titre of th egroup (n=5), bars indicate 1 standard deviation.

5.4.3. The influence of soluble adjuvants on the antibody response to PRP and TT

To study the effect of soluble adjuvant on the CaP-PCMCs' immunogenicity, the same combination of formulations were used as in the previous section except for the addition of soluble adjuvants singly or in combination as shown Table 5.5.

5.4.3.1. The anti-PRP response

The anti-PRP IgG responses are shown in Figure 5.8. The figure shows the CaP-PCMC suspensions remain poorly immunogenic even in the presence of different well-known adjuvant, whether these were used singly or in combination. In all groups immunised with the CaP-PCMCs, the geometric mean responses were low, ranging from 0.07-0.17, compared to a mean of 0.8 in the positive control, and 0.09 in the negative control group immunised with soluble PCMCs and antigens, where PRP and TT are co-

immobilised. Similar responses for the CaP-PCMCs and the negative control further confirms that the CaP-PCMCs are not acting as a virtual conjugate and the PRP is being presented to the appropriate immune cells (B cells) as an antigen.

There were few animals which had a substantial anti-PRP IgG responses (circled on Figure 5.8). These rats were from the groups which had both MPLA and Pam2Csk4, QuilA and MPLA, Poly(I:C) and Pam2Csk4 as well as Poly(I:C) and MPLA combinations. As seen in Figure 5.2, a few animals respond and show that in isolated instances, the CaP-PCMCs act as a virtual PRP-TT conjugate.

CpG was present in all the CaP-PCMCs used in the immunisation, however it is evident that there is no adjuvant effect of this. The responses were low, and similar to the group dosed with soluble PCMCs and antigen without CpG. To properly control for the adjuvant effect of CpG, however, a group immunised with CpG-less CaP-PCMCs should be used.

Sera that was collected after the first and second immunisations of animals which showed high anti-PRP responses (circled in orange in Figure 5.8) were further analysed. The kinetics are shown in Figure 5.9. The highest response was seen in an animal from a group which was administered with a combination of MPLA, Pam2Csk4 with the CaP-PCMC suspension; this immune did show a very clear anti-PRP booster response after the booster doses. The OD increased after each dose, from 0.4, to 1.8, to 3, which indicates the successful generation of PRP specific memory B cells. This may suggest that the stimulation of the TLR 4 (by MPLA), TLR2 and TLR 6 (both by Pam2Csk4) and TLR9 (from the CpG adsorbed to the crystal) might be useful to induce the production

of a T cell-dependent anti-PRP response. However, the results are not conclusive as only one animal from the group of five responded.

Another responding sera was from an animal immunised with the CaP-PCMC suspensions with Poly(I:C) and MPLA, the OD increased 8-fold from 0.17 after the first immunisation to 1.4 after the third. The next highest responder was from an animal immunised with QuilA and MPLA. The increase was from 0.1 after the first dose to 0.4 after the third. In these two animals however, these increases may not be the formation of a memory response, as after the second dose the OD values are close to the background level. If it is the case that there is a very low anti-PRP response after the second dose, the addition of the TLR agonists could help to boost a low, but existing memory B cell pool.

The anti-PRP responses shown in Figure 5.2 demonstrate that there are only a few responding animals even in the group administered the same formulation as that in the pilot study (Formulation 1, Table 5.2).
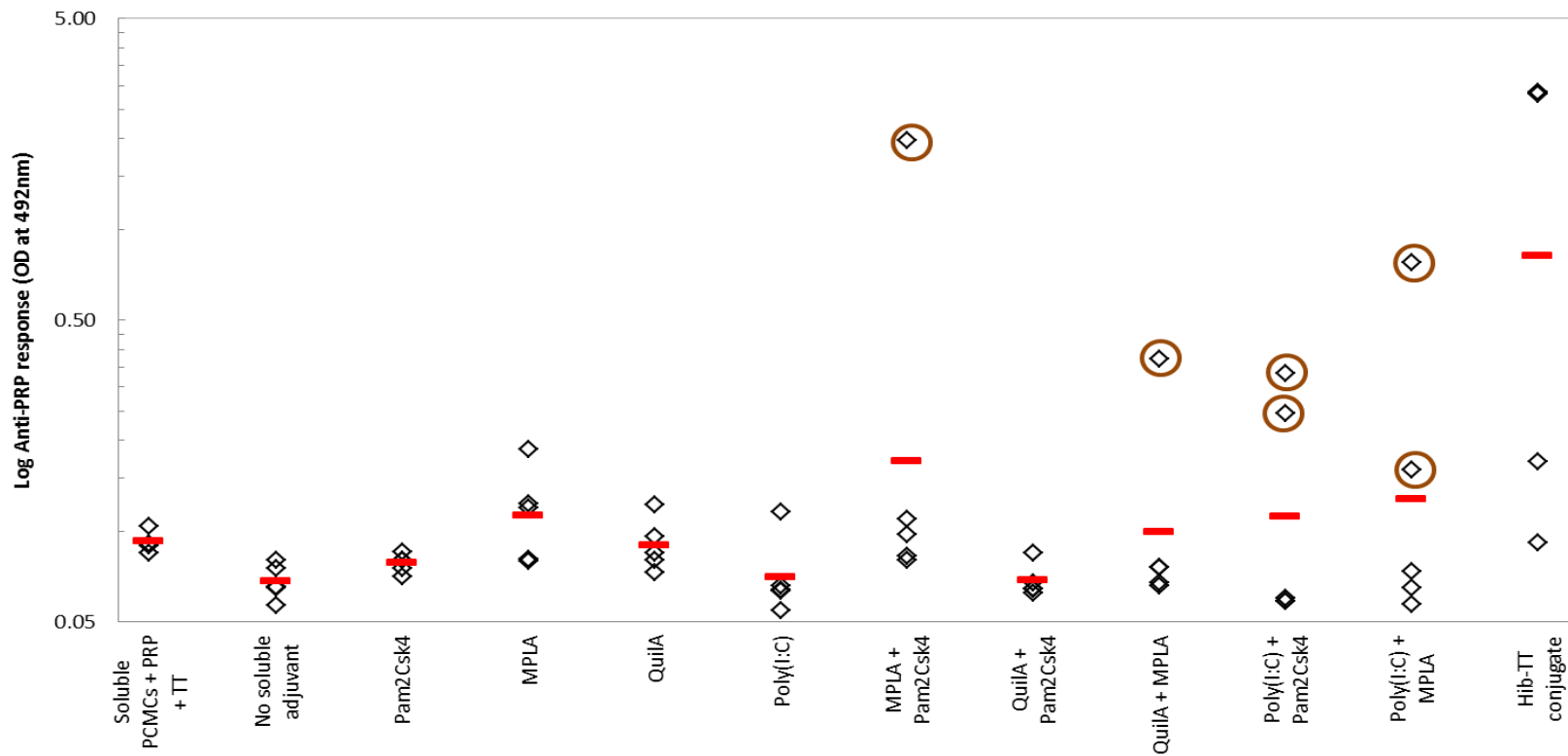
Figure 5.8. Anti-PRP responses after immunisation with CaP-PCMC formulations in combination with different soluble adjuvants.

Animals were immunised with 3 doses of various CaP-PCMC formulations with the equivalent PRP dose of 6µg of PRP/rat for the initial dose and 1µg/rat for the booster doses. Animals in the control group were immunised with a Hib-TT conjugate containing 0.4µg PRP/dose, a negative control group were immunised with soluble PCMCs with 6µg of PRP, followed by 1µg in the following two doses. Sera were tested after 3 immunisations at a dilution of 1/25. The anti-PRP response is presented as OD of individual animal sera and geometric mean response of the group (red horizontal bars; n=5). Data was tested by two-way ANOVA and it was found that there was no significant difference between each group. The orange circles show the IgG titres of the highest responders which were selected for further analysis described in the following section.
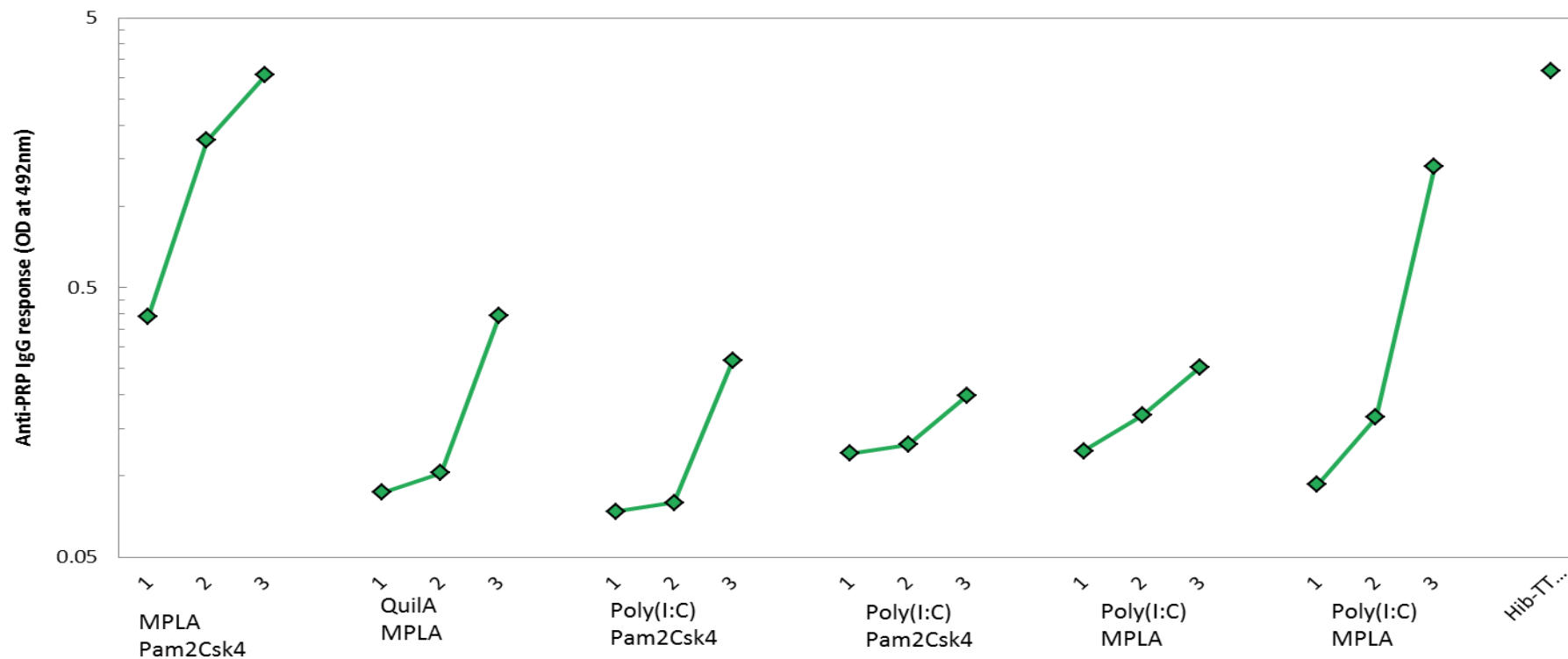
Figure 5.9. The kinetics of the anti-PRP IgG response in the high responding animals administered with a combination of CaP-PCMC suspension and soluble adjuvants

Animals were immunised with 3 doses of various CaP-PCMC formulations containing 6µg of PRP/rat for the initial dose and 1µg/rat for the booster doses. Animals in the control group were immunised with a Hib-TT conjugate containing 0.4µg PRP/dose. Sera were tested after each of 3 immuniseations at a dilution of 1/25. The anti-PRP response is presented as mean OD of two readings form the ELISA plate.

5.4.3.2. The anti-TT response

The anti-TT titre in the sera of animals immunised with CaP-PCMC suspension in combination with the soluble adjuvants is shown in Figure 5.10.

The titres of anti-TT antibodies were high in every group tested, with titres ranging from 21500 to 362000. It was found that there was a significant effect of subsequent immunisations on the anti-TT response which showed significant increases in titre after each immunisation (repeated measures ANOVA, $p<0.05$), indicative of the generation of a memory response. However, there were no significant differences between the groups given different adjuvants or groups given no adjuvant (ANOVA, $p<0.05$).

It is interesting that the same group which had the highest anti-PRP responder in the previous section was in the group immunised with Pam2Csk4. This would confirm that the generation of the anti-TT response may positively correlate with the generation of the anti-PRP response.

The anti-TT responses were higher in the animals immunised with the CaP-PCMCs compared to the two groups which were administered the soluble PCMCs and the Hib-TT conjugate (neither containing the TLR9 agonist, CpG). This result shows that the CpG may increase the anti-TT response generated, even though it does not increase the anti-PRP response, although the results are not significant.

A big difference between the CpG-less control groups is the low titre after the priming dose. The mean titres from the groups given the Hib-TT conjugate and the soluble PCMCs with TT had mean titres of 87 and 8 after the first dose. However this then increased 90-fold and 642 fold with the booster dose, indicating that even with a low

titre generated by animals in the control group, a memory response was still evident, demonstrating that CpG is not crucial for the development of the memory response.

There has been a lot of previous studies that use the encapsulation of TT in particles to reduce the number of doses needed (179-181), and it was found that the sustained release of TT from particles was able to boost the antibody response. In this case, a slower release of TT from the CaP-PCMCs, which may be determined by the quality of the CaP-layer, may have acted to increase the anti-TT titre compared to the soluble PCMCs or the Hib-TT conjugate.
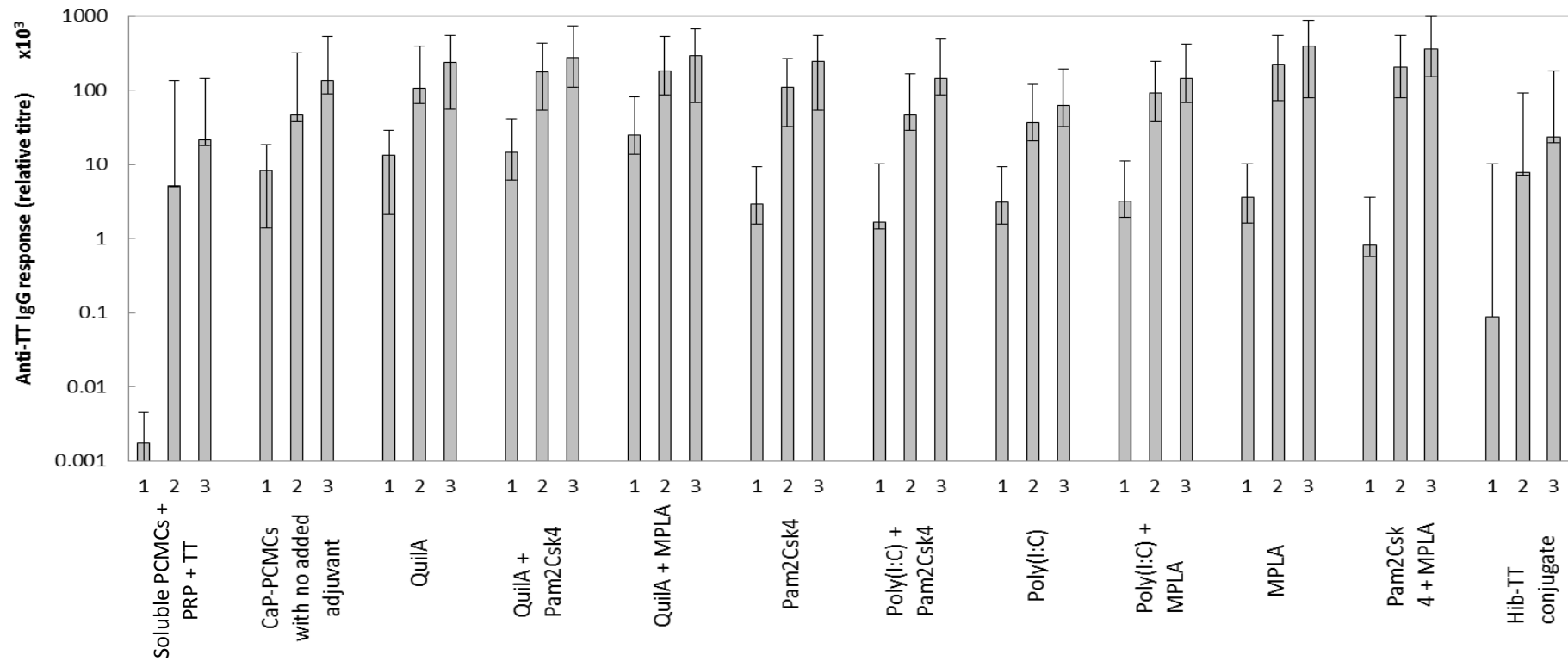
Figure 5.10. Anti-TT responses induced by immunisation with CaP-PCMC formulations in combination with different soluble adjuvants after 1, 2 and 3 immunisations.

Rats were administered with the TT equivalent dose of 0.6µg for the first, followed by two doses of 0.1µg. Soluble adjuvants were mixed with CaP-PCMC suspension. Sera was tested at a 1/1000 dilution after 1, 2 and 3 immunisations (1, 2 and 3 on x axis). Grey bars represent the geometric mean titre of the responses from each group (n=5), and error bars represent a 95% confidence interval. Two-way ANOVA was carried out on the data and it was found that there was a significant difference between sequential immunisations, but not between groups. Pediacel vaccine Hib component was run as a positive control (Hib-TT conjugate).
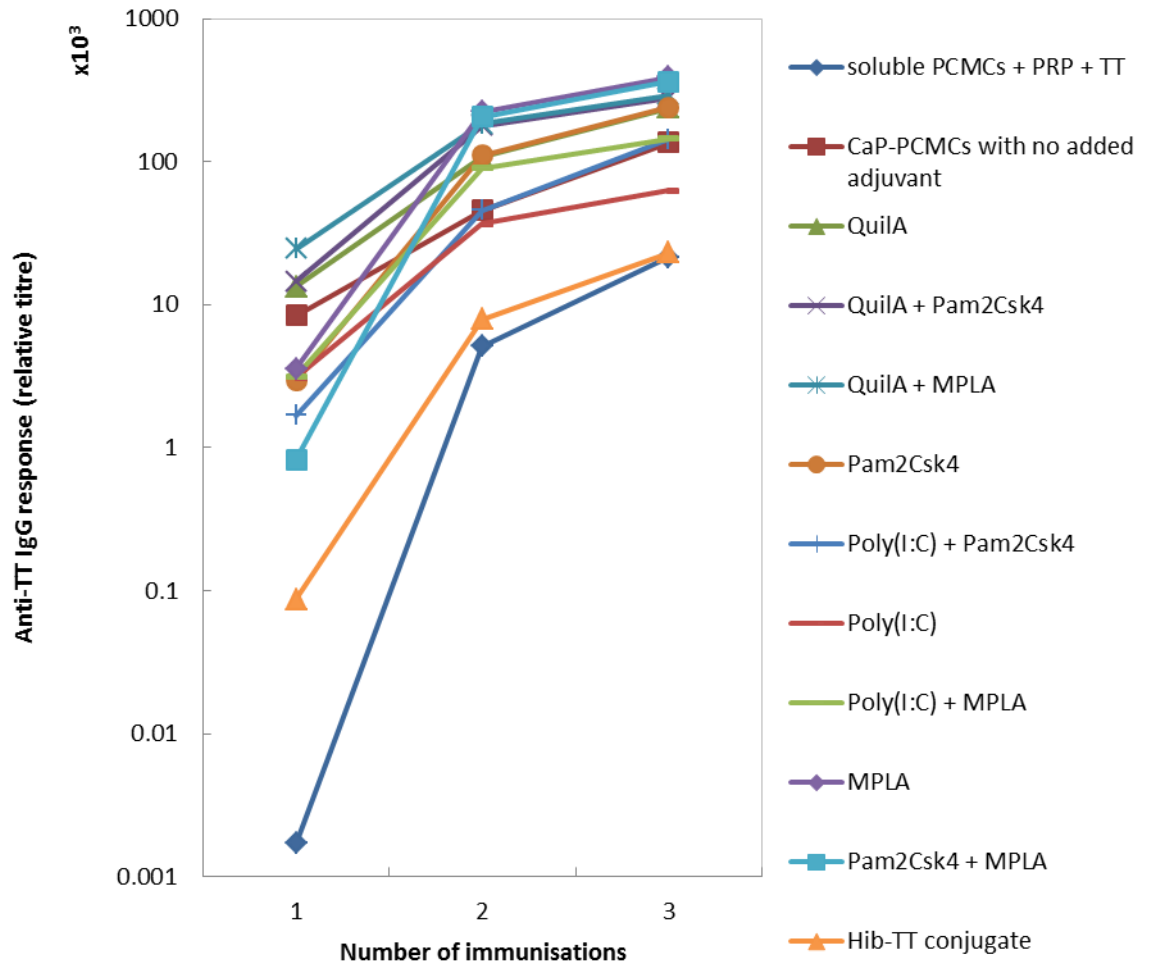
Figure 5.11. Overlayed Anti-TT responses induced by immunisation with CaP-PCMC formulations in combination with different soluble adjuvants after 1, 2 and 3 immunisations.

The data is the same as in Figure 5.10 but presented to visualise the differences among the different groups. Rats were administered with the TT equivalent dose of 0.6µg for the first, followed by two doses of 0.1µg. Soluble adjuvants were mixed with CaP-PCMC suspension. Sera was tested at a 1/1000 dilution after 1, 2 and 3 immunisations (1, 2 and 3 on x axis). Points represent the geometric mean titre of the responses from each group (n=5). Pediacel vaccine Hib component was run as a positive control (Hib-TT conjugate).

5.4.4. Investigation into PRP antigenicity

The low anti-PRP responses seen in Figure 1.8, Figure 5.6 and Figure 5.8 could be due to either a low PRP response generated by immunisation with the CaP-PCMCs, or possibly due to a change in the PRP that occurs during the CaP-PCMC preparation. Any change in the PRP conformation could lead to generation of different anti-PRP antibodies not detectable by antibodies generated against the Hib-TT conjugate.

To investigate this, PRP was exposed to the various conditions it encounters during the CaP-PCMC preparation process. After exposure, its antigenicity was assessed through its ability to bind to anti-PRP antibodies which were generated against the Hib-TT conjugate. To mimic the exposure of PRP to different conditions it was necessary to use HbO-HA instead of PRP which is a carbohydrate, and therefore lacks the ability to adhere to the plate surface without conjugation to a protein.

Immune sera from the Hib-TT immunised animals as well as two immune sera from CaP-PCMC immunised animals that are known to have high anti-PRP antibody titres were used.

The binding of antibodies generated against Hib-TT conjugate to the HbO-HA coated plate exposed to IPA and $CaCl_2$ are shown in Figure 5.12. It can be seen that the titration curves of the antibody binding were similar whether or not the plate was treated with $CaCl_2$, IPA or the combination of the two. This suggests that the exposure did not result in a different antigenicity of the Hib oligosaccharide (HbO-HA) on the plate. However, it must be noted that the anti-PRP antibodies used were those that had been generated against Hib-TT conjugate.

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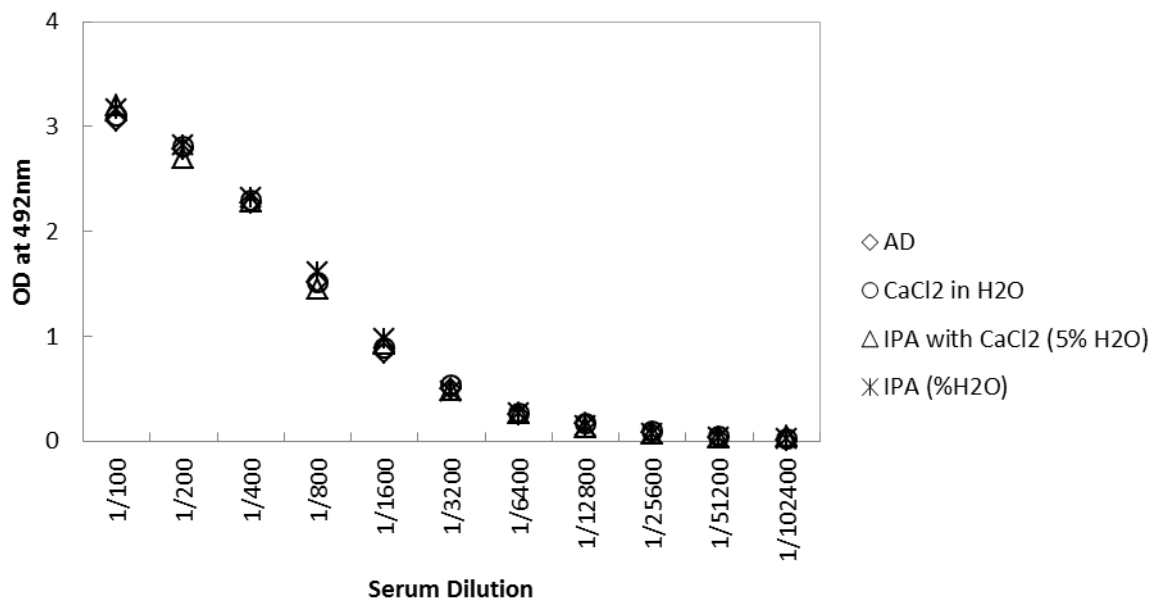
Figure 5.12. The effect of exposure of HbO-HA to different conditions on its binding to anti-PRP antibodies

ELISA plates were coated with HbO-HA and then duplicate rows were treated with $CaCl_2$ in H2O, $CaCl_2$ in IPA or IPA for 16 min. Duplicate control rows were treated with assay diluent. Sera from immune animals immunised with Hib-TT conjugate was used at a 1/100 dilution. Points shown are means of duplicate wells on the plate.

It is possible that during the CaP-PCMC preparation, the conformation of the PRP exposed to IPA and $CaCl_2$ was changed and that this altered epitope induced a different antibody response. In this case, the new antibody would bind less to untreated HbO-HA and more to HbO-HA treated with the substance that altered the epitope during the preparation process.

In order to test this hypothesis, the binding between HbO-HA exposed to IPA and $CaCl_2$ individually and in combination and antibodies in sera from animals immunised with the CaP-PCMC suspension was measured and is shown in Figure 5.13. The sera from

CaP-PCMC immunised rats bound at similar levels to the HbO-HA when exposed to any of the treatments, at all dilutions of sera. There is a small decrease in the readings from wells treated with either IPA or IPA and $CaCl_2$, although the difference is small and disappears at higher serum dilutions.

These results indicate that the exposure of the PRP to the IPA and $CaCl_2$ does not alter the PRP sufficiently to change the binding of anti-PRP antibodies. However, it must be remembered that as PRP could not coat the ELISA plates by itself HbO-HA was used. Therefore, HbO-HA may be more resistant to changes than the unconjugated PRP molecules to IPA or $CaCl_2$ in the production process, which would explain the results.
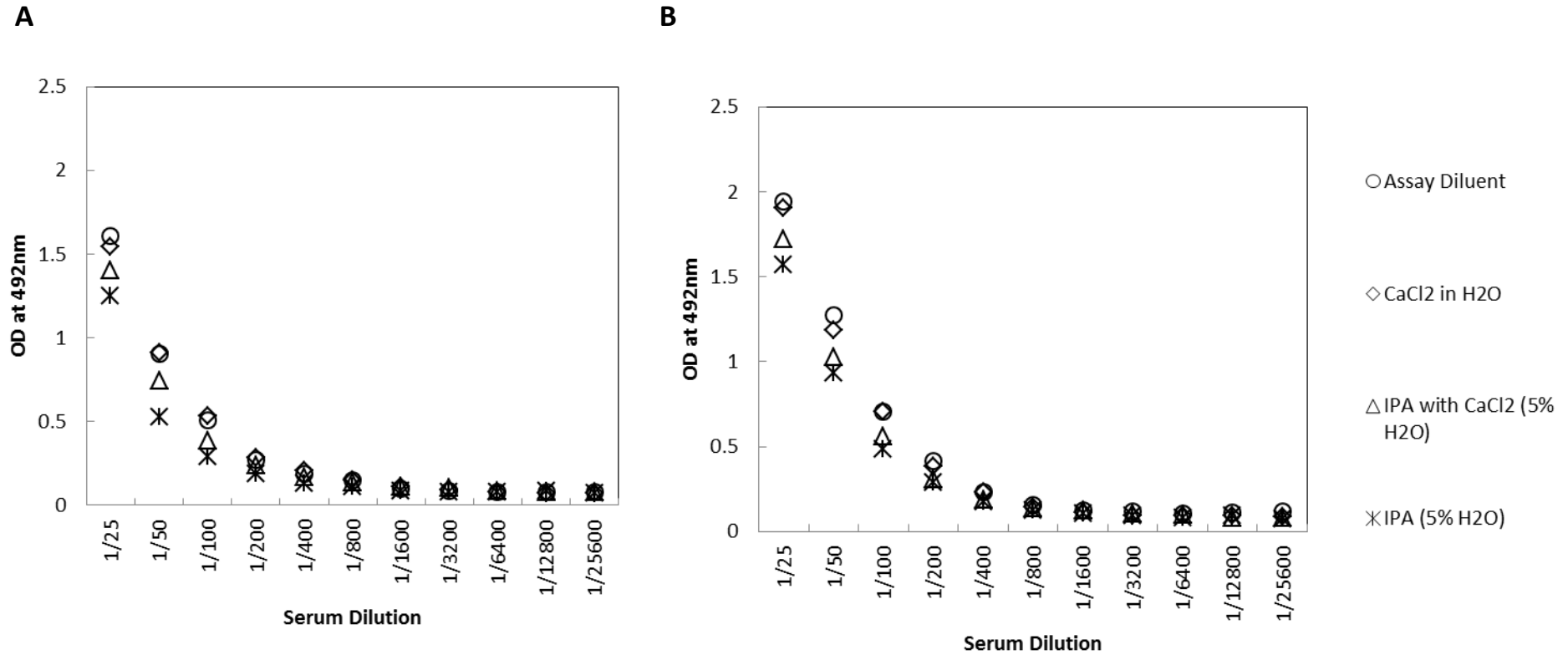
Figure 5.13. The effect of different treatments of HbO-HA on binding of anti-PRP antibodies from CaP-PCMC immunisation.

Duplicate rows of HbO-HA coated wells were treated with IPA, $CaCl_2$ and the combination for 16 min before addition of immune sera from animals immunised three times with CaP-PCMC suspension. The two sera used (A and B) were from an animal immunised with CaP-PCMCs with 7% water content, 2 x $CaCl_2$ excess and 9.4% CaP loading (A) and an animal immunised with CaP-PCMCs with 7% water content, 2 x $CaCl_2$ excess and 9.4% CaP loading alongside soluble MPLA and Pam2Csk4 (B) . The data represent the mean of duplicate readings from ELISA plates

5.4.4.1. Investigation into specificity of anti-PRP antibodies generated by CaP-PCMC immunisation

The ability of antibodies generated by immunised with the CaP-PCMCs to bind to PRP itself was tested in an Inhibition ELISA. Both the native PRP and the PRP forming part of a CaP-PCMC formulation were used. The results are shown in Figure 5.14. The data shows a slightly lower inhibition of HbO-HA binding by the PRP in the CaP-PCMCs was shown at all but one of the serum dilutions

The decrease in binding, however, is not low enough to indicate that there would be an anti-PRP response generated by CaP-PCMC immunisation which is undetectable in the anti-PRP ELISAs from the immunogenicity studies.
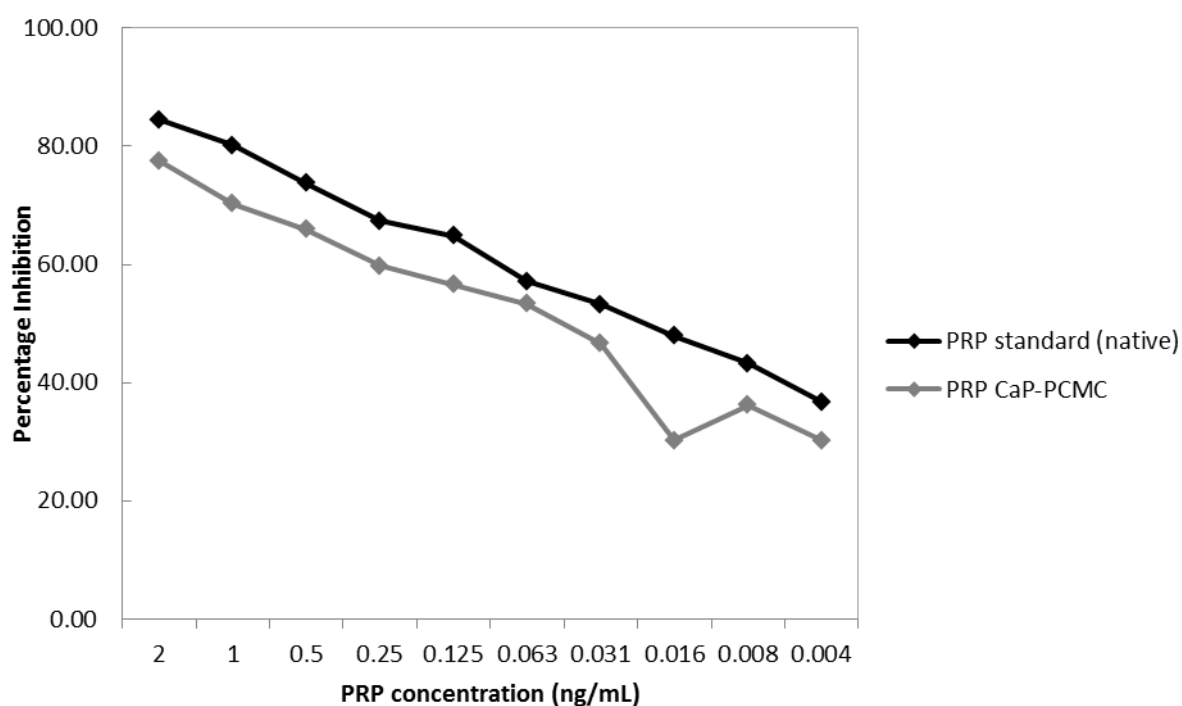
Figure 5.14 Specificity of the binding of anti-PRP generated against the CaP-PCMC formulation to formulated and native PRP.

The percentage inhibition of the anti-PRP antibody from a Hib-TT immunised animal to the HbO-HA at different dilutions of unformulated PRP (black line) or as a component of a CaP-PCMC (grey line). Values shown are mean of duplicate readings on plate.

5.5. Conclusions

Generally, the immune response to the CaP-PCMC (prepared using different experimental conditions) was low. Only a few animals from a few groups responded. It seems that the PRP is not being delivered to or recognised by the follicular B cells when present on the CaP-PCMCs.

CpG, a commonly used TLR9 agonist, was present in the CaP-PCMC. Despite this, the CaP-PCMCs were not immunogenic for PRP. Other adjuvants tested; MPLA, Pam2Csk4, Poly(I:C) and QuilA were also unsuccessful in enhancing the immunogenicity of the CaP-PCMCs..

In contrast to the low anti-PRP responses, anti-TT titres were high, whether the TT was administered in the soluble form, adsorbed on CaP-PCMCs or whether soluble adjuvants were used. It is clear that the generation of the memory response is not dependent on the TT being adsorbed to a CaP-PCMC formulation.

It was hypothesised that the poor immune responses to PRP could be due to the alterations in PRP conformation (and thereby epitope), during the CaP-PCMC preparation process, such that different anti-PRP antibodies were generated *in vivo* and that these antibodies would not bind to the detection antibody used in the antigen assays. However, exposure of HbO-HA to IPA and $CaCl_2$ did not show any altered binding by immune serum from animals immunised with the Hib-TT sera or sera from animals immunised with CaP-PCMCs. It was also demonstrated that the anti-PRP antibodies were still specific for the PRP in the CaP-PCMCs and the standard 'native' conformation of PRP.

Having established that the PRP in the crystal formulation is not inducing an anti-PRP response, the next investigations need to focus on the PRP delivery *in vivo* to the B cells. This may not be occurring for a number of reasons, and the retention of the PRP on the crystals needs to be confirmed to increase the likelihood of the CaP-PCMC acting as a virtual conjugate.

Chapter 6.   Immunogenicity of CaP-PCMC formulations in differing pH and with varying administration routes

6.1. Introduction

The low levels of anti-PRP response induced by the CaP-PCMC formulations indicate that there may be a problem with the antigenicity of the crystals. In chapter 5, it was shown that the changes in the PRP, if any, do not affect the specificity of the antibody response generated by the crystals. This would indicate that the low immunogenicity may be due to an inability of the formulation to deliver the antigens to the PRP specific B cells in a fashion similar to what happens with the conjugate vaccine.

Previously used formulations were tested for PRP release by XstalBio and results indicated that less than 5% of total PRP is soluble and released into water in the formulation used in the pilot study. As the testing conditions for the PRP did not mimic physiological conditions as they were tested in water, it was important to find out if results were different when the free PRP released when the free PRP was measured from suspensions of the CaP-PCMCs in more physiological buffers such as PBS. For this reason, the formulations prepared under the same conditions as those used in Chapter 4 were tested in PBS.

However, when a similar formulation was tested in an isotonic solution, PBS, the results indicated that over 70% of the total PRP content was released as soluble PRP in the supernatant when the CaP-PCMC suspension was pelleted by centrifugation. This test was repeated four times and the release ranged between 70-81% with a mean of

77%. This high level of free PRP might be the reason for the low immunogenicity seen in the studies in Chapter 4.

Therefore, the high rate of soluble PRP in the PBS was addressed by XstalBio and various CaP-PCMC preparation parameters were screened and two new formulations with low levels of soluble PRP were prepared. The difference being the change in the $CaCl_2:NaH_2PO_4$ ratio, from 1:1 to 5:2. The two formulations varied in the form of Sodium Phosphate used and both were investigated for the ability to induce an anti-PRP response. The details of the formulations are presented in Table 6.1 .

Table 6.1 Preparation parameters of the two formulations prepared at XstalBio

	Percentage Antigen Loading		CpG loading	CaP loading	Water content	$CaCl_2:NaH_2PO_4$ ratio	Sodium Phosphate form
	PRP	TT					
Formulation 1	0.5%	0.05%	0.5%	9.4%	5%	5:2	Monobasic
Formulation 2	0.5%	0.05%	0.5%	9.4%	5%	5:2	Dibasic

All percentages are w/w. Soluble PRP was measured from a CaP-PCMC suspension in PBS.

The formulations were evaluated for the ability to induce an anti-PRP antibody response in the immunogenicity studies using different dosages. The effect of the administration route, either subcutaneous or intraperitoneal was also evaluated.

Intra-peritoneal routes of immunisation of the CaP-PCMC suspensions were investigated because this route may offer better trafficking of a particulate suspension. Subcutaneous immunisation may results in a 'depot' of crystals due to physical inability of the particulate form to move from the site of injection. This may result in the release and trafficking of only the soluble antigen from the formulation which will fail to induce a T cell dependent antibody response. Intra-peritoneal routes may be advantageous because the structure of this space is less restrictive to larger sized

particles. Previous studies have shown increased immunogenicity from particulate vaccines administered intraperitoneally (182).

In addition, the adjuvants used in the previous chapter (MPLA, QuilA and Poly(I:C)), the effect of aluminium-based adjuvant insoluble or "Alum" on the CaP-PCMC immunogenicity was investigated. Aluminium salts have been shown to enhance the generation of a Th2 response, due to the provision of local inflammation and recruitment and activation of antigen presenting cells at the site of injection (183). It has also been shown that alum induces chemokine secretion and results in priming of Th2 cells, and secretion of IL-4, IL-5 and IL-10 (184).

The effect of Addavax, a squalene-in-water based adjuvant similar to MF59 (Novartis) (185), on the immunogenicity of the CaP-PCMC was also investigated. MF59 has been widely used in Europe with the seasonal flu vaccines and has a good safety profile (186, 187). The MF59 emulsions, as well as the similar adjuvant AS03 (GlaxoSmithKline) stimulate stronger antibody responses, permit fewer doses and antigen dose sparing, and generate marked memory responses, with a mixed Th1-Th2 cell phenotype (188).

6.2. Aims and Objectives

In this Chapter, it was important to determine not only if the CaP-PCMCs were immunogenic, but if they failed to induce an IgG response against the PRP.

Two formulations were selected by XstalBio with low free PRP content. The assumption that with high retention of the PRP on the crystal, it is more likely that the crystals will behave like a virtual conjugate and deliver the PRP and TT on the crystals

to PRP specific B cells leading to induction of an anti-PRP antibody response. The two formulations were tested in the rat model at different dosages administered through both the subcutaneous or intra-peritoneal routes.

Combinations of the CaP-PCMC suspensions and adjuvants that can improve the 'depot' effect of the crystals such as Aluminium Hydroxide. Addaxax (MF59) and Aluminium Phosphate as well as the soluble adjuvant QuilA were also tested in an immunogenicity study.

To investigate the effect of buffer on the immunogenicity of the formulation, a phosphate buffer was used which did not contain any saline to try to reduce any changes in pH after injection, this could increase the soluble PRP. Water was also used in two immunogenicity studies to try to reduce the release of soluble PRP from the crystal formulation.

The antibody response to PRP is dependent on T cell help and therefore a study was designed to investigate if the poor antibody response to PRP is due to a deficient T cell response. Splenocytes from animals immunised (intraperitoneally) with the CaP-PCMC suspension were also analysed for a proliferation response to stimulation with TT, important for determining if the absence of a an adequate T cell help is responsible for the lack of antibody response. Sera from four animals immunised with the CaP-PCMC suspension were also tested for bactericidal function.

6.3. Materials and Methods

6.3.1. Materials

The following materials were used in the study:

Phosphate Buffer for injection (pH 7.4)
Phosphate Buffered Saline (pH7.4)
RPMI for cellular proliferation (Invivogen, UK)
Hank's Buffered Saline Solution (HBSS, supplemented with 5% FCS, 1% Penicillin Streptomycin, 1% L-glutamine)
RPMI (Sigma, UK)
TT (NIBSC 04/150)
Concanavalin A (Sigma, UK)
Tritium Thymidine in RPMI
Chocolate Agar plate (Blood Agar base no. 2, Oxoid, UK)
Mueller Hinton Broth (supplemented with 10 µg/ml hemin and 1 µg/ml nicotine adenine dinucleotide (NAD))
Bactericidal Assay Diluent (1x Hanks Salt Solution, 10% FCS in H_2O)
Hanks Salt Solution (Sigma, UK)
Thymidine Tritium (stock at 1mCi/mL in ethanol, Perkin Elmer, UK)

6.3.2. Preparation of CaP-PCMCs

Two formulations were selected by XstalBio by screening of a range of preparation parameters for crystals with a low soluble PRP content. The details of the formulations are seen in Table 6.2.

Table 6.2. Details of preparation parameters used to produce the two CaP-PCMC formulations with low soluble PRP content, as tested by XstalBio

	CaP-Loading	$CaCl_2$:NaH_2PO_4 molar ratio	Number of process steps	Sodium Phosphate form
Formulation 1	10	5:2	1	Monobasic (NaH_2PO_4)
Formulation 2				Dibasic (Na_2HPO_4)

6.3.3. Influence of adjuvants on immunogenicity of CaP-PCMCs

Details of animal studies are shown in Chapter 5.

Adjuvants MPLA, QuilA, Addavax, $Al(OH)_3$ and $AlPO_4$ were mixed with CaP-PCMC suspensions immediately prior to immunisations. All suspensions were prepared immediately before immunisation times, with the exception of Aluminium Hydroxide and Aluminium Phosphate which were prepared as a suspension with the CaP-PCMCs 1 h prior to immunisation at a concentration of 1mg/mL.

6.3.4. Influence of dosage of CaP-PCMC immunisation on induction of anti-PRP response

To determine the optimum dosage of the two low-soluble PRP CaP-PCMC formulations (detailed in Table 6.2), three doses were investigated:

- 0.2µg of PRP per dose (0.02µg TT per dose,)

- 1µg of PRP per dose (0.1µg of TT per dose)

- 5µg of PRP per dose (0.5µg of TT per dose)

In another study, one formulation was selected and tested and used in combination with Aluminium hydroxide at the following doses:

- 0.4µg of PRP per dose (0.04µg TT per dose,)

- 1µg of PRP per dose (0.1µg of TT per dose)

- 2.5µg of PRP per dose (0.25µg of TT per dose)

6.3.5. Influence of CaP-PCMC immunisation on the boosting of an existing anti-PRP response

Five groups of five female Sprague-Dawley rats were injected subcutaneously with two 0.4μg PRP equivalent doses of Hib-TT conjugate (Menitorix) three weeks apart. Test bleeds were taken on days 51, 62, 83, 104. Animals were immunised a third time on day 126 with a dose of CaP-PCMC suspension at a PRP equivalent of 1μg/rat. This dosage was selected from immunogenicity studies to optimise the dosage. Control groups were administered soluble PRP only, soluble PRP, TT and CpG, soluble PMCs formulated with PRP, TT and CpG and Menitorix (this group was given a dose of 0.4μg/rat).

6.3.6. Influence of CaP-PCMC immunisation on Splenocyte Proliferation in response to TT stimulation

Cell-mediated immune function was assessed by measuring splenocyte proliferation in response to incubation with TT. Animals were killed two weeks after the third immunisation and spleens were harvested. A single cell suspension was prepared with the use of a cell sieve (Corning). The single cell suspension was washed in HBSS. Red blood cells were lysed with H2O and the resulting suspension washed in HBSS. The suspensions were adjusted to 2×10^5 cells/mL and 100μL added to rows on 96 well plates (Falcon). 100μL of TT in RPMI, RPMI without antigen was added to wells with cells and incubated for 4 days at 37°C and 5% CO_2. Volumes of 10μL/well of Tritiated Thymidine in RPMI were added to the cells and incubated for 4 h. The cells were then harvested using a cell harvester onto glass fibre filtermats. The radioactive count for each well was recorded using radioactive count machine.

6.3.7. Investigation of Bactericidal Ability of serum from CaP-PCMC immunised animals

The bactericidal assay was used to assess the ability of the sera collected from immunised animals to kill a Hib culture, which would indicate protection against infection.

Haemophilus influenzae type b strain (supplied by NIBSC) was stored in vials at -80°C and were streaked on chocolate agar plates after thawing. The streaked plates were incubated overnight at 37°C. The next day, 20 colonies were collected from the plate with a sterile loop and suspended together in 20mL of Mueller-Hinton broth in a 25mL conical flask and incubated for 4 h at 37°C. Terminal Serum from animals immunised with CaP-PCMC suspensions or with Hib-TT conjugates were incubated in 300μL volumes at 55°C for 30 min to deactivate complement. The sera was then diluted in two-fold in triplicate, 9 times across a round bottomed 96-well plate into Mueller Hinton broth in 50μL volumes (50μL of broth added to wells in final column). Complement was then diluted 1:1 in broth and added in 30μL volumes to each well, leaving a final column without complement.

20μL of the Hib bacterial suspension in Bactericidal assay diluent is then added to every column. The plate is then incubated for 1 hr at 37°C. 10μL volumes were taken from the triplicate wells on the plate and pipetted into a tilted chocolate agar plate. The three droplets were then allowed to run down the plate to evenly distribute the viable bacteria across the plate surface in three lines. The plates were then incubated overnight at 37°C. The following day, colony forming units (CFUs) were counted. The complement dependent bacterial killing was calculated as a percentage drop in the

CFUs of the serum mixed bacterial suspension compared to the CFUs on plates where only bacterial suspension had been used without complement.

6.3.8. Measurement of tissue pH at the site of injection

The pH at the site of the injection was measured following injection with 500μL of PBS into female Sprague-Dawley rats. The animals were terminated and the site was dissected 5 min and 30 min after injection via the intra-peritoneal and subcutaneous route. A contact pH probe (Mettler Toledo, Ohio, USA) was used to measure the pH in the space where the liquid had been injected.

6.4. Results and Discussion

Two formulations prepared by XstalBio which showed low PRP soluble PRP release into PBS were prepared and tested at NIBSC for the low soluble PRP. The preparation parameters of these formulation is shown in Table 6.2. Both formulations were tested three times for the soluble PRP content. The results are shown in Table 6.3. The mean free PRP was found to be 13.2% and 25.3% in the two formulations. The upper limit for free PRP in the manufacture of the current conjugate vaccines is 20% (189). This would indicate that Formulation 2 may contain too much free PRP to initiate a T cell dependent response, however due to the different nature of the CaP-PCMC suspension, the formulation 2 was tested in an immunogenicity study.

Table 6.3. Release of soluble PRP from CaP-PCMC suspensions as percentage of total PRP

	Soluble PRP (%)	Mean (%)
Formulation 1	10.1	13.2
	5.7	
	23.7	
Formulation 2	22.2	25.3
	32.6	
	21.1	

Formulation 1 was selected to be tested for release of soluble PRP in three buffers, PBS, Saline and H_2O. The percentage release can be seen in Table 6.4. The levels were found to be lower in Saline than in PBS and lower in H_2O than in Saline. These results indicate that the presence of both phosphate and Sodium Chloride increase the release of the soluble PRP from the new crystal formulation.

Table 6.4. Release of PRP from a new samples prepared with a 5:2 ratio of CaCl$_2$:NaH$_2$PO$_4$, a 9.4% CaP, a 5% water content and a 1 step process

CaCl$_2$:NaH$_2$PO$_4$ ratio	CaP loading (%, w/w)	Process Step	Buffer	Release
5:2	10	1	PBS	13.2
			Saline	9.8
			H$_2$O	1.8

The low level of release of soluble PRP indicates that at least 86.8% of the PRP remains as part of the crystal. This level was thought to be enough to ensure that the PRP as part of the same crystals contained

6.4.1. Immunogenicity of the CaP-PCMC formulation

To investigate the optimum dose for the new CaP-PCMC formulation, three doses were investigated in an immunogenicity study. Each CaP-PCMC suspension tested at 0.2, 1 and 5µg of PRP equivalent dose for all three immunisations. Anti-PRP responses after the second and third immunisations are shown in Figure 6.16.

It was found that the responses induced in the animals given the Hib-TT conjugate vaccine were significantly higher than those in the animals immunised with the CaP-PCMC formulation (repeated measures ANOVA, $p<0.05$). It was found that there was a significant increase of anti-PRP IgG titre between the second and third immunisation in all groups (repeated measures ANOVA, $p<0.05$). However, there were no differences in the anti-PRP IgG response generated among the groups immunised with the CaP-PCMC formulations (ANOVA, $p<0.05$). The highest anti-PRP response was seen in one animal from the group given 1µg of PRP dose of CaP-PCMC suspension (Formulation

1). After three doses this sera produced an OD of 1.3. The second highest anti-PRP response was also seen from this group, with a final OD of 0.3.

It can be seen that the only group that had a responding animal produce an OD greater than 1 was immunised with Formulation 1 at a dose of 1µg of PRP, although it only induced an anti-PRP response in one animal. The lack of this response seen in groups immunised with formulation 2 may be due to the higher levels of soluble PRP seen in Table 6.3. As well as a decrease in the PRP able to act as part of the crystal structure like a virtual conjugate, the free PRP may act to decrease the response by inducing a T cell independent response.

Low ODs in groups given 5µg PRP doses of the CaP-PCMC suspensions may be due to a higher than optimum dose, a similar effect to that seen with the conjugate vaccine. The lower dose of 0.2µg induced similar levels of low anti-PRP responses to the higher dose. Formulation 1 was therefore selected at a dosage of 1µg of PRP per dose (0.1µg of TT) for future immunogenicity studies with subcutaneous immunisations formulation and dosage in combination with routes and soluble adjuvants.
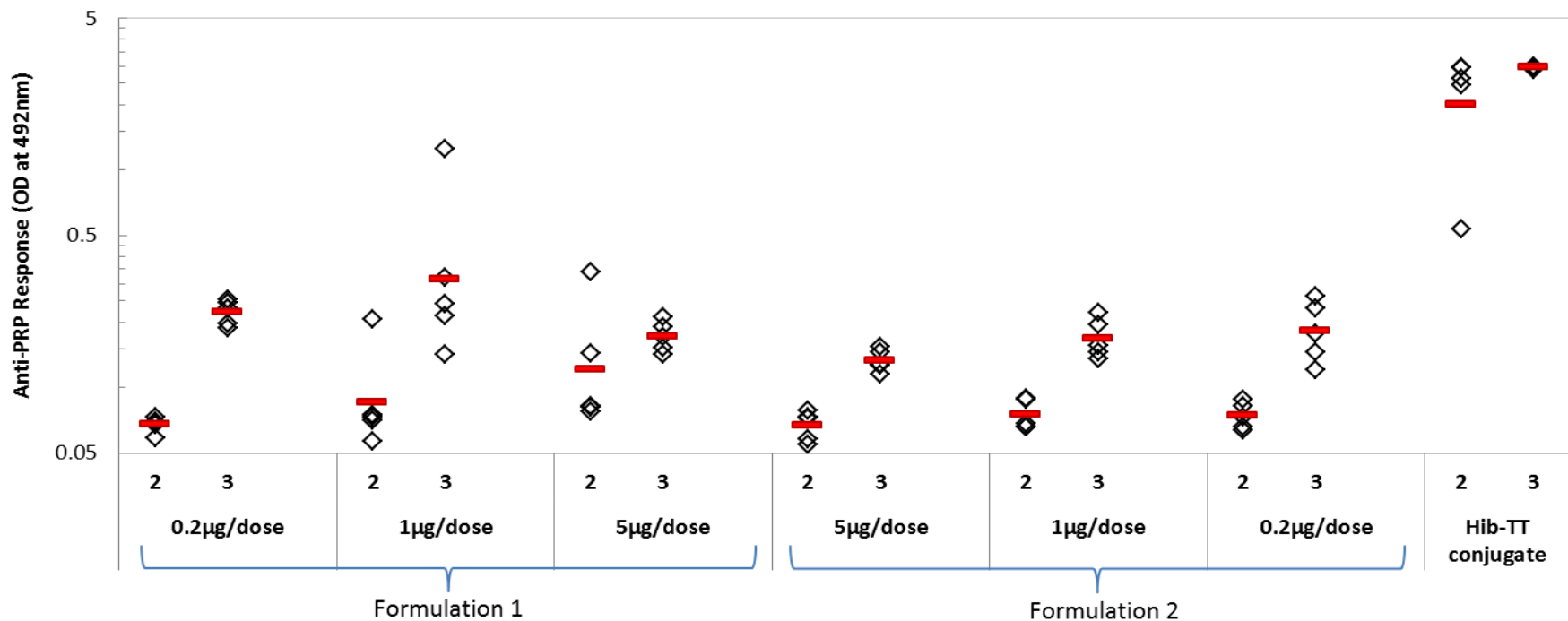
Figure 6.16 Anti-PRP IgG responses induced after the second and third immunisation (2 and 3) of two CaP-PCMC formulations at three dosages.

Groups of rats were immunised subcutaneously with the CaP-PCMC suspension and sera was collected after three CaP-PCMC immunisations. Individual responses from sera measured at 1/25 dilution shown as green diamonds. The anti-PRP in response in animals given the Hib-TT conjugate (positive control) of a PRP equivalent of 0.4μg/dose of the Hib-TT conjugate. PRP dose was either 0.2, 1 or 5μg PRP/rat for all three immunisations. Geometric Mean of responses within the group shown as red horizontal bars (n=5). Two-way ANOVA was carried out and it was found that there was a significant effect of dose on all groups, however, there were no significant differences between groups.

6.4.2.   Investigation of dose, route, and Aluminium Hydroxide on the immunogenicity of the CaP-PCMC formulation

The combination of the intra-peritoneal route and the effect of aluminium phosphate were investigated in an immunogenicity study. Hib-TT conjugate (Menitorix) was also tested in three groups with the same PRP dosages. Sera from the animals after three immunisations were tested by ELISA and the results are shown in Figure 6.2. Although aluminium salts have a good safety profile, they can occasionally be associated with severe tissue reactions, induction of IgE and related allergic and hypersensitivity reactions (190).

It was found that there was a significantly higher anti-PRP IgG response in the group immunised with the Hib-TT conjugate vaccine compared to the groups immunised with the CaP-PCMCs (ANOVA, $p < 0.05$). However, there was no significant difference in the anti-PRP IgG response among the groups immunised with the CaP-PCMC. The highest responding animals were seen in the groups that were administered 1µg of PRP per dose administered without alum and intra-peritoneally. Within this group, two sera samples produced an OD of over 0.2, which is low, but might indicate the generation of a memory response. The highest responding animal in this group produced an OD of 0.49. The 1µg PRP dose of CaP-PCMC administered intra-peritoneally without Aluminium Hydroxide did not show any responding animals, however the 5µg dose did induce one sera to give an OD of 0.27, similar to the second highest responder in the group administered the 1µg dose.

The group that was immunised intra-peritoneally in combination with Aluminium Hydroxide showed even lower numbers of responding animals and lower ODs. The 1μg dose of the CaP-PCMC with this route and Aluminium Hydroxide combination had a serum sample produce an OD of 0.23. The lower dose of 0.4μg PRP of CaP-PCMCs had a sera produce an OD of 0.18, which is higher than the equivalent dose without Aluminium Hydroxide. This small difference indicates there is no adjuvant effect of the aluminium hydroxide on the anti-PRP response.

had sera samples with ODs greater than 0.2 was at the lower dosage of 0.4μg of PRP in CaP-PCMC suspension given without Aluminium Hydroxide. The sera produced an OD of 0.22. 4 animals produced an OD greater than this in the groups that were administered the CaP-PCMC suspension intra-peritoneally.

The Hib-TT conjugate vaccine groups showed a high level of responses at all three dosages. The PRP dosage that had the highest geometric mean was the 1μg PRP dose, the mean OD was 2.7 compared to 2.2 in the 0.4μg dose and 2.5 in the 4μg dose. In each group there was one animal that had a sera OD that was much lower than the mean. This heterogeneity may be due to differences (genetic or environmental) between the animals within the group.

This immunogenicity study indicates that the CaP-PCMC suspension may be able to act more immunogenically when administered through the intra-peritoneal route. However the number of responding animals and the low anti-PRP responses indicate that these CaP-PCMCs are not immunogenic, at least at these dosages and routes. There does not seem to be any effect of using Aluminium Hydroxide in combination with the CaP-PCMC suspension.
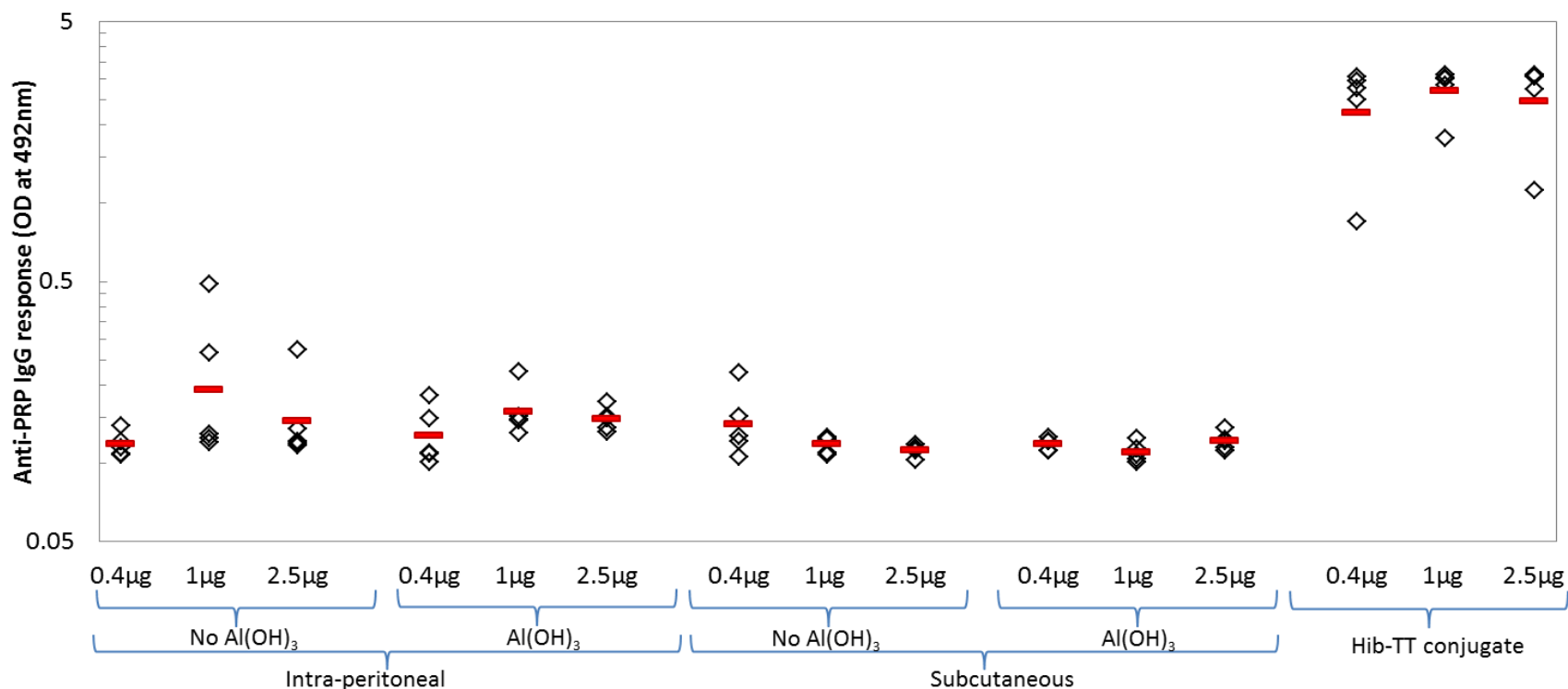
Figure 6.2. Anti-PRP IgG responses after 3 doses of CaP-PCMCs administered either intra-peritoneally, or subcutaneously, with and without aluminium hydroxide at three different doses.

Groups of rats were immunised with either 2.5, 1 or 0.4µg of CaP-PCMC suspension in water. Six groups were given the CaP-PCMC suspension mixed with Aluminium Phosphate (Alum). Rats were immunised either intra-peritoneally or subcutaneously. Rats in the control group were given the PRP equivalent doses of the Hib-TT conjugate vaccine (Menitorix) subcutaneously. Individual responses from sera tested after three doses at a dilution of 1/50 The anti-PRP response is presented as OD of individual animal sera and geometric mean response of the group (green diamonds and red horizontal bars respectively, n=5). Two-Way ANOVA was carried out and it was found that there were no significant differences between groups.

6.4.3. Investigation of bactericidal ability of immune serum

To confirm at the anti-PRP response generated is able to kill the Hib bacteria, a bactericidal assay was carried out on sera from responding animals. The immune sera from the immunogenicity study shown in Figure 6.2 was selected for testing. The percentage killing by 'Positive serum' from a Hib-TT immunised animal, non-immune serum and serum from four animals selected from the CaP-PCMC immunised groups are shown in Figure 6.3.

The positive serum showed a reduction in the percentage killing of over 60% at a 1:1 dilution of serum to 0% at a 1/1024 dilution. The bactericidal effect of the sera from two animals immunised with the CaP-PCMC suspension intra-peritoneally are shown in graphs C and D. The percentage killing from these sera has a maximum of 40-50% at a ½ sera dilution, which is surprising due to the low anti-PRP antibodies seen in the immunogenicity study. In animals which had been immunised with CaP-PCMC suspension with Aluminium Hydroxide are shown in parts E and F. The maximum killing of these sera at a ½ serum dilution is around 50%. The differences between the animals immunised with and without aluminium hydroxide are not great, but the serum from the Hib-TT immunised animal had a higher percentage killing, which is not surprising as the positive sera from the Hib-TT immunised animals shows a much higher anti-PRP titre (Figure 6.2).
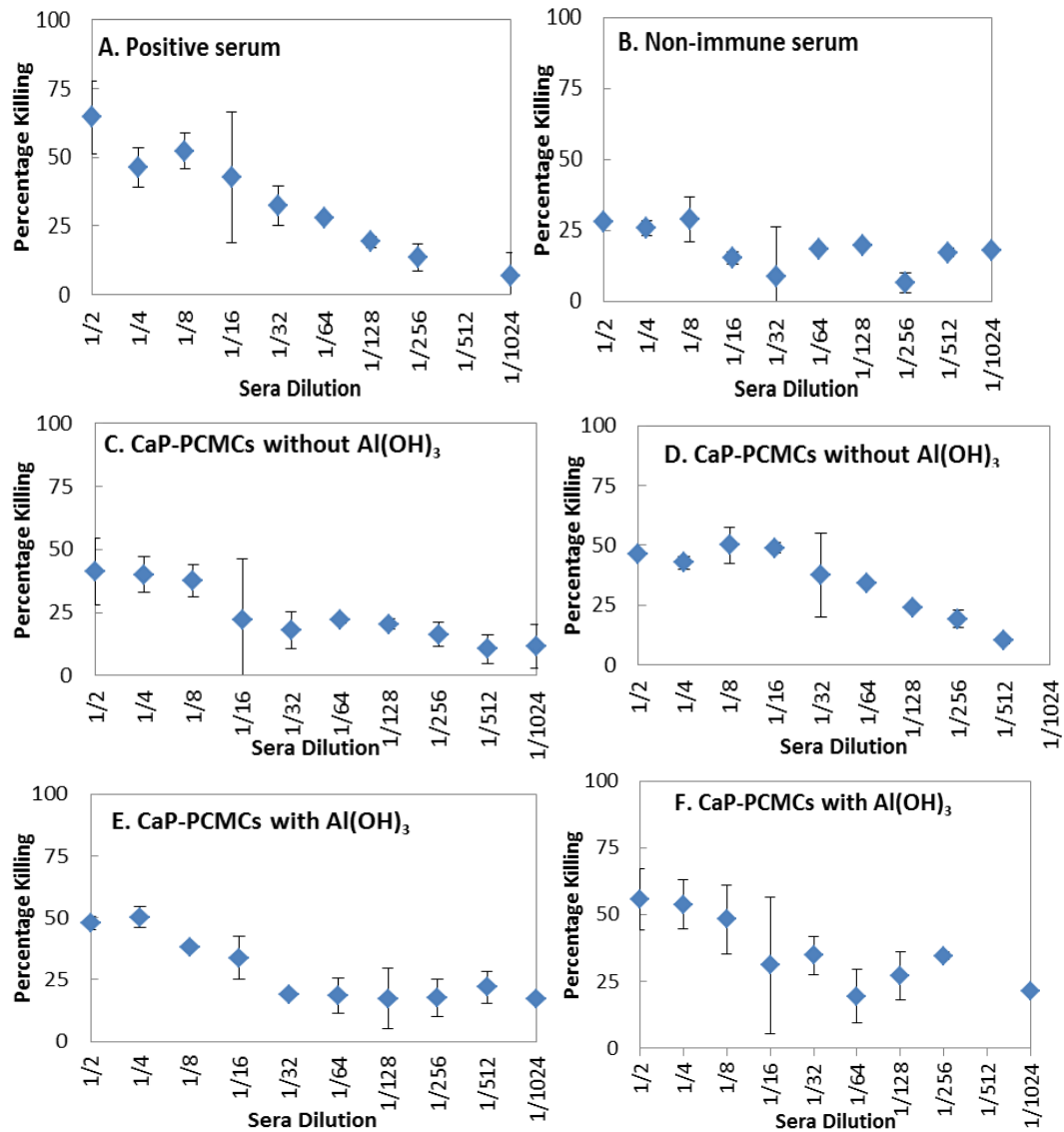
Figure 6.3. Bactericidal effects of immune sera from CaP-PCMC immunisations were tested.

The immune serum from animals immunised with Hib-TT conjugate (A) and non-immune serum (B) were tested alongside immune serum from animals immunised intra-peritoneally with 1μg of PRP equivalent of CaP-PCMC suspension without and with aluminium hydroxide (C and D or E and F). Blue diamonds represent means of triplicate colony counts and error bars show standard deviation.

6.4.4. Investigation of immunogenicity of CaP-PCMC formulations administered intraperitoneally

To confirm immunogenicity of the CaP-PCMC suspension administered at 1µg PRP of CaP-PCMC dose intraperitoneally, a larger group size was used. Negative controls included groups immunised with soluble PRP, soluble antigens (and CpG), soluble antigens (and CpG) with soluble PCMCs with soluble antigens (and CpG). The anti-PRP IgG responses can be seen in Figure 6.4.

Similarly to previous immunogenicity studies, the Hib-TT conjugate showed significantly higher anti-PRP IgG responses to those in the groups immunised with CaP-PCMCs (ANOVA, $p<0.05$). The animals in the positive control group in this experiment were immunised with a dose of Hiberix, a monovalent Hib conjugate vaccine. All 9 animals showed a high anti-PRP response with a geometric mean of 2.3 within the group.

There was no significant difference among the anti-PRP IgG responses in the groups immunised with CaP-PCMCs. The negative controls in this experiment all showed low anti-PRP responses. The highest response from a negative control group was from an animal immunised with soluble PCMCs formulated with PRP, TT and CpG. The OD on this animal was still low, at 0.141. The highest responder in this group showed an OD of 0.17, however, the 9 other animals showed lower ODs than the geometric mean of the group at 0.09. This response was much lower than the ODs from the previous immunogenicity study shown in Figure 6.2, which confirms the low immunogenicity of the crystals.
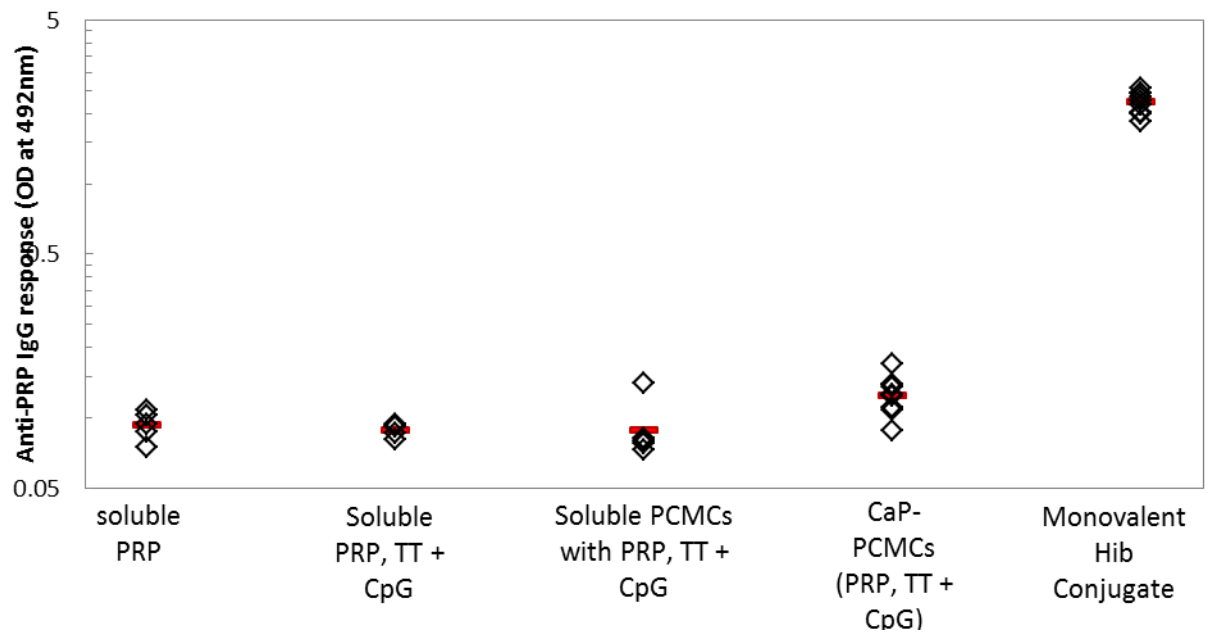
Figure 6.4. Anti-PRP IgG response after 3 CaP-PCMCs immunisations administered intra-peritoneally.

Rats were immunised intra-peritoneally with three doses of CaP-PCMC suspensions (in water) containing 1µg of PRP per rat. Animals in control groups were given equivalent PRP doses of soluble PRP, soluble PRP with 0.1µg of TT and 1µg of CpG with and without soluble PCMCs (n=5) and a monovalent Hib conjugate (Hiberix, n=9). Sera were tested after three immunisations at a dilution of 1/25. The anti-PRP response is presented as OD of individual animal sera and geometric mean response of the group (red horizontal bars; n=10). Two-Way ANOVA was used and it was shown that there was a significant increase in the group immunised with the Hib-Conjugate and all other groups.

6.4.5. Investigation of cellular response to CaP-PCMC immunisation

The proliferation response of splenocytes in response to stimulation with TT was tested to determine if the low anti-PRP responses were due to a lack of cellular response to the TT. The indexes calculated from the tritium counts are shown in Figure 6.5. Index values greater than 1 indicate an increase in the proliferation in splenocytes stimulated with TT compared to unstimulated splenocytes. There was no significant difference between the indexes measured among the different groups.

The high indexes in the group immunised with CaP-PCMC suspensions indicate that there is a successful induction of a T cell response to the TT in the CaP-PCMC formulation. Furthermore, all of the responses in this group were higher than 4 of the 5 indexes from the group immunised with Hib-TT conjugate. This increased response is most likely due to the inclusion of CpG in the CaP-PCMC formulation.

In the 10 animals from the two negative control groups, all showed proliferation responses. The high index shown from one animal in the group immunised with soluble PRP, TT, and CpG was 16.6. The responses in these groups indicate that there is a response to soluble TT, which is due to the high immunogenicity of TT even when not part of a crystal formulation. However, there was a slight increase in the indexes from the CaP-PCMC immunised animals, the geometric mean was 5.3 compared to 3.5 and 3.4 in the two negative control groups.
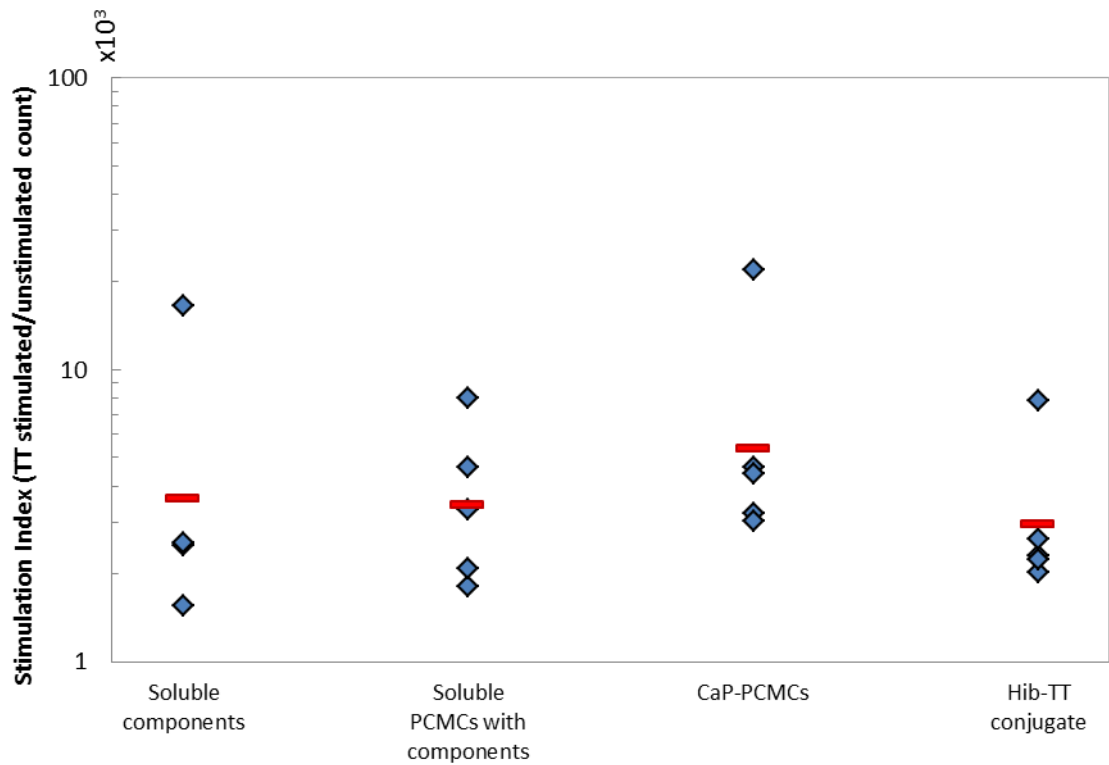
Figure 6.5. Stimulation index of splenocytes harvested after three immunisations of CaP-PCMC suspension.

Rats were immunised intra-peritoneally with three doses of CaP-PCMC suspensions (in water) containing 1µg of PRP per rat. Animals in control groups were given equivalent PRP doses of soluble PRP, soluble PRP with 0.1µg of TT and 1µg of CpG with and without soluble PCMCs (n=5) and a monovalent Hib conjugate (Hiberix, n=9). Splenocytes were harvested after three immunisations and incubated for 4 days with and without Tetanus toxoid and then for 5 hours with Tritium-thymidine and uptake of tritium measured.

6.4.6. Investigation of soluble adjuvants on the immunogenicity of the CaP-PCMC suspension with and without CpG

The immunogenicity of Formulation 1was investigated in combination with Addavax (MF59), AlPO4, MPLA and QuilA to improve the immunogenicity of the CaP-PCMC formulation to induce anti-PRP responses. Each adjuvant was made into a suspension with the CaP-PCMCs formulated with and without CpG to assess the adjuvant effect of CpG included in the formulations. The anti-PRP IgG responses from the groups are shown in Figure 6.6.

The group immunised with the Hib-TT conjugate showed a significantly higher anti-PRP response than all of the groups immunised with the CaP-PCMC formulations (ANOVA, $p<0.05$). There was found to be no significant difference among any of the groups which were immunised with either CaP-PCMC formulations even in combination with the different adjuvants.

The groups that were given the CaP-PCMCs without CpG or soluble adjuvant had one animal show a response over 0.2. The other four animals showed low anti-PRP responses. In the groups immunised with the CaP-PCMC suspensions with the adjuvants, only one animal given the QuilA in combination with the CaP-PCMC formulated with CpG, this animal produced an OD of 0.21. The highest OD from the other groups given the adjuvants with the CaP-PCMCs was given Aluminium Phosphate with a non-CpG formulation, and had an OD of 0.61. These results indicate that the CaP-PCMC formulation is still not immunogenic enough to induce

an anti-PRP response, and this cannot be improved by use of any of the selected

adjuvants.
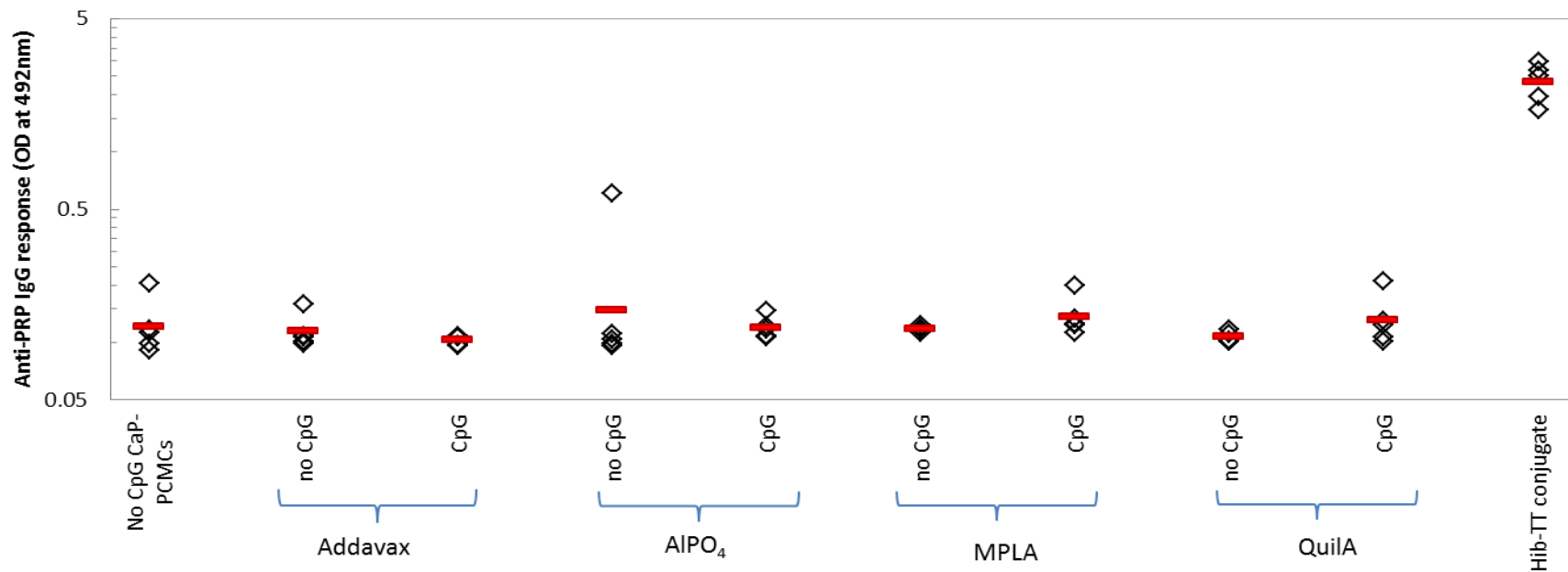
Figure 6.6 Anti-PRP responses induced before and after 3 doses of CaP-PCMC suspension with and without formulation with CpG and in combination with soluble adjuvants .

Animals were immunised with 3 doses of various CaP-PCMC formulations containing a 1µg of PRP/rat. Animals in the control group were given CaP-PCMCs formulated without CpG at a dose of 1µg/rat and the positive control group were immunised with Hib-TT conjugate (Menitorix) at a dose of 0.4µg/rat. Sera were tested after three immunisations at a dilution of 1/25. The anti-PRP response is presented as OD of individual animal sera and geometric mean response of the group (red horizontal bars; n=5). ANOVA was used and it was shown that there was a significant increase in the group immunised with the Hib-Conjugate and all other groups.

6.4.7. Investigation of the T cell proliferation response in animals immunised with soluble adjuvants.

Three groups immunised with Formulation 1 (formulated with CpG) in combination with Addavax (MF59), Aluminium Phosphate and QuilA were analysed for the proliferation of splenocytes in response to stimulation by TT. Splenocytes form the group immunised with the formulation without any adjuvant as well as the group immunised with the Hib-TT conjugate were included as controls. The stimulation indexes are shown in Figure 6.7.

It was found that there were no significant differences among any of the groups tested (ANOVA). The groups given the adjuvants Addavax, Aluminium Phosphate and QuilA all showed proliferative responses with indexes over 1, indicating the induction of a cellular response. In both the Addavax and the Aluminium Phosphate groups, there was one animal that showed a high index over 5, with 10.09 from the group immunised with Addavax and 9.35 from the group immunised with Aluminium Phosphate. The rest of a group had stimulation indexes below 2, which although less proliferative, indicates that all the animals induced a TT-specific pool of T cells in the spleen. The group given the QuilA showed a higher geometric mean index of 3.1, compared to 2 and 1.6 in the other groups. There were two animals within this group that had a stimulation index over 5. The highest index was 16.17 and was from this group. Although the QuilA was not able to improve the anti-PRP IgG response induced by the CaP-PCMCs, it may be able to slightly increase the cellular response to the TT with its adjuvant properties.

Both of the groups immunised with the CaP-PCMCs formulated without CpG and

the Hib-TT conjugate vaccine showed similarly lower indexes. The indexes from

these groups were 1.6 and 1.44 respectively. The lower values maybe due to the

lack of adjuvant present in the formulation.
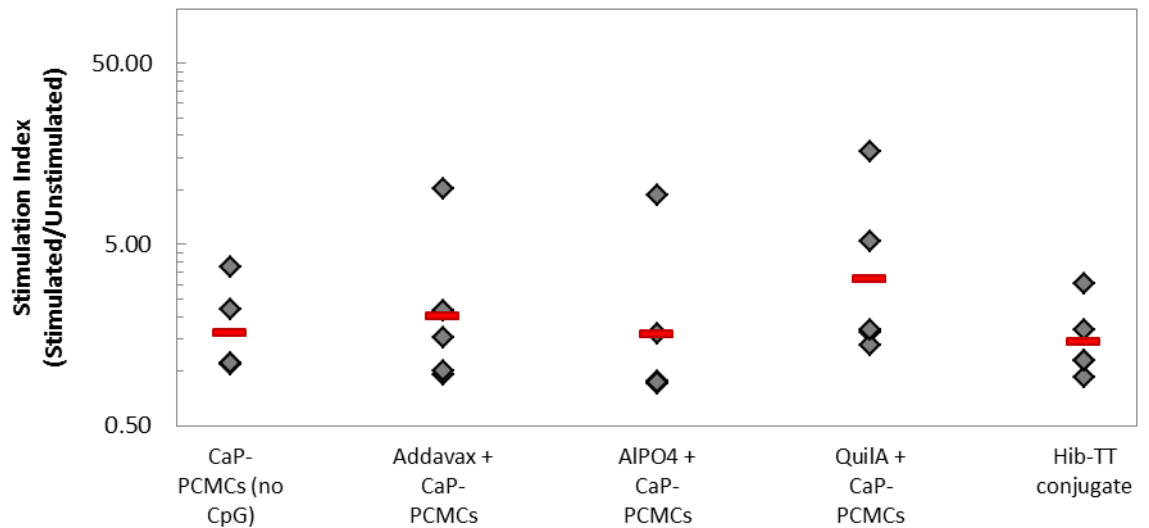
Figure 6.7. Stimulation index of splenocytes harvested after three immunisations of CaP-PCMC suspension with combinations of adjuvants.

Animals were immunised subcutaneously three times with CaP-PCMC suspensions containing 1μg of PRP and CpG per rat with various adjuvants. Animals in control groups were given equivalent PRP doses of CaP-PCMCs without CpG and 0.4μg PRP of Hib-TT conjugate vaccine, menitorix. After the third immunisation, splenocytes were harvested and incubated for 4 days with and without Tetanus toxoid and then for 5 hours with Tritium-thymidine and uptake measured.

6.4.8. Investigation of soluble adjuvants on the immunogenicity of the CaP-PCMC suspension with and without CpG

The anti-TT response was measured in all the groups. In combination with soluble adjuvants and CaP-PCMC formulations with and without CpG. The resulting titres calculated from ELISA results are shown in Figure 6.8.

In the immunogenicity studies in Chapter 4, the Hib-TT conjugate group had lower anti-TT titres than the CaP-PCMC formulations containing CpG. In this case, the Hib-TT conjugate showed a higher anti-TT IgG titre than the negative control group with the CaP-PCMC formulation formulated without CpG. This is due to the high levels of TT included in the Hib-TT conjugate group, as the CaP-PCMC group was only administered 0.1µg/dose compared to 4µg/dose of conjugated TT. All of the test groups showed high levels of anti-TT response, although statistically, they fall within the confidence intervals, and therefore a conclusion on the most immunogenic combination is hard to decide.

Between each CpG and 'no CpG' formulations given the same adjuvants. There is no clear pattern as to whether the inclusion of CpG makes a significant difference in the anti-TT response. One difference that can be noted is between the two groups administered Aluminium Phosphate; the group which was given the Aluminium phosphate without CpG showed a slightly higher geometric mean titre of 250530 compared to the group that was given Aluminium Phosphate with CpG which had a geometric mean titre of 147552, which may indicate that this adjuvant does not

work synergistically with the CpG. This was seen before in the anti-PRP responses shown in Figure 6.5.
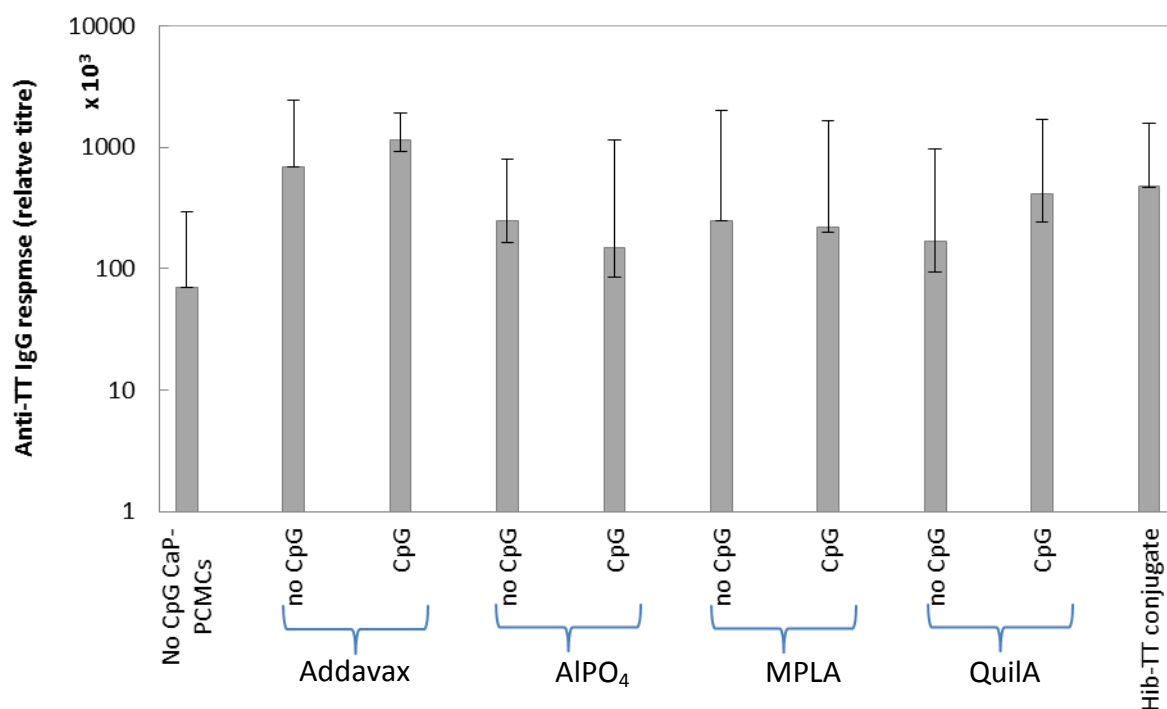
Figure 6.8. Anti-TT IgG response after 3 doses of CaP-PCMCs formulated with and without CpG administered with and without soluble adjuvant.

Animals were immunised with three doses of various CaP-PCMC formulations (with and without CpG) containing 1µg of PRP per rat. Animals in the negative control group were given CaP-PCMCs formulated without CpG. A positive control group was immunised with three (0.4µg PRP equivalent) doses of Hib-TT conjugate (Menitorix). Sera were tested after the third immunisation at a dilution of 1/1000. The anti-TT response is presented as relative titre and error bars show 95% confidence intervals (n=5). Two-Way ANOVA was used and it was shown that there was no significant difference between groups.

6.4.9. Investigation of change of buffer and use of Aluminium Hydroxide on immunogenicity of CaP-PCMCs

It had been suggested that the injection of the formulation into the intraperitoneal cavity could cause a drop in the pH of the tissue, which may negatively affect the

immunogenicity of the CaP-PCMC by altering the release of soluble PRP. The use of the phosphate buffer which did not contain any saline may act to prevent a drop in pH at the site of injection. Two groups were also immunised with the CaP-PCMC suspensions with Aluminium Hydroxide, as this may act to improve the 'depot' effect of the suspension within the intra-peritoneal cavity. The anti-PRP responses after the three immunisations are shown in Figure 6.9.

All four of the groups given the CaP-PCMCs in this study shown low anti-PRP responses, and all the responses even between the group immunised with the Hib-TT conjugate and the groups immunised with the CaP-PCMC formulations did not significantly differ (ANOVA). The lack of anti-PRP responses indicate that there was no effect of either the phosphate only buffer, or the inclusion of Aluminium Hydroxide on increasing the induction of an anti-PRP response by the CaP-PCMCs.

The group immunised with the Hib-TT conjugate showed a high variation in anti-PRP IgG responses. Three of the five animals in this positive control groups showed a PRP response of less than a 0.14 OD. This heterogeneity might have been due to genetic or environmental variations in the animals within the group.
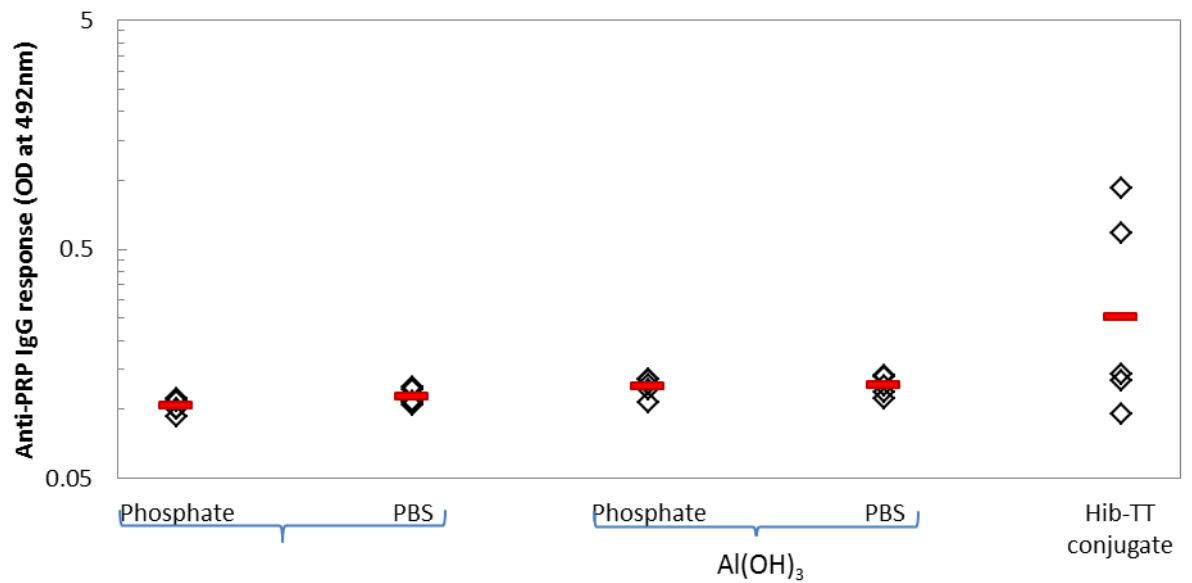
Figure 6.9. Anti-PRP responses induced after 3 immunisations of CaP-PCMC suspensions in phosphate or phosphate saline buffer with and without Aluminium Hydroxide.

Animals were immunised intra-peritoneally with 3 doses of CaP-PCMC suspensions (in either Phosphate Saline Buffer or Phosphate only buffer) containing 1µg of PRP/dose. Animals in the control group were immunised with a Hib-TT conjugate containing the equivalent of 0.4µg PRP/dose. Sera were tested after 3 immunisations at a dilution of 1/25. The anti-PRP response is presented as OD of individual animal sera and geometric mean response of the group (red horizontal bars; n=5). ANOVA was used and it was shown that there was a significant increase in the group immunised with the Hib-Conjugate and all other groups.

6.4.10. Investigation into pH at site of injection

The immunogenicity results indicated that the phosphate buffer without saline does not increase the immunogenicity. The mechanism that this was thought to operate was that the reduction in pH was prevented with the use of this buffer.

To determine whether the pH in the intra-peritoneal cavity drops after an injection of PBS, the pH was measured in the cavity at 5 min and 30 min after injection. The pH measurements are shown in Figure 6.10. The pH at the starting point was measured at around 7 in both the subcutaneous and the intraperitoneal spaces. Five min after the injection with the PBS, the pH was found to be 7.35 in both the subcutaneous and the intraperitoneal space, this is similar to the original pH seen at the starting point. After 30 min the pH at the subcutaneous site had increased to 7.8 whilst the pH at the intra-peritoneal space remained at 7.35.

These results indicate that there was no decrease seen in the pH measured at the sites of injection at either 5 min or 30 min. Although there is a small increase in the pH measurement at the subcutaneous site, the difference (0.45) was small given that the variation between the same site on different animals is 0.18.
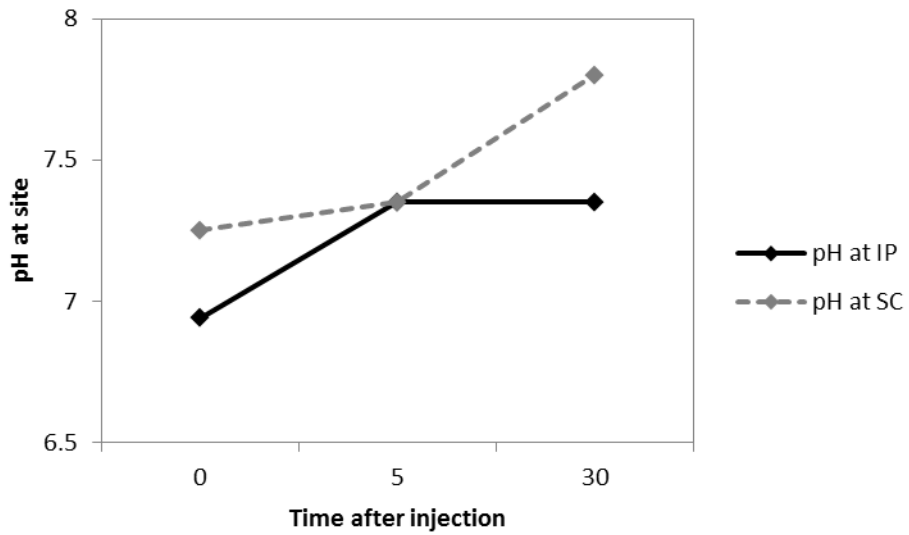
Figure 6.10. pH measured at Intraperitoneal and Subcutaneous sites post injection.

Animals were immunised intra-peritoneally or subcutaneously with 500µL of PBS. The pH of the intra-peritoneal cavity was measured with a probe after either 5 min or 30 min. A control rat was measured at both sites with no injection.

6.4.11. Investigation into the ability of the CaP-PCMC formulation to boost an existing anti-PRP response

From the various immunogenicity studies using the CaP-PCMC formulations, it is clear that the combinations of dose, adjuvants and formulation used were not able to improve the immunogenicity of the crystals enough to induce a memory anti-PRP response. However, it is possible that when an already established pool of PRP specific memory pool cells exists, the PRP-CaP-PCMCs may be able to boost the response.

To investigate the ability of the PRP-CaP-PCMCs to boost an existing anti-PRP response, animals which were immunised twice with the (0.4μg of PRP equivalent dose of) Hib-TT conjugate were then boosted with a CaP-PCMC suspension (using Formulation 1). There were also control groups boosted with soluble PRP, soluble PRP, TT and CpG, and soluble PRP (at a 1μg PRP dose per animal). There was also a positive control group given the Hib-TT for a third dose (0.4μg of PRP equivalent dose).

The anti-PRP responses were measured from day 51 to day 104 and it was expected that a decline in the response would be seen after the second dose over time as the pool of anti-PRP antibody secreting plasma cells decline in number. In the positive control group it can clearly be seen that all 5 animals had an increase in their anti-PRP response after the third immunisation with Hib-TT conjugate on day 126. Therefore, as expected, it is clear that the Hib-TT is able to boost the anti-PRP response.

In the groups immunised with the CaP-PCMC suspension as well as the soluble antigens, and the soluble PCMCs, the anti-PRP response also increased after the booster dose on day 126. The boosting effect is clear in each of the five animals in all apart from one animal in the group boosted with the soluble PCMCs with the TT, PRP and Cpg, which had the lowest anti-PRP response in the group, and also decreased at day 104 from an OD of 0.15 to 0.09, and appears to be a poor responder.

The group immunised with soluble PRP indicate that the all animals have responded to the soluble PRP with a boost in the anti-PRP response. The minimum increase in this group was from 2.3 to 3, a difference of 0.6. These results indicate that the increase in the anti-PRP response in the other groups is due to the presence of the PRP, whether soluble or as part of the CaP-PCMC formulation. It also indicates that the PRP even when formulated as part of a CaP-PCMC is able to be recognised by the PRP-specific B cells, which confirms the specificity of the anti-PRP response shown in the inhibition ELISAs (Chapter 4).

These results indicate that although the PRP from the CaP-PCMC formulation is able to reach the B cells it is meant to target, the formulation is not able to induce a T cell response. The group immunised with the soluble PRP showed a similar boost in the anti-PRP response to the group immunised with the CaP-PCMC suspension, which may indicate that the PRP may not be retained as part of the CaP-PCMC structure when it is *in vivo*. It is not known how long the PRP stays adsorbed to the crystal without doing *in vivo* imaging of labelled antigens. The uptake by the crystal

by certain cells of the immune system such as neutrophils or dendritic cells might

lead to destruction of the crystal structure.
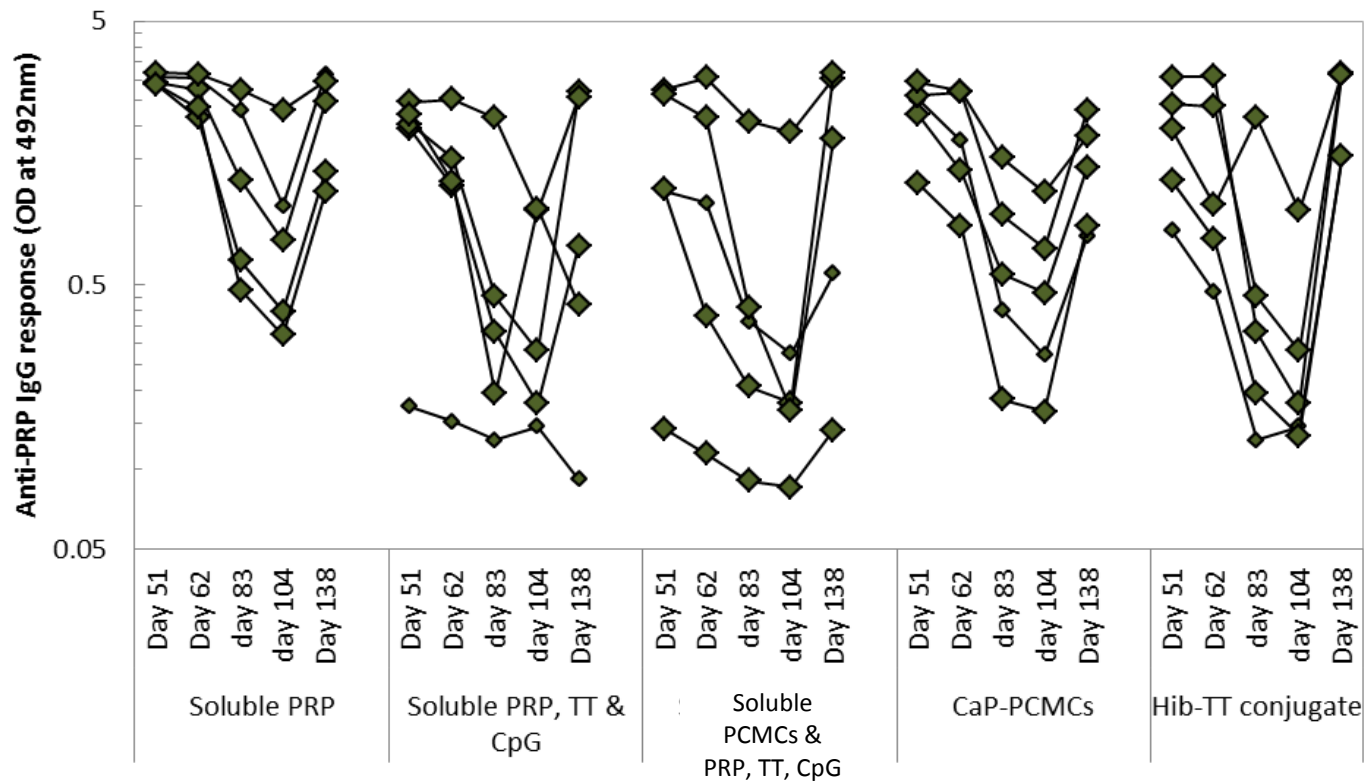
Figure 6.11 Anti-PRP responses after two doses of Hib-TT conjugate and boosted with CaP-PCMC suspension.

Animals were immunised subcutaneously with two (0.4µg of PRP) doses of Hib-TT conjugate (Menitorix) on days 0 and 21 and boosted with a CaP-PCMC suspension on day 126. Animals in the control group were immunised with; (i) soluble PRP only, (ii) soluble PRP, TT and CpG and (iii) soluble PCMCs containing PRP, TT and CpG all at a PRP dose of 1µg (and a TT dose of 0.1µg) and a positive control of Hib-TT conjugate (Menitorix) given at 0.4µg/dose. The anti-PRP response is presented as OD of individual animal sera.

6.5. Conclusions

The preparation of the CaP-PCMC formulations with a low release of PRP in PBS was important, as previous immunogenicity studies in Chapter 4 showed low anti-PRP responses after immunisation of the CaP-PCMCs in a PBS suspension. The two formulations selected by XstalBio were used in these immunogenicity studies to reduce the levels of soluble PRP after injection. However, the results in this chapter indicate that even with these formulations, low numbers of animals were generating an anti-PRP response. The low immunogenicity may be due to larger proportions of the soluble PRP being released from the crystal than is measured by the Orcinol assay.

A peritoneal route of injection was also used to increase the immunogenicity of the formulation. The route has previously been used for injection of particulate vaccines with some success for the T cell antigen, TT (191). Although none of the animals from the CaP-PCMC immunised group mounted an anti-PRP IgG response, the stimulation indexes of the splenocytes from this group are on average higher than those of the control groups that were tested, including the Hib-TT conjugate. This result is interesting as it may indicate that the particulate formulation may enhance the cellular response generated against the TT when administered intraperitoneally, something that might enhance the anti-PRP response should one be induced in the first place.

The immunogenicity of the CaP-PCMC formulation combined with adjuvants Addavax, QuilA and Aluminium Phosphate were also investigated and found that

these adjuvants did not increase the anti-PRP response. A combination of a phosphate only buffer and aluminium hydroxide with the CaP-PCMC suspension was also tested in the model and neither was found to increase the immunogenicity, although it was later found that the pH of the site of injection may not change after injection. The effect of the injection of phosphate only buffer compared to phosphate saline on the local environment may require further investigation by injection of larger volumes of the solution. The effect of the phosphate buffer on the release of the soluble PRP also needs to be determined.

The final investigation to determine if an existing PRP response induced by two doses of the Hib-TT conjugate could be boosted by a third dose containing the CaP-PCMC suspension. The anti-PRP responses measured in these groups did show a boosting effect in the group immunised with the CaP-PCMCs, however, the same increase was shown to occur in the negative control groups which were administered soluble PRP with and without soluble PCMCs. These results may indicate that the boosting effect from the CaP-PCMC dose is not dependent on the retention of PRP on the crystal structure. The similarity of the responses between the CaP-PCMCs and the soluble PRP boosting responses may even indicate that the PRP boost may be solely from the free PRP released from the CaP-PCMCs, even if this is a small quantity.

As it has been determined, that the PRP in the formulations is retained as part of the crystals in a suspension, the trafficking of the CaP-PCMCs route *in vivo* might be responsible for the low immunogenicity. The next step is to investigate the uptake and transportation of the crystals by cells of the immune system.

Chapter 7. Uptake of CaP-PCMC formulations by cells of the immune system

7.1. Introduction

Upon immunisation, inflammatory signals are produced at the site of injection after tissue damaged caused by the insertion of the needle. The signals increase the migration of antigen presenting cells to the area, and therefore also the interaction with, and transportation of the antigen through the lymphoid system. The interaction between the antigen and the antigen-specific lymphocytes is limited to secondary lymphoid tissues such as the lymph nodes or the spleen (192). Therefore, antigen needs to reach the lymphoid organs to be able to interact with the cells of the immune system.

It can be assumed that for the particulate CaP-PCMCs to get to the secondary lymphoid tissues, there needs to be trafficking from the site of injection. Immature Dendritic cells and macrophages catch particulate (as well as soluble) antigenic material and carry it to lymph nodes via lymphatic vessels (182). Due to the size of the CaP-PCMCs, the particles are likely to be internalised by endocytosis or phagocytosis into the antigen presenting cells in the tissue (193), or be bound to the surface of the cell. Studies have shown that T cell responses are dependent on the dendritic cell migration through the lymphatic vessels, indicating that the transport is crucial for a successful immune response (194).

Naïve B cells circulate through the blood to the secondary lymphoid organs. It is here they encounter antigens and T cell clones specific for the same antigen. Within the lymphoid organs, naïve B cells and T cells are distributed in distinct zones; lymphoid follicles for B cells and the paracortex in the lymph nodes and the peri-arteriolar lymphoid sheaths in the spleen for T cells (195). A study by Liu et al (196) showed B blast cells were to be found in three sites; around interdigitating cells in the T cell-rich zones in the lymphoid organ; in the follicular dendritic cell network and in association with macrophages in the red pulp.

Memory B cells, which are not in cell cycle, accumulated in the marginal zones in response to T cell dependent and T cell independent antigens. It is these locations that are key for the B cell to act as an APC and present the T cell antigen for both induction of the T cell response, as well as induce full activation of the B cell, thereby enabling the development of memory B cells, Ig class switching and long-lived plasma cells (97).

Recent studies have investigated the B cell activation process *in vivo* by demonstrating that within minutes of injection, small soluble antigen diffuses directly through the SCS, leading to activation of follicular B cells according to their proximity to the SCS (197).

Studies of the delivery of particulate antigens, immune complexes and viruses, to follicular B cells were investigated with multiphoton microscopy (198-200). These larger antigens are found to localise at the SCS where macrophages can present the antigen and mediate the retention of specific B cells. This allows the B cells, having acquired antigen, migrated towards the boundary between B cell and T cell zones

to recruit cognate CD4+ T cell help for full activation, crucial for the T cell dependent memory response, as induced by the conjugate vaccine.

A schematic representation of the dynamics of antigen trafficking within the lymph node is shown in Figure 7.1. Lymphatic fluid containing soluble antigens or particulate antigens carried by immune cells and chemokines (red and green respectively) flow through the afferent lymph vessel into the lymph node. Soluble antigens may be able to diffuse from the pores in the SCS to access the antigen-specific B cells. Particulate antigens, such as CaP-PCMCs (diamonds) are taken up by SCS macrophages and can be directly presented to the Ag-specific B cell in the follicle. Particulate antigens can also be transported by nonspecific B cells to Follicular dendritic cells in a complement-dependent manner.
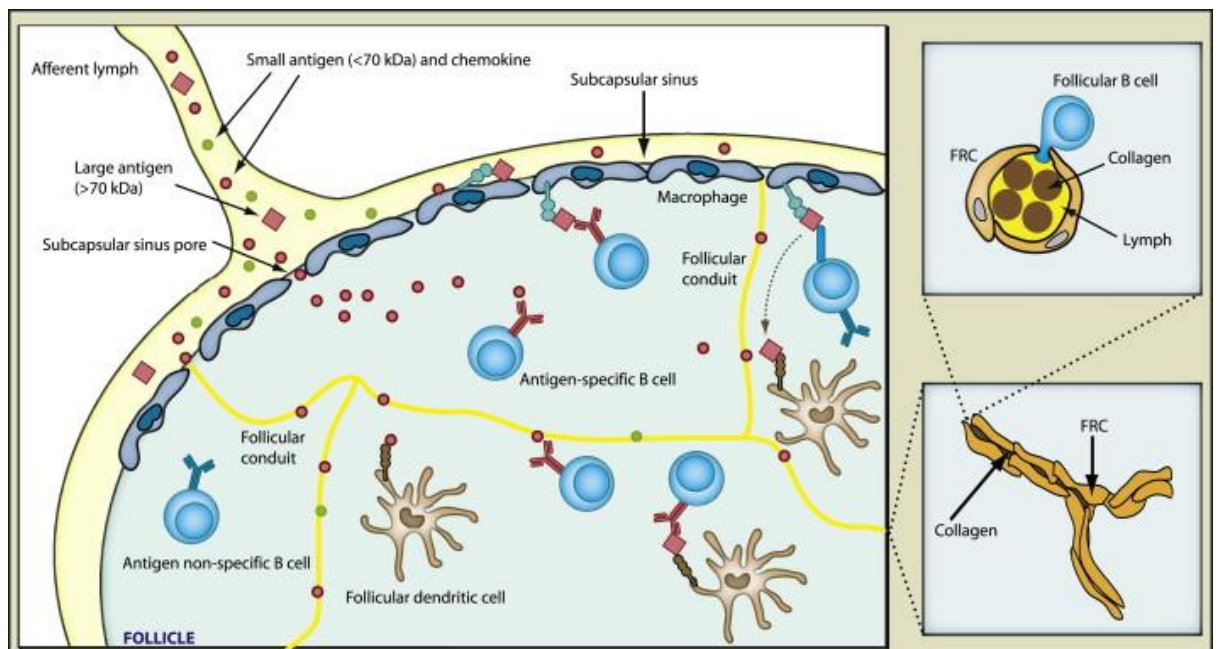
Figure 7.1. Antigen presentation to follicular B cells in the Lymph Node

Image taken from Batista and Harwood 2009 (192).

It has been shown that dendritic cells (DCs) are capable of capturing soluble antigens, whole microbes, apoptotic bodies and latex micro-particles (201-203), and therefore may be capable of taking up the CaP-PCMCs. It has been observed by Suzuki et al (204) that various antigens accumulating on the surface of follicular dendritic cells (FDCs) were detectable on the cell surface up to one week. Thiele et al (205) used micro-particles as an antigen delivery system to target macrophages and dendritic cells and found that positively charged particles became attached to the cell surface. They also found that the differently charged particles were phagocytosed by macrophages and dendritic cells at different rates, with negatively charged bovine serum albumin (BSA) coated particles taken up by macrophages at a slower rate than dendritic cells.

It has been previously demonstrated that macrophages take up CaP-PCMCs formulated with BSA-FITC sample antigen following 1 h of incubation (206). Confocal imaging clearly showed the intracellular location of the CaP-PCMC and flow cytometry data indicates that a large number of the macrophage population took up the BSA-FITC when formulated as part of the CaP-PCMC compared to the cells incubated with soluble antigens which did not show any uptake.

B cells have also been shown to capture antigen through their B-cell receptor (BCR). The cross-linking of the BCR triggers a cascade of signalling events which results in the activation of the B cell and initiate the development of germinal centres (GCs), required for the production of a high affinity antibody response. For this to occur, activated B cells must process and present the Ag on MHC class II molecules to

primed CD4 T cells. This process is known as T-cell-B-cell cooperation (207). The BCR is essential to modify the endocytic capacity of B cells. Resting mature B cells perform minimal endocytosis, but are efficient at specific Ag uptake, which optimises the presentation of the antigen at low concentrations (208). Binding of the BCR activates Src kinases which then act to phosphorylate clathrin and induces the association of the BCR to the lipid rafts which can then target the MHC class II containing intracellular compartments (209) where the antigen can be processed, bound to and presented by the MHC class II.

Results from the immunogenicity studies in Chapters 4 and 5 show that the CaP-PCMC formulation is not sufficiently immunogenic to induce an anti-PRP response comparable to that of the Hib-TT conjugate vaccine. Although it is shown that the PRP is retained on the crystal structure on the CaP-PCMC *in vitro*, it is possible that the crystals fail to reach the lymphoid organs due to poor uptake by innate immune cells or the recognition and uptake by B cells.

7.2. Aims and Objectives

The aim of this chapter is to investigate the ability of DCs and neutrophils to bind and take up CaP-PCMCs, which is necessary for trafficking of the CaP-PCMCs to B cells. This was carried out by incubation of dendritic cells and neutrophil-like cells with CaP-PCMCs containing fluorescent antigens and uptake of the crystals was measured by flow cytometry. Interactions between PRP-specific B cells and the PRP-CaP-PCMCs were investigated by isolation of splenocytes from Hib-TT conjugate and PRP-TT-CpG-CaP-PCMC immunised animals and incubation of the cells with fluorescently labelled PRP-CaP-PCMCs. The B cell uptake study will

determine if the crystals are able to be recognised through the PRP specific B cell

receptor and if any cellular interaction follows. In addition we investigated the

ability of B cells to interact with the antigens on the crystals.

7.3. Materials and Methods

7.3.1. Materials

Phosphate Buffered Saline (PBSA, 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl, 5% Bovine Serum Albumin, w/v)

Bovine Serum Albumin (BSA, Sigma, UK)

75cm2 flasks (Costar, Cambridge, MA USA)

Supplemented Culture Medium (RPMI-1640 (Sigma), 10% FCS, 1% L-glutamine, 1% Penicillin)

All-Trans-Retinoic Acid (ATRA, Sigma)

1α,25-Dihydroxyvitamin D3, (Vitamin D3 ,Sigma)

Granulocyte Colony Stimulating Factor (G-CSF, Chemicon)

Fetal Calf Serum (FCS, Invitrogen Life Sciences)

Trypan Blue Stain (Sigma)

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF; NIBSC; 91/658)

Interleukin-4 (IL-4, NIBSC, 91/656)

FACS tube (BD Falcon)

PBA buffer (PBS with 0.1% BSA, 0.1% Sodium Azide)

Hanks' Balanced Salt Solution (HBSS; Sigma)

OptiPrep™ Density Gradient Medium (Sigma)

FITC conjugated Anti-MHC class II monoclonal IgG1 antibody (AMS-32.1, Pharmingen)

PE conjugated Anti-mouse anti-CD11c monoclonal IgG1 antibody (HL3, Pharmingen)

PE conjugated Anti-rat anti-MHC class II monoclonal IgG2 antibody (eBioscience)

FITC conjugated BSA (eBioscience)

Alexa-fluor 647 conjugated OVA (eBioscience)

7-Aminoactinomycin D (7-ADD, eBioscience, UK)

FITC labelled PRP (prepared by Innova, UK)

Texas Red labelled TT (prepared by Innova, UK)

6-well tissue culture plates (Greiner, UK)

24 well tissue culture plates (Greiner, UK)

96 well black plastic plates (Greiner, UK)

7.3.2. Preparation of CaP-PCMCs with fluorochrome-labelled antigens and determination of total and soluble antigen content

Five different CaP-PCMCs were prepared as described in section Chapter 2, with 9.4% CaP, a 5:2 $CaCl_2$:NaH_2PO_4 ratio and a 1 step process. Formulations were prepared with only PRP-FITC, a combination of PRP-FITC and TT-Texas red, BSA-FITC and OVA-Alexa Fluor 647, PRP (unlabelled) and OVA-Alexa Fluor 647 and OVA alone (Table 7.1).

Table 7.1. Antigen Loading on formulations prepared

Formulation	Antigen	Loading (%, w/w)
1	PRP-FITC	0.5
2	PRP-FITC	0.5
	TT-Texas Red	0.05
3	BSA-FITC	0.5
	OVA- Alexa Fluor 647	0.5
4	PRP (unlabelled)	0.5
	OVA- Alexa Fluor 647	0.5
5	OVA- Alexa Fluor 647	0.5

To determine of the level of total and soluble antigens in the CaP-PCMC formulation, the crystal solution and supernatant (after centrifugation of the CaP-PCMC suspension) are collected as in chapter 3. However, the only difference was that the CaP-PCMC concentration was 4mg/mL (a theoretical antigen concentration of 20μg/mL), instead of 10mg/mL.

In addition, as the antigens used were covalently conjugated to fluorochromes, measurement of the fluorochrome signal was used to detect the level of total and free antigen on the various CaP-PCMC formulations.

To quantify the fluorochrome-conjugated antigens, 200μL of supernatant and dissolved CaP-PCMCs were added to wells in the first column of a black 96-well plate. Multiple 1:1 dilutions were performed across the plate in PBS. The fluorescence level was read at 512nm (excitation at 488nm) for FITC, at 615 (excitation at 596) for Texas-red and at 665 (excitation at 650) for Alexa-fluor 647 fluorescence. Levels of total and soluble antigen on the CaP-PCMC crystals were calculated from a standard curve generated from serial dilutions of the soluble labelled antigen. The level of soluble antigen was expressed as percentage of the total antigen content of the CaP-PCMCs.

7.3.3. Differentiation of HL-60 cells into neutrophil-like cells

HL-60 cells were differentiated into neutrophil-like cells using a method previously described (210) .This method was optimised for the cell culture at NIBSC and the results can be seen in section A.1. HL-60 cells were seeded at 1×10^5 cells/mL in $75cm^2$ flasks in supplemented culture medium and incubated at 37°C and 5% CO_2. The culture medium was changed every 48-72 hr and cells were passaged upon reaching confluence.

When the cell number reached 10^8 cells per flask, cells were collected and centrifuged to remove culture medium. Cells were differentiated by culturing them in 80mL of RPMI supplemented with 2.5μ M ATRA, 9.6fM Vitamin D3 and 30ng/ml G-CSF in a flask for 3 days at a seeding density of 10^5 cells per flask.

The cells were then harvested and assessed for level of differentiation by measuring the level of expression of CD11b, using a CD11b specific monoclonal antibody, immuno-fluorescent staining method and flow cytometry analysis.

7.3.4. Isolation, differentiation and enrichment of murine bone marrow-derived dendritic cells

Dendritic cells were generated from mouse bone marrow precursors using a method previously described by Inaba et al (211). .This method was optimised for the cell culture at NIBSC and the results can be seen in section A.2. Mouse tibias and femurs were collected from female 6-8 week old Balb/c mice and dipped into 70% ethanol to reduce contamination. The bones were then washed in RPMI and placed in RPMI containing 100U/mL Penicillin, 100µg/mL Streptomycin, 2mM L-glutamine and 10% heat inactivated FCS.

To collect the bone marrow cells, a 25-guage needle was used to flush cold RPMI through the bone shafts into a container. The collected cells were washed twice by centrifugation at 1200rpm for 10 min. Cell viability was determined using trypan blue stain and cell number was adjusted to $1x10^6$ cells/mL in RPMI supplemented with 20ng/mL of GM-CSF, and 20ng/ml of IL-4. Cells were cultured in 24-well tissue culture plates in a final volume of 2mL/well at 37°C and 5% CO_2. The culture medium was changed every 2-3 days by exchanging 50% of the old medium (while cells are settled at bottom of well) with the same volume of freshly made GM-CSF and IL-4 supplemented culture medium. Cultures were maintained for a total period of 4-11 days.

To enrich for dendritic cells at the end of the culture period, cells were harvested by gentle pipetting and centrifugation for 10 min at 1000 rpm. The cells were then washed three times in HBSS by centrifugation for 5 min at 1000 rpm. Pellets were resuspended in 3 mL of HBSS and cell viability was determined. Dendritic-like cells

were then enriched using an OptiPrep™ Density Gradient Medium (60% (w/v)

solution of iodixanol in water, ρ=1.32g/mL) as follows: 3mL of cell suspension

containing dendritic cells were gently mixed with 1mL of Optiprep stock (giving a

15%(w/v) iodixanol solution; ρ=1.085g/mL). 5mL of an 11.5% (w/v) iodixanol

solution (ρ=1.065g/mL), was carefully overlaid on top of the cell suspension and this

was carefully overlaid with 3mL of HBSS. Following centrifugation for 20 min at

1000 rpm, the rotor was allowed to decelerate without the break and dendritic cells

were visible as a band at the interphase between the 11.5% iodixanol and HBSS.

Cells at the interphase were harvested and washed twice in culture medium to

remove iodixanol.

To determine the number of dendritic cells in the cell population, the level of

expression of MHC class II molecules and of CD11c were measured using specific

monoclonal antibody, immuno-fluorescent staining method and flow cytometric

analysis.

7.3.5. Preparation of B cells

Six to eight week old female Sprague-Dawley rats were immunised on days 0, 21

and 42 with either the conjugate Hib-TT vaccine or a CaP-PCMC formulation

prepared with 0.5% PRP, 0.05% TT and 0.5% CpG at a PRP dose of 1μg PRP per

animal. Animals were sacrificed 10 days after the 3rd immunisation and the spleens

were harvested.

Single cell suspensions were prepared from individual spleens and lymph nodes as

described previously in chapter 4. To remove macrophages, cells were then

incubated in RPMI (supplemented with 1% Penicillin/Streptomycin, 1% L-glutamine

and 10% FCS) in 6 well plates at a concentration of 2 x 10^6 cells per mL in 4mL

volumes for 1 hour at 37°C and 5% CO_2. The supernatant was then harvested

leaving the adherent macrophage population on the plate.

To determine the number of B cells in the cell population, the level of expression of

MHC class II was assessed using specific monoclonal antibody and immuno-

fluorescent staining method and flow cytometric analysis. The results of this

process can be seen in section A.3.

7.3.6.   Uptake of the CaP-PCMCs by immune cells

HL-60, DCs or B cells were adjusted to 2 x 10^6 cells/mL. The cell suspension was

dispensed in 1mL volumes into wells of a 24-well plate. One mL of the different

concentrations of freshly prepared CaP-PCMC suspension or control (soluble

antigen) were then immediately added to the wells. The plate was incubated at

37°C and 5% CO_2 for various time periods. In one experiment, HL-60 cells were co-

cultured with CaP-PCMCs that had been pre-incubated in a RPMI suspension for the

different time periods. The cells were then harvested and prepared for flow

cytometric analysis

7.3.7.   Immuno-fluorescent staining and FACS analysis

Cells were adjusted to approximately 2x10^6 cells/mL, 100µLs of the cell suspension

is added to a FACS tube. 2µl of the fluorescent monoclonal antibody or appropriate

isotype control was added to each tube and cells incubated for 40-60mins on ice.

The cells were then washed twice in PBA buffer and fixed in 0.5ml of 2%

formaldehyde in PBS.

In the case of the study of uptake by B cells, the cell population was also stained with a monoclonal antibody specific for MHC Class II before being fixed in the 2% formaldehyde.

When cells were co-cultured with CaP-PCMC, all samples were subject to an additional two washes in Tris/Citrate/EDTA buffer to dissolve the CaP-PCMCs remaining in the culture medium to allow analysis by flow cytometry, before being finally resuspended in 500μL of 2% formaldehyde in PBS.

Flow cytometric acquisition was performed using a BD FACSCanto II flow cytometer machine, 10^6 events were collected and analysed for frequency of stained cells and the intensity of the staining using BD FACSDiva Software.

7.4. Results and Discussion

7.4.1. Uptake of CaP-PCMCs with both BSA-FITC and OVA-Alexa-fluor-647

The differentiation of a HL60 line into neutrophil-like cells was optimised. The optimum number of days was determined to be 3 days (see Appendix), which was shorter than the -9 days seen in previous studies (212), a difference that may be due to slight differences in the age and condition of the cell line.

Both TT and PRP were difficult to label with fluorescent tags, it was for this reason that other labelled antigens with improved stability were chosen to use as model antigens adsorbed to the CaP-PCMC. A study was designed with CaP-PCMCs containing two fluorescent antigens, OVA-Alexa-fluor 647 and BSA-FITC.

The total and soluble antigen content in the CaP-PCMCs were measured, to demonstrate that there would be enough fluorescent signal from the CaP-PCMCs to be detected at the location of the crystals. Analysis of the total and soluble content of the BSA and OVA, as measured by fluorescence assays showed that 36.2% of the BSA-FITC is adsorbed to the crystals in this formulation, and of this, 1.6% was soluble. The OVA content was 23.3% and of this, 14.4% was found to be soluble. Although the antigen content was not as high as intended, the level was considered appropriate to detect antigen uptake within the cells by flow cytometry.

Table 7.2. Total and soluble antigen content in CaP-PCMCs containing 0.5% BSA-FITC and OVA-Alexa Fluor 647

	BSA-FITC	OVA-Alexa Fluor 647
Total antigen (%)	36.2	23.3
Soluble antigen (%)	1.6	14.0

The total and soluble antigen content of two antigens on the CaP-PCMC formulations (with 0.5% BSA-FITC and OVA-Alexa Fluor 647 loading) was determined by measurement of fluorescent intensity.

The differentiated HL-60 cells when incubated with the crystals for 10, 30, 60 and 120 min at CaP-PCMC concentrations of 1 or 2mg/mL. Control cells were incubated within culture medium alone, or soluble BSA-FITC and OVA-Alexa Fluor 647. The flow cytometric analysis is shown in Figure 7.2.

Analysis of the live cells only showed that the cells incubated with the soluble antigens had a low level of uptake of OVA-Alexa Fluor 647 or BSA-FITC with few cells taking up both soluble antigens (6.8%) after two hr. Cells incubated with CaP-PCMCs showed that following a 10 min incubation, the percentage of cells positive for both antigens are 82.4% and 92.2% at the 1mg/mL and the 2mg/mL concentrations of CaP-PCMCs. In cells incubated with 1mg/mL of CaP-PCMCs, the effect of time seems to decrease the percentage of cells with both antigens from 82.4% to 67% at 2 h. This effect could be due to apoptosis in cells after uptake of the crystals. At the 2mg/mL concentration, the cells positive for uptake of both antigens remain over 90% for 1 h. The time independent response seen at the

higher CaP-PCMC concentration could be due to early uptake and retention of CaP-PCMCs for the duration of the experiment. The high number of double positive cells are a good indicator that both the antigens are taken up by the cell and are likely to be retained onto the crystal at least up the point of uptake by the cell.

The fast uptake of the crystals within 10 min of incubation seen in this experiment may not be representative of the *in vivo* environment. Although neutrophils arrive at the site of inflammation within minutes (213), the concentration used for immunisation in the immunogenicity studies are much lower and the concentration will be much more diluted when encountering the immune cells.

To more accurately simulate the *in vivo* environment where the crystals may remain in a suspension, for example within the interstitial space, for a longer period of time before cellular interaction, the CaP-PCMCs were incubated in the media at 37°C for various time periods. The CaP-PCMC suspension was then added to the differentiated HL-60 cells and incubated for another 10 min. If the incubation time of the CaP-PCMC in the cell media resulted in any disintegration of the CaP-PCMCs or loss of antigens from the crystal, this would be seen in lower levels of uptake with increased incubation times.
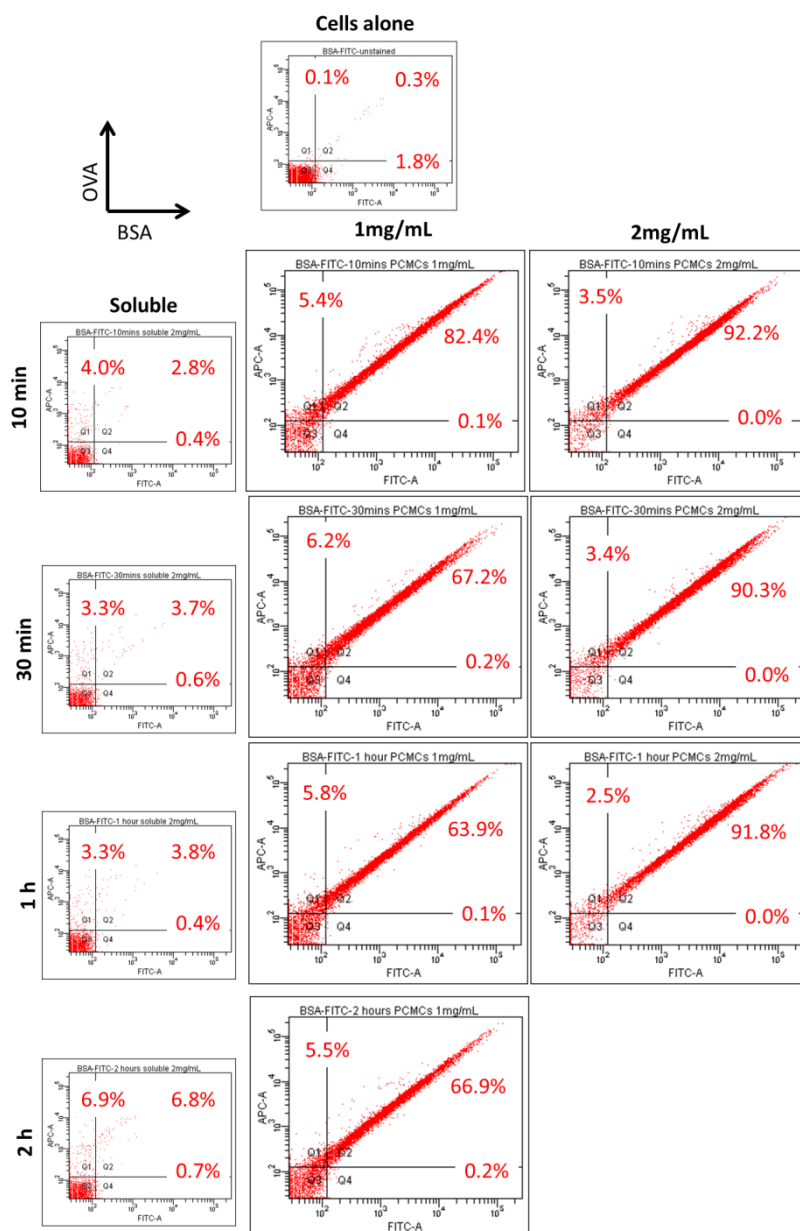
Figure 7.2. Uptake of BSA and OVA CaP-PCMCs in differentiated HL-60 cells.

Differentiated HL-60 cells were incubated with CaP-PCMCs contained 0.5% loaded BSA-FITC and OVA-Alexa-Fluor-647, control cells were incubated in culture or medium or with 10µg/mL of soluble BSA-FITC and OVA-Alexa-Fluor-647 (equivalent to theoretical on 2mg/mL).

Results resented in Figure 7.3 show that cells positive for CaP-PCMC uptake at each time point. However, the plots show high levels of double positive cells, starting at 76.1% after 10 min and peaking at 2 h at 99.9%. As shown before, the cells

incubated with the combination of the soluble control antigens and the blank-PCMCs showed a very small increase of uptake of both antigens with time, increasing from 1% of the total population after a 10 min pre-incubation, to a 2% at 30 min, a 7.7% at 50 min, and 13% of the population at 110 min.

These results indicate that the pre-incubation of the CaP-PCMCs in culture media at 37°C for up to 50 min did not reduce the uptake of particles compared to those seen in Figure 7.2. This may indicate that the crystal incubation can cause the cells to die after longer than 10 min incubation, or the incubation in the media could be decreasing the crystal size, thereby enabling easier cellular uptake. It also indicates that the crystals remain intact in media, as the majority of the cells have taken up both antigens at similar intensities.
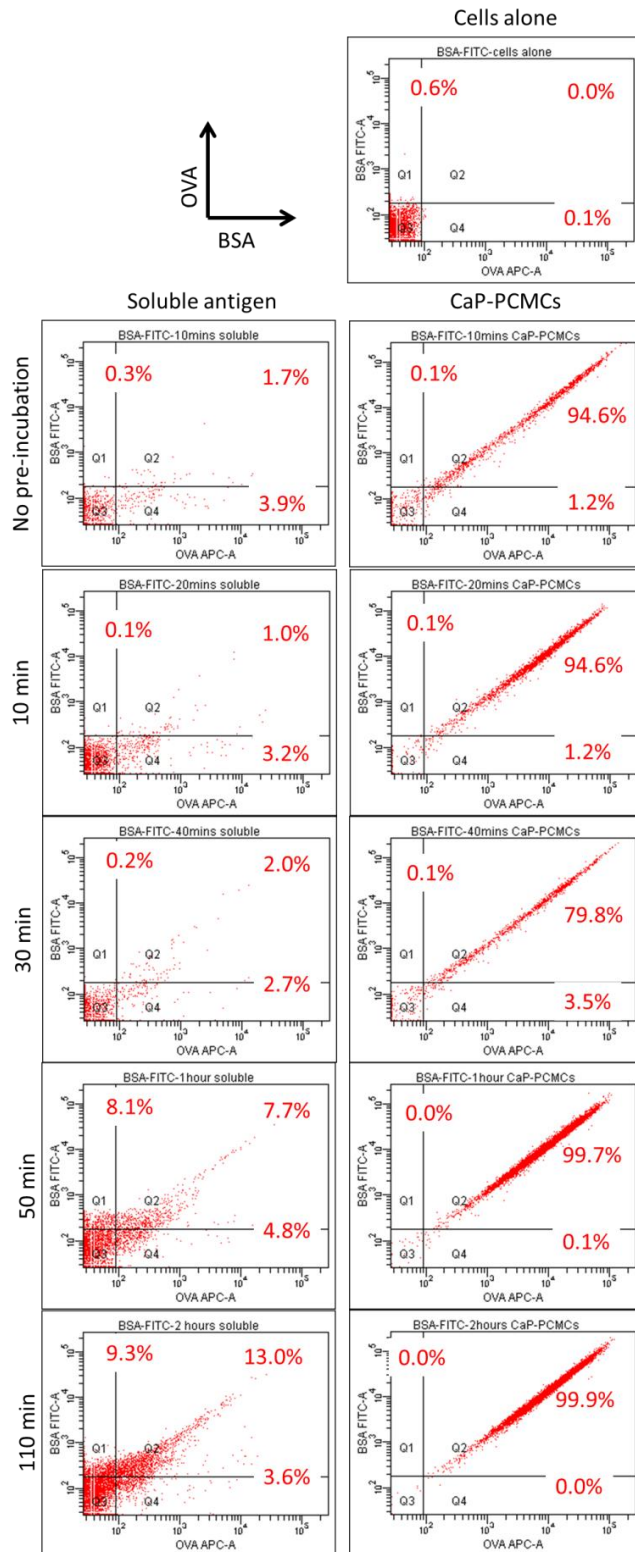
Figure 7.3. Uptake of OVA and BSA by neutrophil-like cells when cells are incubated with CaP-PCMC suspension pre-incubated in media.

BSA-FITC and OVA-Alexa-Fluor-647 loaded CaP-PCMCs were incubated in cell media at 37°C for 0, 10, 30, 50 and 110 min before addition to neutrophils for 10 min, control cells alone or cells incubated with a solution of 10µg/mL of BSA-FITC and OVA-Alexa-Fluor-647.

7.4.2.   Interaction of DC cells with CaP-PCMCs

The optimisation of isolation and enrichment of DC cells from a primary cell culture was carried out, the details of the experiments can be seen in the appendix. The method used was based on that described in a study by Inaba et. al. (211), and similar findings of differentiation time were confirmed.

To investigate interaction between the CaP-PCMC and the DCs, the cells were incubated with the OVA-Alexa-Fluor 647 and the BSA-FITC formulation in a similar experiment to that used with the differentiated HL-60 cells. The uptake of either antigen into the DCs is shown in Figure 7.4. Control cells were incubated with soluble antigens or with soluble antigens and blank CaP-PCMCs.

It can be seen that uptake of either antigen was less than 1% of the total population in cells incubated with the soluble antigen. At 10 min, the cells incubated with soluble Ag only had 36% of the population positive for OVA, and had 43% of the cells positive for OVA when incubated with both soluble antigens and the blank PCMCs. Over time, this only increases to 56.3% at 30 min, and 68.4% at 1 h in the soluble antigen group, and 62.1% at 30 min and 71.6% with 1 h incubation of the soluble Ags with the blank CaP-PCMCs. In the same groups, the soluble BSA- FITC was also taken up by the cells, although at a slightly lower level and more delayed kinetics, 27.8% and 36.5% of BSA positive cells at one hour incubation with soluble antigens or soluble antigens and blank PCMCs respectively. These high rates of uptake of antigen in the soluble form may show that DCs are capable of taking soluble antigen.

When all cells are cultured with CaP-PCMCs (at 2mg/mL) containing BSA-FITC and OVA-Alexa Fluor 647 a much higher proportion of the cells have taken up the antigens (72.7% after 10 min). These values increase over a time period, reaching 89.2% double positive cells at 1 h. This timescale is longer than that of the neutrophils, which is not surprising as dendritic cells may either take up or bind to the antigens slower than neutrophils which take up antigen by phagocytosis (203) It is also clear from the results that there is a high correlation between uptake of BSA and OVA in the CaP-PCMC incubated cells compared to the cells with soluble antigen. This result indicates that the uptake must be from crystals which contain both antigens.
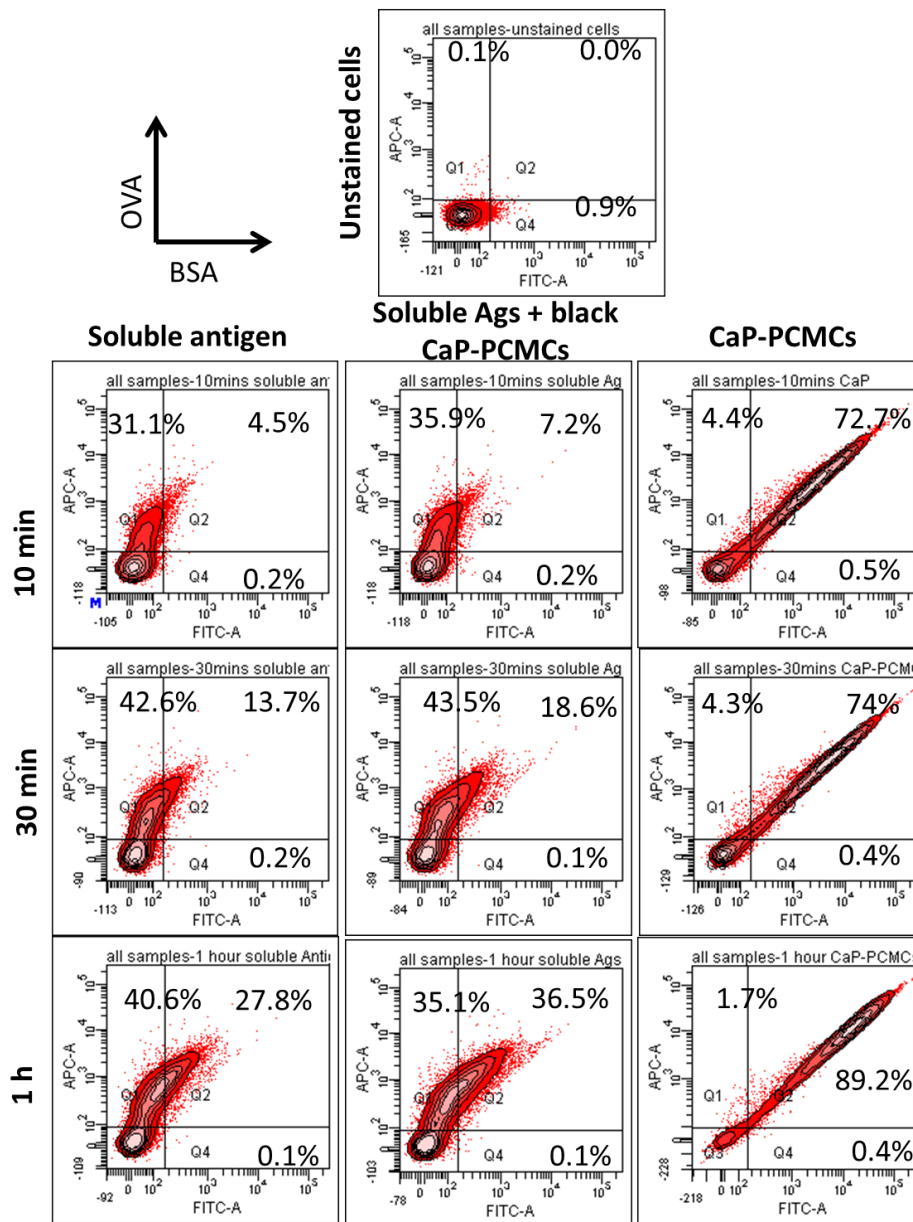
Figure 7.4. Uptake of two antigens on CaP-PCMCs into Bone Marrow Derived Dendritic Cells

Dendritic cells were incubated with 2mg/mL BSA-FITC and OVA-Alexa-Fluor-647 (read through APC channel) labelled CaP-PCMCs in cell media for 10, 30 and 60 min. Control cells were incubated alone or with 10µg/mL of soluble BSA-FITC and OVA-Alexa-Fluor-647 with and without blank CaP-PCMCs which are not loaded with any antigen

7.4.3.   Uptake of CaP-PCMCs by B cells

For successful induction of an anti-PRP response following immunisation with the crystals, it is important that, given successful trafficking of the CaP-PCMCs to the lymphoid organs, the crystals containing both antigens would still be recognised, taken up and processed by the PRP specific B cell. To investigate if crystals could be recognised by PRP-specific B cells, the uptake of PRP-CaP-PCMCs was measured in splenocytes isolated from rats immunised with either the Hib-TT conjugate or the PRP-TT-CaP-PCMC to increase the frequency of PRP-specific B cells. As it was not possible to label PRP successfully with a fluorochrome, CaP-PCMCs which were prepared with unlabelled PRP and OVA-Alexa-Fluor-647. The uptake of these CaP-PCMCs by B cells could be measurable by detection of OVA-Alexa Fluor 647. Non-specific uptake by non-PRP specific B cells will be measured with the use of CaP-PCMCs prepared with just OVA-Alexa Fluor 647 (without PRP).

7.4.4. Interaction of CaP-PCMCs with B cells

A similar CaP-PCMC formulation was prepared, but with only fluorescently labelled OVA, as well as non-labelled PRP. The retention of both antigens was found to be satisfactory, as can be seen in the Appendix. To perform the uptake study, (macrophage depleted) splenocyte cultures were incubated with 1mg/mL of the CaP-PCMC suspension for 1 h. Cells were then analysed by flow cytometry after staining for MHC class II expression.

The cells incubated with the soluble OVA-Alexa Fluor 647, showed high levels of OVA uptake in both the MHC class II positive and negative populations (26.8% and 30.5% respectively in the Hib-TT conjugate immunised group). This was similar to results obtained from cells of animals immunised with CaP-PCMC immunised group (35.7% and 28.8% in MHC II positive and negative cells respectively). The uptake into the MHC class II positive group may have been due to pinocytosis of the soluble antigen, as has been shown to occur in CD4+ T cells (214).

It was seen that in both the groups immunised with the OVA-CaP-PCMCs and the soluble OVA-Alexa-Fluor-647, there were two populations, one with higher MHC CLASS II levels and one lower. However, within each of these populations there appears to be continuous spread of levels of OVA-Alexa-Fluor-647, which may indicate cells from both populations taking up varying quantities of soluble antigen or OVA-CaP-PCMC non-specifically.

Interestingly, the splenocytes from both groups, when incubated with the PRP-OVA-CaP-PCMCs showed lower levels of OVA uptake, with only 19.2 and 18.2% of the cells from the Hib-TT conjugate and the CaP-PCMC immunised group (respectively) showing fluorescence from the OVA-Alexa-Fluor 647, which may be due to the size of the crystals. In both the groups, a large proportion of MHC class II positive cells (65%) have taken in CaP-PCMCs. About two thirds the number of the cells which had taken up particles were MHC class II negative, which might indicate at that least some proportion of the B cells taking up the crystals through BCR-dependent binding and endocytosis. The lower level of fluorescence intensity for OVA positive cells may also indicate that fewer crystals were taken up by the cells, but it is not known whether this uptake is mediated by BCR induced endocytosis or not.
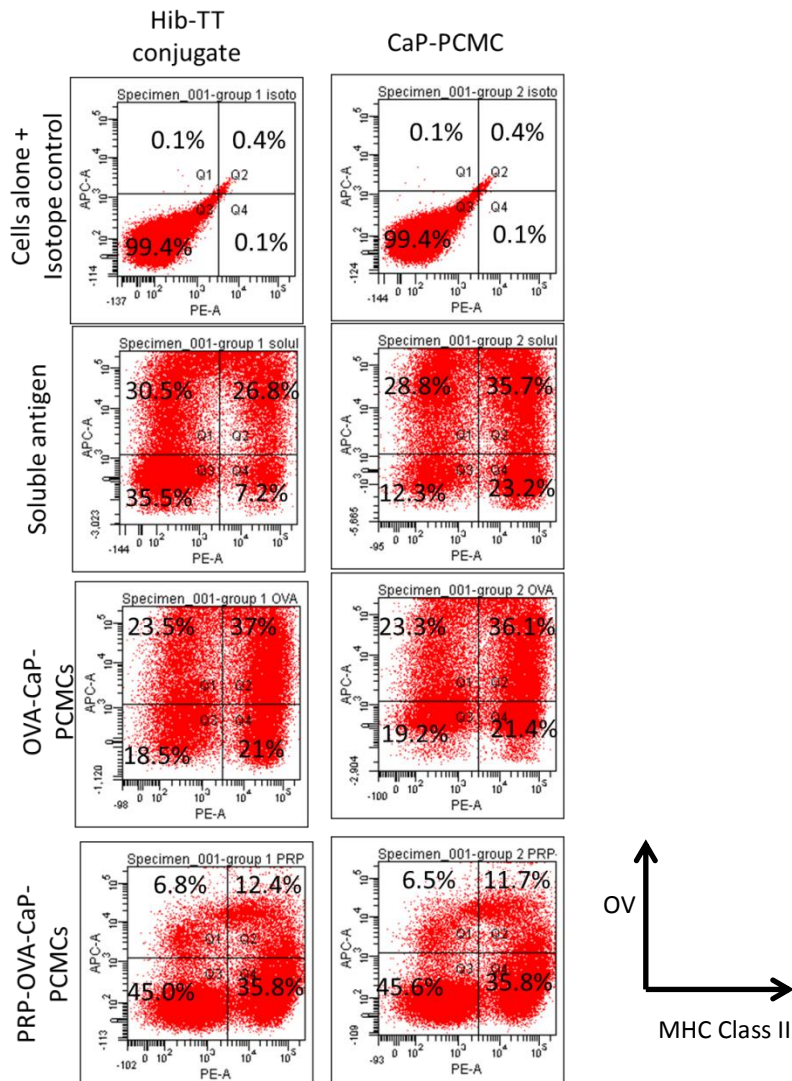
Figure 7.5. Uptake of CaP-PCMCs and soluble antigen by splenocytes from rats immunised with a Hib-TT conjugate and a CaP-PCMC suspension

Splenocytes from rats immunised with 3 doses of CaP-PCMC suspension or Hib-TT conjugate were harvested negatively selected for macrophages and incubated with 1mg/mL of CaP-PCMCs formulated with both PRP and Ova-Alexa-Fluor-647 CaP-PCMCs. Control cells were incubated with CaP-PCMCs formulated with just Ova-Alexa-Fluor-647 or soluble Ova-Alexa-Fluor-647. Representative plots from one animal out of group of 5 shown.

7.5. Conclusions

The results from this chapter suggest that cells of the innate immune system, such as neutrophils and DCs are able to bind and take in the CaP-PCMCs, which may help to transport the antigen to the lymphoid organs which happens with intact crystal structure. Neutrophils were not able to take in the soluble antigen, but did take up the crystals, whilst dendritic cells

Dendritic cells also appear to take up CaP-PCMCs with two adsorbed antigens. However it is not clear if the same will happen *in vivo*. To better investigate the trafficking route of the CaP-PCMC *in vivo*, a live-imaging system could be utilised with infra-red labelled antigens. The measurement and tracking of two antigens *in vivo* would also be used to assess the stability of the crystal and the delivery of both antigens as a virtual conjugate.

The B cell uptake results may indicate that B cells are able to bind the soluble and CaP-PCMC antigens, but there is non-specific uptake of the CaP-PCMCs. This uptake may act to reduce the pool of CaP-PCMCs available if the uptake is high enough by the non-specific B cells. The uptake could also be occurring through remaining macrophages or dendritic cells, although the numbers positive for OVA and MHC CLASS II appear too high for this to be possible. Previous studies have focussed on using imaging techniques to track an antigen of interest through the lymphoid organ. It may be possible to use a fluorescently labelled antigen adsorbed to the crystal formulation to track if the crystal can be trafficked through the lymph vessel to the B cell zone.

Chapter 8. General Discussion and Further Work

The development of a thermostable and more cost-effective virtual protein-polysaccharide conjugate will provide a cheap vaccine for the immunisation of infants in areas where the current vaccine is not utilised due to the high current cost of production, purification and the requirement of the cold-chain (104, 215). Polysaccharides require stable conjugation to a protein antigen in order to induce a protective antibody response, however this process is costly and therefore has proved prohibitive for use in poorer areas in the past (27). CaP-PCMCs have been used to stabilise several antigens and have been investigated as a potential for vaccine delivery (206), as they have been shown to be thermostable over long periods of time (149). The aim of this project was to use this technology to prepare crystals that act to virtually conjugate the polysaccharide and the protein.

A vital part of the crystal formulation is the insoluble Calcium Phosphate (CaP) layer that theoretically coats the crystal and prevents the crystal core from dissolving. To act as a virtual conjugate, both PRP and TT antigens need to remain adsorbed on the surface of the CaP-PCMC as the crystals are trafficked to follicular B cells. The quality of the CaP layer was only measurable by the quantification of the soluble PRP/TT release upon suspension of the crystals in Phosphate Buffered Saline (PBS).

The crystals were prepared using two main methods to alter the formation if the CaP-layer. The single/one-step method involved the rapid precipitation of the glutamine, the antigens and the CpG, in the Isopropyl Alcohol which contains the Calcium Chloride ($CaCl_2$). The two-step method involves adding the $CaCl_2$ in a

second step, giving a delayed formation of a CaP-layer and therefore potentially changing the structure of the crystal. Other factor such as temperature and CaCl2 concentration were also thought to be able to alter the rate at which the CaP layer on the PCMC formed and therefore the stability of the crystals in an aqueous suspension.

The aim of the initial experiments described in Chapter 3 was to characterise the shape of the CaP-PCMCs through the use of Scanning Electron Microscopy (SEM). The CaP-PCMCs were formulated using various preparation parameters to determine how these parameters might influence the morphology, and thereby the immunogenicity. An optimal vaccine delivery system should mimic a pathogen in size and shape (216), and it has been shown that for the best antibody responses, a particle in the micron range of size would be desirable (178, 217). Following the SEM imaging of the CaP-PCMCs, the water content during the preparation process was found to alter the morphology and size of the crystals. Cap-PCMCs with water contents of 5, 7 and 10% in the preparation mix were therefore selected to be tested for immunogenicity.

Investigation into antigen retention on the CaP-PCMCs was therefore carried out in Chapter 4. Chemical assays including Orcinol and BCA protein assay showed that both the PRP and the TT were efficiently adsorbed to the crystal during the preparation process. However, the quantity of either antigen on the CaP-PCMCs was not effectively measured using ELISA. These results sparked concern about the antigenic quality of the PRP on the crystals. By using ELISA on various formulations, we found that the presence of calcium from the calcium phosphate in the

formulation was changing the PRP in a way that was not allowing its detection by ELISA. However the mechanisms by which this may have occurred is unknown and require further investigation.

The immunogenicity of the first formulations were carried out by subcutaneous immunisation in the rat model with the CaP-PCMC suspensions in Chapter 5. The various crystal properties (resulting from different preparation parameters) were tested and the crystals were shown to be poorly immunogenic in terms of inducing anti-PRP antibodies. The three responding animals were found to be from groups immunised with the CaP-PCMCs prepared with a 7% or 10% water content. These water contents are higher than in the formulation prepared with 5% water content and was used in the pilot study. However, the levels of anti-PRP IgG responses in our study were lower than those in the pilot study. The use of the adjuvants MPLA, Pam2Csk4, Poly(I:C) and QuilA were also investigated with the aim of increasing the immune response to the crystals. However, the increased stimulation of the immune system by the adjuvants was not sufficient to induce a robust anti-PRP IgG antibody response. In contrast to the low anti-PRP responses, high anti-TT responses were induced in all groups of rats, and indicate that the TT on the crystal formulation remained immunogenic.

The low immunogenicity of the CaP-PCMC formulation could be due to changes in the in the PRP during formulation, release of adsorbed PRP from the formulation or ineffective trafficking of the entire crystals to follicular B cells. Some changes in the antigenicity of the PRP was indeed shown to occur when it was exposed to calcium chloride during the preparation process, through the use of ELISA on various

soluble PCMC formulations. However, the anti-PRP antibody response induced in some of the animals immunised by Cap-PCMC was higly specific for both the unformulated (native) PRP and when the PRP was part of a CaP-PCMC (Chapter 4), indicating that the changes seen in the antigenicity of the PRP during formulation have not completely changed its ability to induce PRP-specific antibody response.

Investigation into the release of soluble PRP from the CaP-PCMC upon the latter's suspension in an aqueous medium (for injection into animals) was not carried out initially, as previous results from XstalBio indicated a PRP release of less than 5% from the CaP-PCMC formulations when the Cap-PCMC was suspended in water (data not shown). Following the low immunogenicity results in Chapter 4, the release of PRP from CaP-PCMC formulation was tested, this time following the suspension of the CaP-PCMC in PBS, rather than water, as PBS was used in our immunogenicity studies. In contrast to earlier results by XstalBio, we found a high release of PRP, 73% as discussed in Chapter 4.

The high PRP release being a problem, screening of various calcium phosphate (CaP) layer preparation parameters by XstalBio resulted in the selection of two formulations with low PRP release, one prepared with monobasic sodium phosphate and another with dibasic sodium phosphate. Both of these formulations had a higher excess of $CaCl_2$ (5 times instead of 2 times reacting with the sodium phosphate), which might have acted to increase the speed at which the calcium phosphate layer is formed on the PCMC, or the efficiency with which the sodium phosphate reacts to form the calcium phosphate layer, and therefore increase the quality of the CaP layer and thereby reduce the release of soluble PRP. The two

formulations were tested for immunogenicity, in Chapter 6, and it was found that, once again, the PCMC formulation was poorly immunogenic.

Having demonstrated that the low immunogenicity of the formulation was not due to changes in PRP during formulation preparation, or high release of adsorbed PRP, it was hypothesised that the trafficking of the formulation to PRP-specific follicular B cells may be inadequate. The formulation was therefore injected intraperitoneally (instead of subcutaneously), as this route has been found to improve the mobility of particulates (218). In addition, aluminium hydroxide was added to the CaP-PCMC suspension to decrease the quantity of soluble antigen entering into the lymph, as has previously been demonstrated (219). Once again, low anti-PRP responses were obtained for the IP route of immunisation with or without Aluminium Hydroxide. There were two high responding animals from the group (of five animals) administered three doses of the CaP-PCMCs prepared using monobasic sodium phosphate and administered intraperitoneally at 1µg PRP per dose. Therefore these conditions were further investigated. The improved immunogenicity of the CaP-PCMC prepared with the monobasic sodium phosphate could have been due to the lower PRP release from this formulation compared to the formulation prepared with the dibasic sodium phosphate. The efficacy of the antibody response was also investigated using a bactericidal assay against Hib bacteria (Chapter 6). Sera from the responding animals did kill the Hib colonies in this assay, demonstrating that when animals respond to the immunisation with Cap-PCMC, the induced response is PRP specific and efficacious

To improve the immunogenicity of these formulations, more adjuvants were tested, including Addavax , aluminium phosphate, QuilA and MPLA with the aim of increasing the trafficking of the CaP-PCMCs to the anti-PRP follicular B cells. However, these adjuvants did not improve the anti-PRP response.

It was possible that the cellular response induced by TT may not be effective at co-stimulating the PRP-specific B cells, however the proliferation responses of the splenocytes from the CaP-PCMC immunised animals in response to TT stimulation indicate that this is not the case. It was also thought that a potential reduction in the pH at the site of injection following the administration of PBS might be causing a degradation of the CaP-PCMC structure with a concomittent release of CaP-PCMC adsorbed PRP. Therefore the use of phosphate buffer without saline was investigated to decrease any such effect. However, immunogenicity results from immunisation with the two buffers did not show any increase in immunogenicity in the absence of saline. *In* vivo, pH at the site of injection was then measured and was not found to decrease following injection of CaP-PCMCs in PBS or phosphate buffer. This may explain the lack of difference in the immune responses when phosphate buffer or PBS were used to administer the CaP-PCMCs.

Having demonstrated that the CaP-PCMCs are not immunogenic enough to prime the anti-PRP response, it was thought that CaP-PCMCs might be able to boost an existing anti-PRP response. This was investigated in Chapter 5 by priming rats with Hib-TT conjugate followed a booster dose of the Hib-TT conjugate or CaP-PCMCs. The CaP-PCMCs were found to be as effective as soluble PRP at boosting the anti-PRP IgG response, indicating that PRP-specific B cells were able to recognise and

respond to the PRP formulated in Cap-PCMC. However, the results also indicate that the formulation of PRP into a virtual conjugate does not confer any advantage in this situation.

Interactions between the cells of the immune system and the CaP-PCMCs were investigated using flow cytometry in Chapter 7. CaP-PCMCs were prepared with two fluorescent antigens to model the two antigens on the PRP-TT-CaP-PCMCs. Both neutrophil-like cells and dendritic cells were found to take up the two antigens effectively. It is possible that uptake into, rather than presence of the crystals on the surface of the cells may result in degradation of the crystal and the delivery of single antigens. The study of the kinetics of the uptake and processing of the crystals by dendritic cells and neutrophils can be further investigated using confocal microscopy of the crystals in cell culture. Live cell imaging would be able to demonstrate the kinetics of this process in real time. Interestingly, B cell uptake of PRP-CaP-PCMCs was shown to be just as effective as the uptake of CaP-PCMCs formulated without PRP. This result indicates that conditions *ex vivo* may not be optimal for specific uptake studies. This uptake study can also be optimised by use of a cell separation technique to enrich the cell population for B cells using specific markers such as B220 as well as MHC class II.

To further investigate the trafficking of CaP-PCMCs *in vivo*, imaging systems such as Li-COR system can be used with fluorescently labelled antigens to track the movement of the two antigens in the animal model. The information gained will indicate whether the delivery of both antigens to the follicular B cells is occurring, or if the particles are not acting as a virtual conjugate. Similar techniques have been

used for lymph node mapping (220-222) and by developing a CaP-PCMC containing an antigen labelled with a dye that fluoresces in the far red region, the location of the antigen within the lymphoid system can be pinpointed.

In summary, the results presented in this thesis have demonstrated that CaP-PCMCs are poorly immunogenic in terms of inducing anti-PRP antibody response. This is likely to be due to a failure of the formulation to deliver both antigens to PRP specific follicular B cells. The CaP-PCMC formulation is not able to provide a virtual conjugation between the TT and the PRP. However it is not known at which stage *in vivo* the crystal fails to act as a virtual conjugate. This will require further investigation to determine if the formulation can be improved in a way that allows the induction of a T cell dependent anti-PRP antibody response.

Appendix

A.1 Differentiation of HL-60 cells into neutrophils

Neutrophils are the first cells to arrive at the site of injection and may act to take up the CaP-PCMCs and traffic them to the lymph nodes. To investigate if there is any interaction between the CaP-PCMC formulation and the neutrophils, differentiated HL-60 cells were co-cultured with the CaP-PCMCs prepared with the fluorescently labelled antigens. The method for this is detailed in section 7.3.3.

Previous studies have found that three days of culture in supplemented media containing retinoic acid produces the highest proportion of cells displaying neutrophil morphology (212). In this project, HL-60 cells were incubated with the differentiation media for three days and analysed for morphological changes and CD11b expression. Changes in the light scattering properties in the differentiated HL-60 cells can be observed, indicating increase in size of the cells (FSC, Figure A.1).

Undifferentiated   **Differentiated**
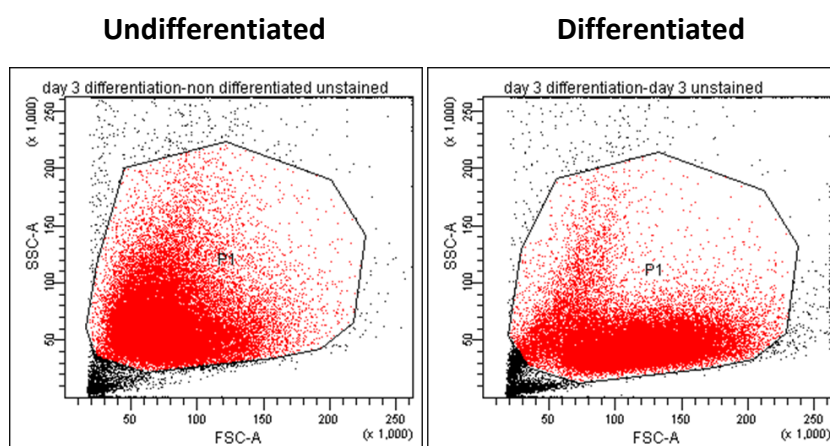
Figure A.1 Changes in the forward and side scattering properties of HL-60 cells following differentiation.

HL-60 cells were incubated in supplemented media for 3 days. Control cells were incubated with normal culture media.

The larger cell population was analysed for CD11b expression, and results showed that while undifferentiated cells do not express CD11b (Figure A.2). Almost all the differentiated HL-60 cell population (97.4%) which had a three day incubation in the differentiation media was found to express CD11b.
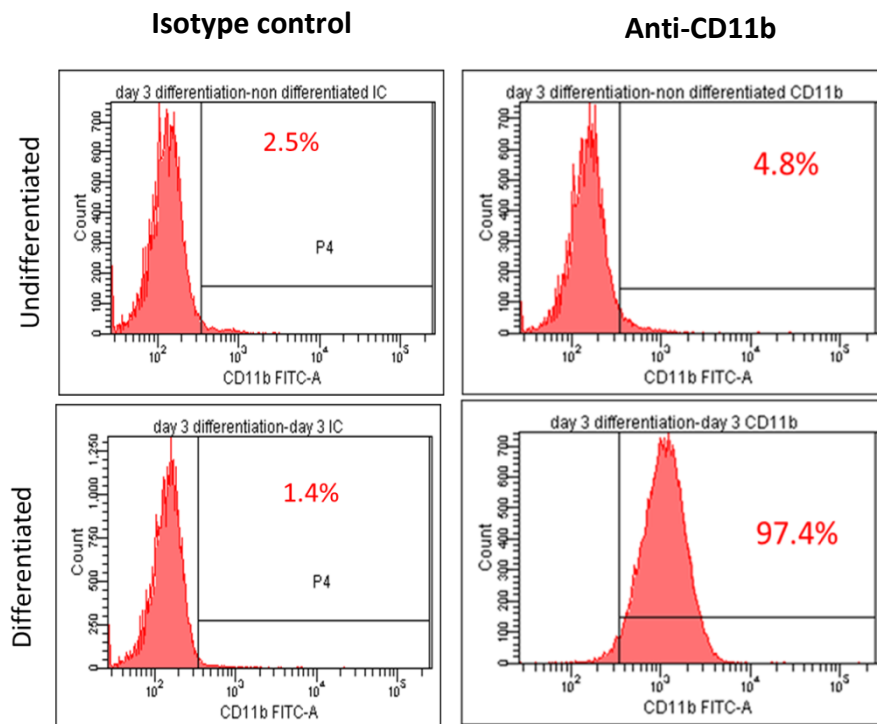
Figure A.2. Expression of CD11b on HL-60 cells before and after differentiation.

HL-60 cells were incubated in differentiation media for 3 days. Histograms show levels of CD11b expression in cell population. Cells were stained for CD11b using a monoclonal antibody and matching isotype control.

A.2 Interaction of BSA-FITC CaP-PCMCs with neutrophil-like cells

To optimise the uptake of CaP-PCMCs, HL-60 cells were incubated with varying concentrations of labelled and unlabelled CaP-PCMCs. The method for these experiments is detailed in section 7.3.3. The differentiated HL-60 cells were incubated with the either 2.5 or 5mg/mL of CaP-PCMCs containing BSA-FITC for 30 min, the resultant changes in the light scattering properties of the cells are shown in Figure A.3. While cells incubated in the culture medium show a low level of small and granular cells (13%), this cell population increased after the incubation with CaP-PCMCs (36 and 52%), which suggests that cells which take up the crystals may undergo apoptosis.
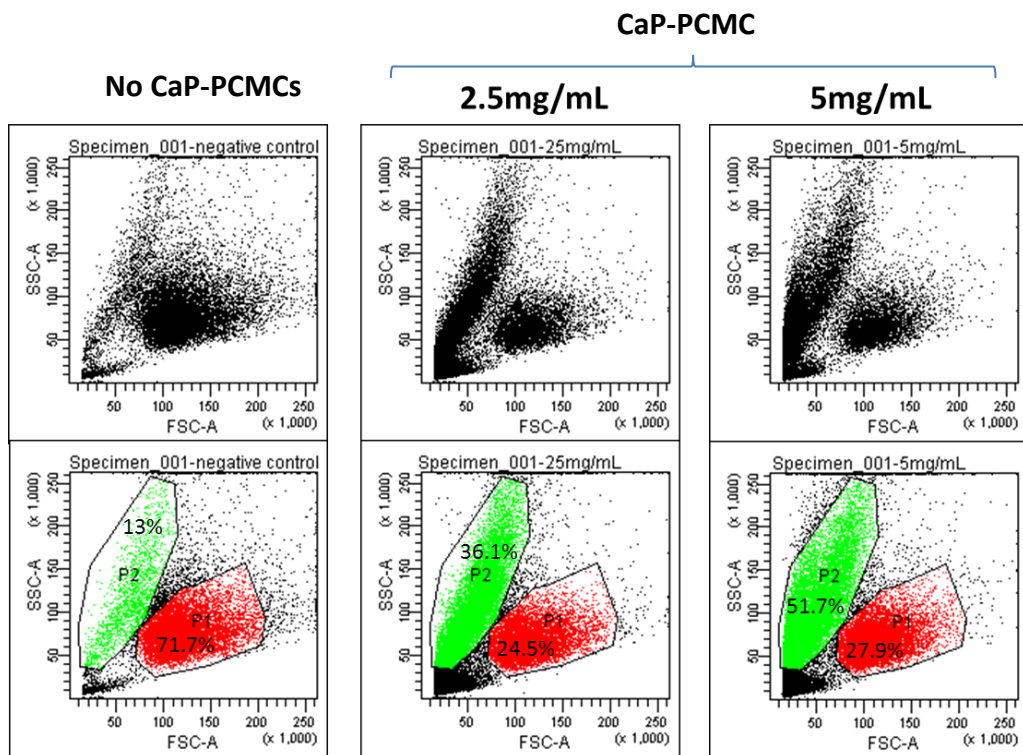
Figure A.3. Size and granularity of neutrophil-like cells incubated with CaP-PCMCs

Neutrophil-like cells were incubated with 2.5mg/mL or 5mg/mL of CaP-PCMCs in media for 30 min. Dot plots showing size (FSC) and granularity (SSC). Gated populations are shown in the bottom row with percentage of total cell population.

To investigate if changes in the cells occur upon incubation with CaP-PCMCs is due to cell death, cells with and without CaP-PCMCs were stained with a fluorescent dye 7-Aminoactinomycin D (7-AAD), which is a dead cell stain (excited at 488nm and emission measured at 647nm) analysed by flow cytometry. The histograms showing relative fluorescence from the stain can be seen in A.4. It is clear that the small and granular cells (green) are highly positive (75%) for the stain when compared to the larger and less granular cells (1.8%, red), indicating that the smaller and granular population contained mainly dead cells.
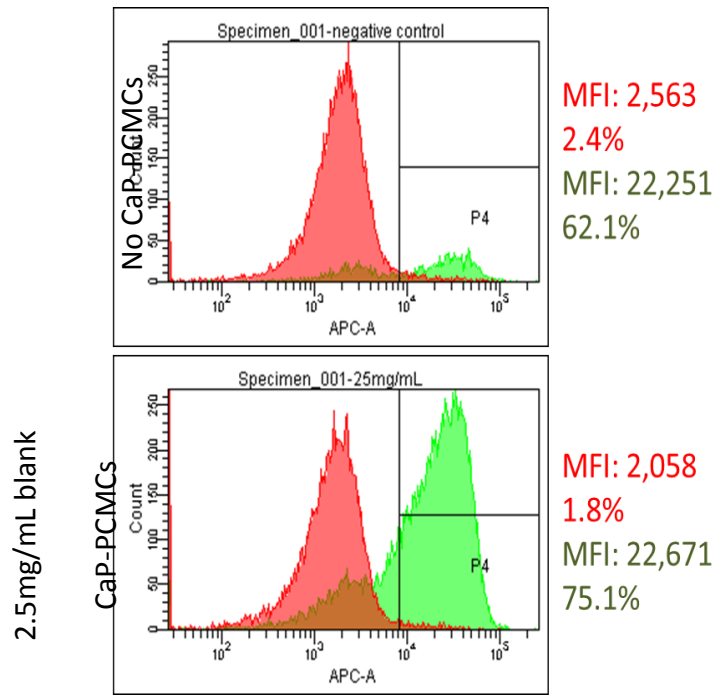
Figure A.4. Effect of CaP-PCMC on the viability of differented HL-60 cells.

Neutrophil-like cells were incubated with CaP-PCMC suspension at 2.5mg/mL for 30 min. The cells were then stained with 7AAD dead cell stain. Mean Fluorescence Intensity (MFI) and percentage of cell population positive for the stain (dead) are shown for large and less granular cells (red) and small and more granular cells (green).

A.3 Differentiation of dendritic cells from murine bone marrow

Bone marrow cells from mice were used to differentiate into dendritic cells by incubation in IL-4 and GM-CSF supplemented media. It has been shown that 4-8 day incubation in the media is optimum for dendritic cell differentiation (203). The method for this section is detailed in section 7.3.4.

In our study, after culture of the bone marrow derived cells for the selected period of time, the cells are harvested and undergo an enrichment step. To confirm the enrichment process results in viable culture, the enriched and non-enriched populations were counted for viability, and the results are shown in Figure A.6. The viability is consistently higher at all three time-points in the enriched population, starting at 90% at day 4, reaching 97.5% at day 7 and finally dropping to 83.3%. These results indicate that the enrichment process is suitable for eliminating dead cells from the population before analysis by flow cytometry. Therefore, cells differentiated for seven days were used for the uptake study.
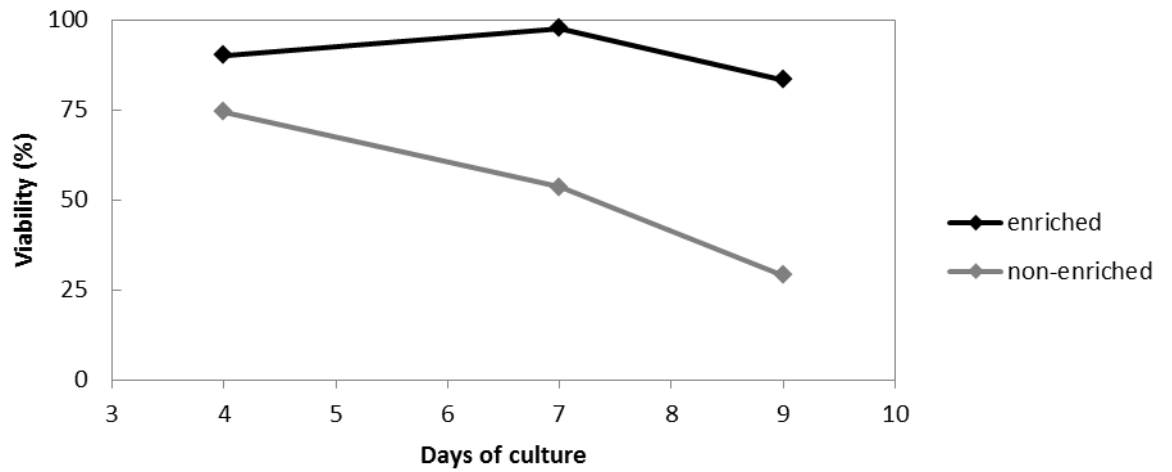
Figure A.6 Percentage viability of enriched and non-enriched cells on days 4, 7 and 9

The cells were enriched and viable cells counted and compared to cells from a non-enriched culture.

To confirm that the cells harvested on day 7 are differentiated, they were analysed for expression of CD11c and MHC class II expression and the results are shown Figure A.7. The results show that 27% of the differentiated cells express MHC class II and of these, 46% also express CD11c.
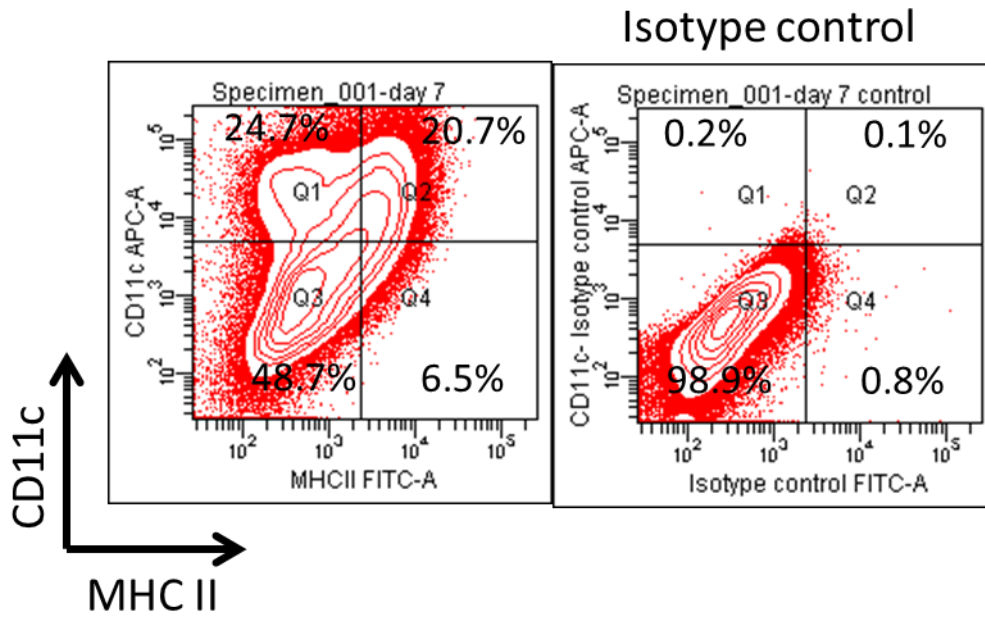
Figure A.7. Expression of CD11c and MHC CLASS II on differentiated DC.

Murine bone marrow cells were incubated in media supplemented to induce differentiation into dendritic cells. The level of differentiation on day 7 were established by analysis of CD11c and MHC CLASS II staining. Control cells were stained with isotype control antibodies.

A.3 Preparation of CaP-PCMCs for B cell uptake

Two formulations were prepared, one with 0.5% PRP and OVA-Alexa Fluor 647 loading (Formulation 1), and one formulation with only 0.5% OVA-Alexa Fluor 647 (Formulation 2) to control for non-PRP specific uptake of the CaP-PCMCs. The method for B cell preparation is detailed in section 7.3.5. The levels of loading as well as free antigen were measured on the formulation using Orcinol assay for PRP and fluorescence assay for OVA and is shown in Table A.1.

Table A.1. Levels of total free antigen in two PRP-CaP-PCMC formulations

	Formulation 1		Formulation 2	
	PRP	OVA-Alexa-Fluor 647	PRP	OVA-Alexa-Fluor 647
Total antigen (%)	64.4	14.4	n/a	16.4
Soluble antigen (%)	34.4	9.3	n/a	7.4

Levels of PRP and OVA-Fluor-647 were measured in the CaP-PCMCs solution in Tris/Citrate/EDTA buffer and the supernatant of a centrifuged CaP-PCMC suspension by Orcinol assay and Fluorescent readings. Free antigen in the suspension was calculated from the measured total loading of antigen and quantities calculated against a various concentrations of the antigens in solution.

The level of PRP detected in Formulation 1 was 64.4% of the theoretical, and 34.4% of this was found to be soluble, which indicates that 65.6% of the total measured PRP remains on the CaP-PCMC in suspension. The OVA-Alexa-Fluor loading was found to be only 14.4% in the CaP-PCMC formulation containing PRP and 16.4% in the formulation prepared without PRP. The release of soluble OVA-Alexa-Fluor-647 was low at 9.3% and 7.4% respectively.

To gain an insight into the nature of the interaction of the CaP-PCMCs with various immune cells, CaP-PCMC were first prepared using PRP and TT covalently conjugated to FITC and Texas Red, respectively. The level of total and soluble PRP and TT in the CaP-PCMC formulation was determined by measurement of fluorescent signal and the results are presented in Table A.2. The total PRP content on the crystal was found to be extremely low, (3.7% of the total PRP-FITC used to prepare the CaP-PCMCs). Furthermore, the release of soluble PRP-FITC from the crystal formulation was high (71.9% of total PRP), leaving on the crystals only 1% of the total PRP used. The total TT content of the crystals was also found to be low (14.5%), which is much lower than expected. However, the level of soluble TT was 23.3% of total TT, which would suggest that, although only 11.1% of the total TT used is associated with the crystals a high proportion of any signal is derived from the TT on the crystals rather than from soluble TT.

Table A.2. Total and soluble antigen content of the PRP-TT-CaP-PCMC.

	PRP-FITC	TT-Texas Red
Total measured antigen (%)	3.7	14.5
Soluble Antigen Release (%)	71.9	23.3

The total and soluble PRP and TT content of the CaP-PCMC were determined by measuring the fluorescent signal intensity for each antigen and compared to the standard curve generated from the different dilutions of soluble fluorochrome labelled- antigen dilutions.

However, as the antigen content of the CaP-PCMC is relatively very low, at least for the PRP when compared to results obtained in the Orcinol assay for previous formulations, it was decided to compare the fluorescent assay with Orcinol to

determine if the low PRP values obtained are real or are caused by loss of the Fluorescein from the PRP during the CaP-PCMC preparation.

The total and soluble PRP content of two CaP-PCMC formulations with a theoretical 0.5% PRP loading (the first batch had a TT loading of 0.5% and the second batch was prepared without TT) were determined by Orcinol assay, and by measuring the fluorescence signal for comparison. The results are presented in Table A.3. The total PRP content as measured by the Orcinol assay was found to be close to 100% of the theoretical value in both batches. Soluble PRP from the CaP-PCMC for both formulations was 46.9 and 38.2% of the total. The total PRP content in both formulations was determined by the fluorescence assay and was found to be similar to the first formulation shown in Table A.2, with only 6.2 and a 5.9% of the expected level. The level of soluble PRP was also found to be high, with 51.8 and 60.3% of the measured PRP content was released into solution. It is clear there is a large discrepancy in the results for total PRP when determined by the Orcinol assay. The fluorescence assays suggest that the low level of PRP detected in the fluorescence assay indicate that the conjugation between the PRP and FITC is either not stable or is affected by the CaP-PCMC preparation process.

Table A.3. Total and soluble PRP content of two CaP-PCMC formulations prepared using PRP-FITC

	Orcinol		Fluorescence	
	Total (% expected)	Soluble (%)	Total (% expected)	Soluble (%)
Formulation 1	109.1	46.9	6.2	51.8
Formulation 2*	117.2	38.2	5.9	60.3

The total and soluble antigen content of two CaP-PCMC formulations (with 0.5% PRP loading) was determined by measurement of fluorescent intensity. *Formulation 2 contains only PRP-FITC and not TT.

Although the precise conjugation method used by Innova to covantly attach the FITC to the PRP is unknown, conjugation of the PRP to a carrier protein, as is the case for Hib conjugate vaccine is done by using cyanogen bromide to activate the polysaccharide, to introduce cyanate (OCN) groups that will react with spacer molecules, or directly with the carrier protein. Another method is the perodite oxidation of polysaccharide which generates aldehyde groups. The conjugation of a fluorophore to a protein however is a slightly simpler process, involving formation of a covalent amide bond with primary amines on the protein to be labelled (223).

Due to the low level of the FITC conjugated PRP in the CAP-PCMC formulation, a CaP-PCMC preparation containing BSA-FITC was used as a model for the uptake studies as an alternative to the PRP-FITC. The total and soluble level of BSA-FITC in the CaP-PCMC formulation were measured to ensure that there were low levels of soluble antigen on the formulation before using them in cellular uptake studies.

Although the total BSA content of the crystals was relatively low (35.2). The low level of soluble BSA-FITC (1.6%), suggests that any fluorescence signal detected by the flow cytometry is likely to be from uptake of the CaP-PCMCs rather than from uptake of soluble BSA-FITC.

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