# IMPROVING DIAGNOSIS AND ORAL VACCINATION STRATEGIES AGAINST BOVINE TUBERCULOSIS

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Doctor of Philosophy

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1

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**Improving diagnostic assay and vaccination strategies against bovine Tuberculosis**

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Along with vaccination, the development of complementary diagnostic tests to differentiate between vaccinated animals and those infected with M.bovis is required so that the test and slaughter control strategies can continue alongside vaccination. In this work peptide antigens [ESAT-6,p45 in water (1ml, 1mg/ml)] have been adsorbed onto 10mg inorganic substrates (hydroxyapatite (MHA P201;P120, CHA), polystyrene, calcium carbonate and glass microspheres) and in vitro release characteristics were determined. The aim of formulation was to enhance the interaction of peptides with antigen presenting cells and to achieve rapid peptide release from the carrier compartment system in a mildly acidic environment. Hydroxyapatite microparticle P201 has a greater surface area and thus has the largest peptide adsorption compared to the P120. CHA gave a further higher adsorption due to larger surface area than that available on microparticles. These particles were incorporated into the BOVIGAM$^{TM}$ assay to determine if they improve the sensitivity. After overnight incubation the blood plasma was removed and the amount of IFN-$\gamma$ in each plasma sample was estimated. CHA and MHA P201 gave a significantly higher immune response at low peptide concentration compared to the free peptide, thus indicating that these systems can be used to evaluate Tuberculosis (TB) amongst cattle using the BOVIGAM$^{TM}$ assay.

Badgers are a source of TB and pass infection to cattle. At the moment vaccination against TB in badgers is via the parenteral route and requires a trained veterinary surgeon as well as catching the badgers. This process is expensive and time consuming; consequently an oral delivery system for delivery of BCG vaccines is easier and cheaper. The initial stage involved addition of various surfactants and suspending agents to disperse BCG and the second stage involved testing for BCG viability. Various copolymers of Eudragit were used as enteric coating systems to protect BCG against the acidic environment of the stomach (SGF, 0.1M HCl pH 1.2 at 37°C) while dissolving completely in the alkaline environment of the small intestine (SIF, 1M PBS solution pH 7.4 at 37°C). Eudragit L100 dispersed in 2ml PBS solution and 0.9ml Tween 80 (0.1%w/v) gave the best results remaining intact in SGF loosing only approximately 10-15% of the initial weight and dissolving completely within 3 hours. BCG was incorporated within the matrix formulation adjusted to pH 7 at the initial formulation stage containing PBS solution and Tween 80. It gave viability of x10$^6$ cfu/ml at initial formulation stage, freezing and freeze-drying stages. After this stage the matrix was compressed at 4 tons for 3 mins and placed in SGF for 2 hours and then in SIF until dissolved. The BCG viability dropped to x10$^4$ cfu/ml. There is potential to develop it further for oral delivery of BCG vaccine.

Key words: - BOVIGAM™, BCG, Microparticles, Nanoparticles, Eudragit

# ABBREVIATIONS

| | |
|---|---|
| G.I.T | Gastrointestinal Tract |
| B-cells | Bone marrow derived lymphocytes |
| T-cells | Thymus derived lymphocytes |
| PMN | Polymorphonuclear neutrophils |
| NK cells | Natural killer cells |
| MHC | Major histocompatability complex |
| APC | Antigen presenting cells |
| IL-1/IL-2 | Interleukin-1/Interleukin-2 |
| Tc | Cytotoxic thymus derived lymphocyte |
| BCGF | B-cell growth factor |
| BCDF | B-cell differentiation factor |
| IgE, IgG | Immunoglobulins |
| *M.tuberculosis* | Mycobacterium tuberculosis |
| TNF-α/β | Tumour necrosis factor |
| PPD | Purified protein derivative |
| *M.bovis* | Mycobacterium bovis |
| *M.avium* | Mycobacterium avium |
| WHO | World Health Organisation |
| HIV | Human Immunodificiency Virus |
| FAE | Follicle Associated Epithelium |
| M-cells | Microfold cells |
| MALT | Mucosa-Associated Lymphoid Tissue |
| GALT | Gut-Associated Lymphoid Tissue |
| CMIS | Common Mucosal Immune System |
| OVA | Ovalbumin |
| PLG | Poly(lactide-co-glycolide) |
| MCC | Microcrystalline cellulose |
| HPMC | Hydroxymethylcellulose |
| CAP | Cellulose acetate phthalate |

| | |
|---|---|
| CMEC | Carboxymethylcellulose |
| DNA | Deoxyribose nucleic acid |
| VLA | Veterinary Laboratory Agency |
| IFN-$\gamma$ | Interferon gamma |
| MHA | Microcrsytalline calcium hydroxyapatite |
| CHA | Carbonate hydroxyapatite |
| PLA | Poly(L-lactide) |
| BCA assay | Bichinchoninic acid |
| BOVIGAM | Bovine Gamma Interferon Test |
| CMI | Cell mediated immunity |
| T.E.M | Transmission Electron Microscopy |
| $Ca^{2+}$ | Calcium ions |
| NaCl | Sodium chloride |
| ELISA | Enzyme-linked immunosorbent assay |
| TB | Tuberculosis |
| BCG | Bacillus Calmete-Guèrin |
| HCl | Hydrochloric acid |
| PBS | Phosphate Buffer Saline |
| cfu | Colony forming units |
| PVP | Polyvinylpyrrolidone |
| PEG | Polyethylene glycol |
| SDS | Sodium dodecyl sulphate |
| SGF | Simulated gastric fluid |
| SIF | Simulated intestinal fluid |
| UV light | Ultraviolet light |
| NaOH | Sodium hydroxide |
| S.E.M | Scanning Electron Microscopy |
| FDA | Food and Drug Administration |
| $Na^{+}$ | Sodium ions |
| $PO_3^{4-}$ | Phosphate ions |
| $Cl^{-}$ | Chloride ions |

# TABLE OF CONTENTS

**CHAPTER 2   MATERIALS AND METHODS**      **76**

# LIST OF FIGURES

14

# LIST OF TABLES

18

# CHAPTER 1

# INTRODUCTION

# 1 INTRODUCTION

## 1.1 Immune response

The immune response is triggered by the introduction of foreign matter (antigen) into the body, which is recognised by the immune system and results in a reaction to eliminate the antigen (Roitt *et al.*, 2001). Three cell types are involved – macrophages, thymus derived lymphocytes (T cells) and bone marrow derived lymphocytes (B cells) (Roitt *et al.*, 2001). These interact directly or indirectly via interleukins. There are two categories of immune response: innate (or non-adaptive) and adaptive (Roitt *et al.*, 2001). The main difference between the two is that an adaptive immune response is specific for a particular pathogen and it has immunological memory, which allows a more rapid and more powerful response on next exposure.

**Innate immunity** is present from birth and is non-specific. The body surface especially the skin forms the first line of defence against invading microorganisms. If the microorganisms penetrate, they encounter the enzyme lysozyme, which is located in secretions and damages bacterial cell walls. Elimination of bacteria also occurs by lysis or by phagocytic cells such as monocytes, macrophages and polymorphonuclear neutrophils (PMN) (Roitt *et al.*, 2001). These cells bind to the microorganisms, internalise and kill them. Other cells involved are the natural killer cells (NK cells), which bind to and kill virus-infected cells and also tumour cells.

**Adaptive immunity** (Fig 1.1) only comes into play when the innate immune response fails to prevent infection. In this case the antigen triggers a chain of events that leads to the activation of T-cells and the production of antibodies, which are specific to the antigen.

**Figure 1.1** <u>Grand scheme of adaptive immune system (Basic and clinical immunology, seventh edition, Stites D.P, Terr I.A)</u>



### 1.1.1 Antigen processing and presentation

T-cells recognise antigen through a cell surface receptor known as the T-cell receptor. The T-cell receptor recognises antigens in the form of peptides bound to the major histocompatability complex (MHC) molecules on the surface of the

body's cells. Thus pathogen-derived proteins must be degraded into peptides to be recognised by T-cells. The binding of a peptide antigen by an MHC molecule and its display at the cell surface is termed antigen presentation.

Microorganisms that invade the body are divided into those that propagate within cells, such as viruses, and those that live in extracellular spaces such as bacteria. Consequently there are two classes of T-cells to combat the invading microorganisms, one is defined by the expression of CD4 glycoprotein on the cell surface and the other contains CD8. The CD8 T-cells are cytotoxic and their main function is to kill cells that have become infected by viruses or other intracellular pathogens, thus preventing the spread of infection. The function of CD4 T-cells is to help other cells of the immune system to respond to extracellular sources of infection and are often referred to as helper cells. There are two types of CD4 helper cells, $T_H1$ and $T_H2$. $T_H2$ cells are mainly involved in stimulating B cells to make antibodies that bind to extracellular bacteria whereas $T_H1$ cells activate tissue macrophages to take up extracellular pathogens by phagocytosis and kill them.

The MHC molecules are crucial in ensuring that the appropriate class of T cells is activated. There are two types of MHC molecules, MHC class I and MHC class II, and each presents peptides from one kind of antigen to one type of T cell. MHC class I molecules present peptide antigens of intracellular origin to CD8 T cells whereas MHC class II present peptides of extracellular origin to CD4 T cells. It is the T cell receptor that recognises its specific peptide MHC molecule ligand thus allowing binding between T cells and antigen presenting cells (APC) via the MHC molecules.

The first step in inducing an immune response to bacteria involves the capture and processing of the antigen following entry into the body by APC such as macrophages. The processed form is presented to TH cells in association with class II MHC molecules (Parham 2000). MHC class II molecules are found on macrophages, dendritic cells in lymphoid tissue, Langerhans cells in the skin,

Kupffer cells in the liver and microglial cells in the central nervous system. These cells phagocytose or pinocytose the antigen, which after modification in the endocytic vacuoles in the cytoplasm is denatured or undergoes proteolysis. If proteolysis, occurs the fragments of the antigen become associated with class II molecules and the complex is transported to the surface of the cell, where it is then accessible by T cells (Fig 1.2). Only a limited number of peptide fragments from the antigen are capable of forming a complex with class II molecules and these are called immunogenic epitopes.

**Figure 1.2** Uptake and processing of immunogen (antigen) by APC. (Basic and clinical immunology, seventh edition, Stites D.P, Terr I.A)

MACROPHAGE
antigen presenting cell (APC)

Peptides generated in the cytosol from viruses or other intracellular pathogens enter the endoplasmic reticulum where they are bound by MHC class I molecules. These peptides are then recognised by CD8 T cells to kill these infected cells. Most of the cells in the body contain MHC class I molecules.

### 1.1.2 Activation of helper T cells

The activation of Tн cells occurs early in the immune pathway and requires at least 2 signals. One signal is the binding of the T cell antigen receptor to the class II

MHC-antigen complex on the APC (Roitt et al., Ch5 2001). The second signal derives from interleukin-1 (IL-1) a soluble protein produced by APCs. Together the two signals induce the expression of another lymphokine IL-2 as well as the production of cell growth and differentiation factors (cytokines) that are important for triggering B cells and activating macrophages (Fig 1.3). IL-2 induces the growth of cells expressing IL-2 receptors that include the same $T_H$ cells that produce it (autocatalytic effect) and cytotoxic T (Tc) cells that do not produce it. Therefore the main function of IL-2 is to amplify the response initiated by contact of $T_H$ cells with APCs.

**Figure 1.3** $T_H$ cell activation (Basic and clinical immunology, seventh edition, Stites D.P, Terr I.A)

### 1.1.3 Activation of cytotoxic T cells

The activation of $T_H$ cells induces further responses along the immune pathway notably the activation of $T_C$ cells (Parham., Ch6, 2000). These cells are mainly involved in the killing of foreign or non-self antigens. The $T_C$ cells are distinguishable from $T_H$ cells by CD8 receptors on the surface rather than CD4 receptors and by the recognition of antigens in the context of MHC class I rather than class II MHC molecules (Parham., Ch6, 2000). The $T_C$ cells also require two activating signals. One is provided by interaction of T cell antigen receptors with a

complex of a foreign epitope and class I MHC on the target cell. These may be virus-infected or tumour cells or foreign tissue grafts. The IL-2 produced by the activated $T_H$ cells provides the second signal. The activated $T_C$ cells then release cytokines that kill the target cell[5].

### 1.1.4 Activation of B lymphocytes

B and T lymphocytes originate from precursors in the bone marrow called haematopoietic stem cells. These stem cells divide and differentiate into more specialised cells called lymphoid, myeloid and erythrocyte precursors. The lymphoid cells divide and differentiate into B and T cells. On activation by infection B cells divide and differentiate into plasma cells whereas T cells differentiate into $T_H$ or $T_C$ cells. B cells complete their maturation in the bone marrow, whereas T cells leave the bone marrow as immature cells and complete their development in the thymus. The bone marrow and thymus are termed primary lymphoid tissues and lymph nodes. Spleen, Peyer's patches, tonsils and appendix are termed secondary lymphoid tissues. This is where mature lymphocytes become stimulated to respond to invading pathogens.

The activation of B-lymphocytes (B-cells) and their differentiation into antibody producing plasma cells leads to antibody production. When $T_H$ cells are stimulated, B cells are also stimulated through antigen binding to antigen receptors on the surface of the membrane of B cells, which are secreted later as antibodies (Parham., Ch4, 2000). The binding of the antigen is followed by endocytosis of the antigen-receptor complex, which acts as one of the activating signals for activation of B cells. Additional signals are required from $T_H$ cells. These are lymphokines called B cell growth factor (BCGF) and B cell differentiation factor (BCDF) (Roitt *et* al., Ch8, 2001). B cells also function as APC's processing endocytosed antigen and transporting immunogenic epitopes complexed with class II molecules to their surface. The complex then activates T cells or the formation of memory T cells.

The BCGF stimulate the proliferation of B cells and BCDF causes the activated B cells to differentiate into antibody-secreting plasma cells (Roitt *et al.*, Ch8, 2001). Therefore the complete process of B cell activation and differentiation require at least three signals, one provided by the antigen and the other two by $T_H$ cells. Some of the activated B cells do not undergo differentiation into plasma cells, possibly due to insufficient BCDF. These B cells form what are known as memory cells, which respond to subsequent encounters with the same antigen (Parham., Ch4, 2000).

## 1.2   Hypersensitivity reactions

Non-infectious agents are seen as foreign, whether they are from plants, animals, food or the environment. Contact with these molecules does not generally produce inflammation or adaptive immunity for most people. However in some people an adaptive immune response is triggered which leads to the development of immunological memory cells. Therefore on subsequent exposure to the antigen the immune memory response produces inflammation and tissue damage. The over reaction of the immune system to harmless environmental antigens is called hypersensitivity or allergic reaction and is grouped into four types.

Type I hypersensitivity reactions result from the binding of antigen to antigen-specific IgE immunoglobulins found on the surface of mast cells connected via Fc receptors on the mast cells. Mast cells are large bone marrow derived cells found in connective tissues. They contain large granules, which store a variety of chemicals including histamine. Examples include allergic rhinitis and asthma.

Type II hypersensitivity reactions are caused by small molecules that covalently bond to the cell surface. This produces modified structures that are perceived as foreign by the immune system. The B cell response is to produce IgG, which bind

to the modified structure and cause their destruction through phagocytosis. Examples include some drug allergies (penicillin).

Type III hypersensitivity reactions are due to small soluble immune complexes formed by soluble protein antigens binding to the IgG made against them. The immune complexes activate complement and initiate an inflammatory response that damages the tissues and impairing their physiological function. Examples include antibodies or other proteins derived from animals.

Type IV hypersensitivity reactions are caused by antigen specific T cells. Most are caused by CD4 $T_H1$ cells in an inflammatory reaction around the site of an insect bite or sting. A minority are caused by cytotoxic CD8 $T_H2$.cells. They arise when small reactive lipid-soluble molecules pass through cell membranes and bond covalently to intracellular human proteins. See section 1.2.1.

## 1.2.1 Type IV hypersensitivity reactions

Type IV hypersensitivity reactions are mediated by T cells specific for the sensitising antigen. They are also known as delayed-type hypersensitivity reactions (DTH) because they take more than 12 hours (Roitt *et al.*, Ch24, 2001) to develop and reach maximal sensitivity between 24 – 72 hours after contact with antigen (Nester *et al.*, Ch24, 1998). When an antigen is applied to the epidermis or injected intradermally in a sensitised individual, T cells stimulate a local inflammatory response over 1 – 3 days. The T cells responsible for the delayed reaction have been specifically sensitised by a previous encounter and they attract macrophages and other lymphocytes to the site of reaction. The process is normally triggered by the uptake, processing and presentation of the antigen by antigen-presenting cells (macrophages).

The Tuberculin skin test is used to determine if a person or animal is sensitive to the organism, *Mycobacterium tuberculosis*, following previous exposure. The skin test is a common example of Type IV hypersensitivity reactions (Parham., Ch10, 2000). Following Tuberculin injection (Tuberculin is a sterile fluid obtained from a culture of *M.tuberculosis*) Tuberculin-specific memory T cells produced during previous exposure to the antigen migrate to the site of injection. Activated $T_H1$ cells release cytokines that act on local endothelial cells which in turn causes the recruitment of more T cells, phagocytes, fluid and protein to the site of injection. It is at this point that the lesions associated with tuberculin skin test become apparent. Macrophages recruited to the site of inflammation by chemokines present antigen and amplify the response.

### 1.3 Tuberculin skin test to determine infection to *M.tuberculosis*

**Figure 1.4** The stages and time course of a type IV hypersensitivity reaction (Parham, Ch10, The immune system, pp 295)

28

People or animals that are sensitive develop redness and a firm swelling reaching its maximum Intensity over 48 – 72 hours at the site of injection (Parham., Ch10, 2000). A strongly positive reaction to the test indicates *M. tuberculosis* bacilli are present in the body of that person or animal. This positive test implies that the person or animal is resistant to acquiring a new infection but, because of the possibility of dormant organisms becoming activated, is probably more likely to develop active tuberculosis than a tuberculin-negative person or animal.

**Figure 1.5**    The role of mediators released by Th 1 cells (Parham, Ch10, The immune system, pp295)



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## 1.4    Tuberculosis

Tuberculosis can be found in many ancient civilisations (Zink *et al.*, 2002)). Deformities of the spine, characteristic of Pott's disease (tuberculosis of the bone) have been found in Egyptian mummies dating from 2000-4000 BC and the clinical symptoms of pulmonary tuberculosis have been described in ancient documents (Willcox., 2002). Tuberculosis was prevalent throughout medieval Europe but it was in the early 1600's that the incidence began to rise sharply. The incidence continued to rise over the next two centuries and at its peak about one in four deaths were due to tuberculosis. Over the last century the incidence of tuberculosis has declined dramatically in more affluent countries, such as in Europe and North America, due to improvements in social environments and the introduction of effective chemotherapeutic treatments in the early 1950's (Robitzek *et al.*, 1952). However this has not been true in less developed countries such as those in the African and Asian subcontinents.

Tuberculosis is a chronic infectious disease usually caused by *Mycobacterium tuberculosis*, an acid-fast bacillus (WHO No. 104, 2000). Other species, such as *M. bovis* found in cattle, can also cause TB in humans. It is mainly prevalent in developing countries where poor housing, over crowding, poverty, poor nutrition and inadequate medical care are involved (WHO No. 104, 2000). Tuberculosis is responsible for approximately 2 million deaths per year and it is estimated that between 2000 and 2020, nearly one billion people will be infected, 200 million will become ill and 35 million will die if control is not strengthened (WHO No. 104, 2000).

### 1.4.1    Structure of Mycobacterium tuberculosis

All types of bacteria have an inner cell membrane and a peptidoglycan wall (Roitt *et* al., Ch15, 2001). The bacterium *Mycobacterium tuberculosis* has a typical gram-

positive cell wall, but bound covalently to the cell wall peptidoglycan is a thick layer composed of complex glycolipids (WHO fact sheet No 104 April 2000). The peripheral (lipid) portion of this layer is bound in turn to other lipids composing the bacterial surface. These lipids include esters of fatty acid with fatty alcohol and mycolic acids. The mycolic acids are extremely long chained fatty acids unique to mycobacteria.

*M.tuberculosis* is a strongly acid-fast, rod shaped bacterium that stains in an irregular (beaded) fashion (Nester *et al.*, 1998). The organism is an aerobe that grows very slowly and thus makes rapid diagnosis of tuberculosis infection difficult (Drobniewski *et al.*, 2003). In comparison to most other bacteria, the tubercle bacillus is unusually resistant to drying, disinfectants, staining and strong acids and alkali (Nester *et al.*, 1998). This resistance is attributed to the structure of its cell wall. However, it is easily killed by pasteurisation.

**Figure 1.6**  Diagram showing components of different bacterial cell walls (Roitt I, Brostoff J, Male D, Immunology 6<sup>th</sup> edition pp230)

There are no toxins and its virulence depends on its ability to survive within host macrophages.

## 1.4.2  Pathogenesis

Infection with *M.tuberculosis* usually occurs by inhaling aerosolised organisms from person to person or animal to animal (www.defra.gov.uk/animalh/tb/point1/plact.shtml). The microorganisms lodge in the lungs where they are ingested by pulmonary macrophages. Here it uses the immune systems cells, in this case macrophages, that are normally used to destroy them. On being phagocytosed the bacterium prevents fusion of the phagosome with the lysosome consequently protecting itself from the bacterial actions of the lysosomal contents (Nestar et al., Ch24, 2001). The bacteria continue to multiply within the macrophages that carry them to the lymph nodes. There they continue to multiply and with lysis of the macrophages are released into the lymphatics and blood vessels. This allows the spread of the organism to other parts of the body.

After about two weeks, delayed hypersensitivity to the organism develops which involve an intense reaction at sites where the bacteria have collected. Further macrophages become activated and collect around the bacilli with some macrophages fusing together to form large multinucleated giant cells. Lymphocytes and macrophages then collect around these large cells forming a barrier between the infected area and the surrounding tissue. This localised collection of inflammatory cells is called a granuloma (tubercle), which is a characteristic response of the body to microorganisms that resist digestion and removal by phagocytosis (Nestar et al., Ch24, 2001). In most cases the growth and spread of the mycobacterium is halted by the granuloma formation and no significant illness develops. The *M.tuberculosis* may however remain in a dormant state within the tubercles for many years and become activated at a later time. The macrophages tend to lyse and then release

32

their enzyme content into the infected tissue. This predominantly results in death of the infected tissue with the formation of necrotic tissue.


## 1.4.3 Epidemiology and aetiology

Tuberculosis is spread by prolonged close contact with an infected individual through inhalation of airborne droplets from sputum. The initial infection is usually silent and may heal with no further symptoms, or it may progress and develop into pulmonary TB (www.defra.gov.uk/animalh/tb/point1/plact.shtml). It is only when it develops to this stage that the person is infectious and able to propel the TB bacilli through coughing, sneezing, etc (www.who.int/vaccines-disease/diseases/TB.html). Ingestion of unpasteurised milk from cows, which can become infected with *M. bovis*, can also lead to TB development in humans. People infected with TB will not necessarily become ill as the immune system has the capability of barricading the TB bacilli from the rest of the body, which can remain dormant for years (www.defra.gov.uk/animalh/tb/point1/plact.shtml).

The disease develops in 5 - 15% of infected people if left untreated and is dependent on various factors (eg. inoculum size, virulence of infecting strain, the host's age, inherent resistance and state of health and nutrition) (www.stoptb.org/tuberculosis/global.regional.incidence.html). It most commonly affects the respiratory tract (pulmonary TB) and is characterised by inflammatory infiltrations, tubercle formation, caseation, fibrosis and calcification (Buddle *et al.*, 2000). It can also spread to other parts of the body by circulation in the blood and lymphatics. These include lymph nodes, heart, gastro-intestinal or genito-urinary tracts, meninges, bones, joints and skin. Several of these organs may be infected simultaneously and this is called miliary tuberculosis (www.who.int/vaccines-disease/diseases/TB.html).

The incidences of TB are once again on the increase, however it is mostly associated with poor countries where poverty is high. New outbreaks are occurring in Eastern Europe where TB deaths are increasing after almost 40 years of steady decline (www.stoptb.org/tuberculosis/global.regional.incidence.html). The biggest burden of TB is in Southeast Asia. Eight million people around the world become sick with TB each year, with 1.6 million cases occurring in sub-Saharan Africa and 3 million cases in Southeast Asia (WHO TB day 2002 pack).

### 1.4.4 Factors influencing the increase in tuberculosis

- *Multi-drug resistance TB*

A major problem that has resulted in an increase of TB is the emergence of strains resistant to single or even combination of drugs. Some strains are resistant to the most important drugs, isoniazid and rifampicin. Multidrug-resistant (MDR) TB is caused by the inconsistent or partial treatment, when patients do not take the prescribed dose for the required time period. Doctors prescribing the wrong drugs or combination of drugs are also a problem. From a public health perspective, poorly supervised and incomplete treatment of TB is worse than no treatment at all. This is because the people still remain infectious and the bacilli in their lungs may develop resistance to anti-TB drugs. Also the people they infect will harbour the same drug-resistant strain, which is more difficult and expensive to treat (www.stoptb.org/tuberculosis/infection.transmission.html).

- *Poverty, Migrants, The homeless and Prisoners*

A close relationship exists between TB and poverty as shown by the number of cases each year in poor countries (WHO TB day 2002 pack). It is widely recognised the poorer the community the greater the likelihood of being infected and developing the disease. Many poor people are forced to live in overcrowded

conditions, which increase the risk of TB transmission. Overcrowding is often associated with poor nutrition, poor housing and health that on their own are all influencing factors (WHO TB day 2002 pack). A lack of basic health services, poor nutrition and inadequate living conditions all contribute to the spread of TB (www.stoptb.org/tuberculosis/global.regional.incidence.html).

The WHO recognises poverty as the biggest problem in defeating TB. An absence of good health care facilities in poor countries leads to no or poor diagnosis and treatment. This can result in a longer delay between disease and cure, which will inevitably contribute to the spread of the disease amongst the population (www.stoptb.org/tuberculosis/global.regional.incidence.html). Poor nutrition and an inadequate diet weaken the immune system and increase the chances of developing TB as does overcrowding. The economic and human impact of TB is many times greater on poor households and poor nations than on the developed world. The high incidence levels of TB in many poor countries can result in an under performing labour force; the extra strain on limited health services weakens economic growth (www.stoptb.org/tuberculosis/global.regional.incidence.html). Studies suggest that the average patient loses three to four months of work, which results in a loss of household income and reduces the countries gross domestic products (www.stoptb.org/tuberculosis/global.regional.incidence.html). Prisoners are at risk of contracting TB because of overcrowding and inadequate diet often found in prisons in poor settings. They are not geared to deal with the large numbers of TB patients and thus access to diagnosis and poor treatment result. There are now also outbreaks of multidrug-resistant TB in prisons in the poorer countries. This can increase the spread of these strains to the community through families, prison staff and released prisoners.

- *HIV and AIDS*

TB is a leading killer of people infected with HIV due to the weakened immune system in this group of individuals.

## 1.5   Tuberculosis infection in cattle

Bovine tuberculosis (TB) is caused by *Mycobacterium bovis*. This is a bacterium closely related to *M.tuberculosis*, the major cause of human TB. *M.bovis* can infect a wide range of animal species and is the major form of TB in cattle (Buddle *et al.*, 2000). Infected cattle can spread the bacteria by aerosol and through infected milk. The introduction of pasteurisation of milk and the test and slaughter of TB positive cattle has decreased transmission from cattle to humans. However in cattle it is still a problem leading to reduced productivity and premature death in cattle and affected farms suffer severe economic loss (Buddle *et al.*, 2000).

**Transmission of infection from wildlife to cattle**

Many animals act as hosts for *M.bovis* including deer and badgers (Phillips *et al.*, 2000). These animals are regularly seen on farmed land, which consequently bring them into close contact with cattle, thus increasing airborne transmission. The badger is an ideal host for *M.bovis* infection and in the UK they are endemically infected (Phillips *et al.*, 2000). They are capable of excreting *M.bovis* in sputum, urine and faeces. A direct link has been established between infection of TB in cattle and the density of badgers in the surrounding areas which is increasing (Phillips *et al.*, 2000).

In the UK cattle to cattle transmission is minimised because infected cattle are regularly removed following a positive tuberculin test. However, diseased and dead badgers are not removed from farms thus increasing risk of transmission by close contact. Other animals, which may be involved in the transmission, are rodents, hedgehogs, ferrets and rats (Phillips *et al.*, 2000). However badgers live longer than these animals, allowing more time for *M.bovis* infection to progress to a highly infectious stage.

*M.bovis* is deposited in faeces and urine of infected badgers, which are often sited at territorial boundaries and are numerous in high-density areas. Cattle, which then graze on urinated grass, can take up the bacteria via ingestion or inhalation. However, respiratory tract infection in cattle is more common than consumption of contaminated grass (Phillips *et al.*, 2000).

The badgers' principal requirements are for shelter and food. The increased badger density in Britain may have forced some badgers to seek refuge in farm buildings where there is food and shelter. If infected badgers do enter farm premises they could transmit their infection to cattle that are housed there. They can also infect feeding troughs through urination and faecal deposits (Phillips *et al.*, 2000).

Studies of cattle housed with reactors have confirmed that indoor cattle-to-cattle transmission is possible. Poorly ventilated cattle housing provides an ideal environment for the spread of pathogens. *M.bovis* bacilli will survive for at least 74 days when protected from sunlight and the high density of cattle and high humidity provide an ideal environment for transmission of the organism, when compared with cattle at pasture.

## 1.6 Action to protect human health

During the 1930's over 50000 new human cases of *M.bovis* were recorded each year due to over 40% of dairy cattle being infected with *M.bovis* (www.defra.gov.uk/animalh/tb/point1/plact.shtml). Cattle were kept in crowded, poorly ventilated cattle sheds, ideal conditions for TB to spread between cows and into the udders. Consequently milk became infected and as it was usually drunk raw (unpasteurised), TB spread easily to people. It has been suggested that endemic tuberculosis in domestic animals became established prior to its occurrence in man and that *M.tuberculosis* derived from *M.bovis*. However since the completion of the genome of, the identification and distribution of deletions amongst the members of

*M.tuberculosis* would suggest that *M.bovis* and *M.tuberculosis* derived from a common progenitor and evolved in parallel (Gordon *et al.*, 2001). Once established in domestic livestock *M.bovis* has probably been a pathogen of man since the time of the earliest agricultural settlements which brought man and domesticated cattle into close contact. The following measures were therefore introduced to minimise the spread of bovine TB to humans: -

- Pasteurisation of milk

In England and Wales, approximately 6% of all deaths from tuberculosis were from *M.bovis* infection in surveys performed in 1931, 1937 and 1944. (Grange., 2001) Fortunately as a result of milk pasteurisation and current bovine control measures, the UK's population is currently considered to be at negligible risk. The latest figures published by the Public Health Laboratory Service reports that in England and Wales, less than 1% of all tuberculosis isolates were due to *M.bovis* (www.phls.co.uk/publications). Since the 1930's most milk available to the public has been pasteurised. Untreated milk is still available to the public in England and Wales, but since 1990 it must carry a health warning about the risks from drinking it (www.stoptb.org/tuberculosis/infection.transmission.html). All cattle herds that produce milk for sale as untreated must receive a TB test annually. If they fail the test, sales of untreated milk must stop immediately. However, the zoonotic impact of bovine tuberculosis remains an important consideration in developing countries where limited or no control measures are available and where the situation is likely to be exacerbated by the rising incidence of HIV.

- Inspection of carcases at slaughterhouses

The Meat Hygiene Service inspects all carcases to make sure meat is fit to eat. This inspection includes a detailed examination for TB. Carcases that are found to be infected with TB are removed from the food chain and destroyed.

## 1.7 Skin test in cattle

All cattle in the UK are regularly tested for TB using the single comparative intradermal tuberculin test (skin test). The test involves injection of a small amount of tuberculin (a sterile extract obtained from *M.bovis*) into the skin of the animal. The test is similar to that in humans, if the animal is infected with TB it will cause the immune system to react to the tuberculin and cause a swelling at the site of injection. However the test is not specific and cattle can become infected with other types of mycobacteria, which may show a positive response in the skin test (www.defra.gov.uk/animalh/tb/point4/p4inter.shtml). Therefore to distinguish *M.bovis* infection and other mycobacteria, cattle are injected with tuberculin from *Mycobacterium avium* found in birds. Hence the test is called the comparative intradermal tuberculin test (www.defra.gov.uk/animalh/tb/point4/p4inter.shtml). The only subsequent change to this test has been the change of using mammalian tuberculin PPD, prepared from *M.tuberculosis*, and replaced by the use of bovine tuberculin PPD prepared from *M.bovis* culture (Pritchard., 1988). It was found to be more specific and potent per weight then the mammalian product.

Robert Koch first noted the usefulness of such a response in diagnosing tuberculosis during his attempts to isolate the components released by the tubercle bacillus during in-vitro culture. When Koch injected his crude heat killed preparation, which he called tuberculin, subcutaneously into tuberculosis guinea pigs or humans, he noted a characteristic skin reaction 24 to 48 hours later. Koch's method for preparing his tuberculin was later refined and became known as purified protein derivative (PPD) tuberculin. This is essentially the method in use today for diagnosing TB in humans and cattle. The latest figures published by the Department of the Environment, Food and Rural Affairs (DEFRA), formerly known as Ministry of Agriculture Fisheries and Food (MAFF), report that 6% of the British national herd was under movement restriction and nearly 3% of tests on previously unrestricted herds resulted in new cases (www.defra.gov.uk/animalh/tb).

The economic costs of disease control for bovine tuberculosis in 1998 was £21.82 million (MAFF., 1998).

### 1.7.1 Skin test procedure and interpretation of results

A vet identifies and tags the animal to be tested. Two sites are selected on the side of the neck, one above the other. The two sites will be on either side of the neck in a small animal. The hair around the site is clipped to a radius of two centimetres and measurements are recorded from folds of skin at the two sites. Tuberculin is injected; the upper site being used for the avian tuberculin. After 72 hours the two sites are remeasured (www.defra.gov.uk/animalh/tb/point4/p4inter.shtml). If the reaction to *M.bovis* is more than 4mm greater than the reaction to *M.avium* the animal is considered to be infected with TB and is called a reactor (www.defra.gov.uk/animalh/tb/point4/p4inter.shtml). The animal is slaughtered and the herd become Tuberculosis positive with restriction applied. If the reaction to *M.bovis* is between 1 and 4mm greater than that of *M.avium*, the animal is considered an inconclusive reactor and will be retested after 42 days or at 60 days if tested as part of a herd (www.defra.gov.uk/animalh/tb/point4/p4inter.shtml).

The current test for TB does not distinguish between vaccinated and infected cows. As a result the cattle from these herds could lose their Official Tuberculosis Free Status and restrictions would be imposed. Therefore it is crucial to develop a specific diagnostic test, which can detect and differentiate between infected animals (including those that have become infected after vaccination) and vaccinated animals.

40

## 1.8  Vaccination

The principle of vaccination is based on two key elements of adaptive immunity, namely specificity and memory (Parham., Ch12, 2000). Memory cells allow the immune system to mount a much stronger response on a second encounter with an antigen. This secondary response is both faster to appear and more effective than the primary response. Antibodies and T cells recognise specific structural elements of antigens, the epitopes, and not the whole organism or toxin (Parham., Ch12, 2000). The aim in vaccine development therefore is to alter a pathogen or its toxins in such a way that they become innocuous without losing antigenicity (Parham., Ch12, 2000). In general the more antigen retained in the vaccine the better, and living organisms tend to be more effective than killed ones (Parham., Ch12, 2000).

The main types of vaccines are Live vaccines and Killed vaccines. Live vaccines are further split into natural live organisms, in which safety is a problem, and attenuated where the virulence of the pathogen is removed but its antigenicity retained. BCG for example is a live attenuated vaccine based on *M.bovis*.

**Attenuated vaccines** render microorganisms less able to grow and cause disease in their natural host (Parham., Ch12, 2000). These changes normally involve mutation.

Attenuated viruses for example are grown in non-human cells until they become fully adapted to the animal cells and thus grow poorly in human cells (Parham., Ch12, 2000). Several mutation cycles result in poor virus growth in humans but a single dose is sufficient to produce immunity (Nester *et al.*, Ch17, 1998). This is because the microorganism multiplies in the body, is present in the body for longer periods and in greater amounts than killed microorganisms. The disadvantage of using living agents for immunisation is that they have the potential for causing disease in immunosuppressed people (Nester *et al.*, Ch17, 1998). Care must also be

41

taken to avoid giving live vaccines to pregnant women, because the vaccines may cross the placenta and cause damage to the developing foetus.

**Figure 1.7**  Production of attenuated viruses (Parham, Ch12, The immune system, pp329)



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**Killed or inactivated vaccines** consist of virus particles for example that have been chemically treated with formalin or physically treated with heat or irradiation so that they are unable to replicate (Nester *et al.*, Ch17, 1998). Examples of such vaccines include influenza and rabies. Only viruses whose nucleic acid can be reliably inactivated make suitable killed virus vaccines. These vaccines are advantageous in that they cannot cause infections or revert to dangerous forms. They do have drawbacks in that large amounts of the pathogenic virus must be produced and since they do not replicate it is usually necessary to give several doses of the vaccine to induce immunity.

### 1.8.1  Other types of vaccines

Isolating the antigens or antigenic fragments of the infectious agent produces **subunit vaccines**. Some subunit vaccines in use are those directed against

meningococci, pneumococci, Haemophilus influenzae type b and pertussis. **Recombinant vaccines** are a form of subunit vaccine produced by genetic engineering. An example is the vaccine against hepatitis B virus. Genes from a part of the viral protein coat are inserted into yeast cells that produce proteins. Recombinant vaccines are safe but require several doses in order to be effective. **Peptide vaccines** are derived from pathogenic organisms that are either isolated or synthesized in the laboratory. Such vaccines are stable to heat and other environmental factors and do not contain extraneous materials which could cause unwanted hypersensitivity reactions. However they are weakly immunogenic and very expensive. **DNA vaccines** comprising fragments of DNA from infectious organisms can be administered in various ways so as to replicate and code for production of microbial antigens that then induce an immune response. This procedure eliminates the possibility of infection with the immunising agent, as only a small part of the microorganism's DNA is used.

### 1.8.2  Bacillus Calmette Guerin (BCG) vaccines and control of bovine tuberculosis through vaccination

The only currently available vaccine against tuberculosis for use in humans or cattle is the attenuated *M.bovis* strain bacilli Camette-Guerin (BCG). BCG was prepared by Calmette and Guerin who attenuated an *M.bovis* isolate, recovered from a cow with tuberculous mastitis, by serial passage on potato-glycine-ox bile agar over a period of 11 years (1908-1919) (Hawgood., 1999). They noted a change in morphology and when investigated in animal models, not only was the strain attenuated but it also induced protection against tuberculosis challenge (Hawgood., 1999). Since its first administration to a newborn baby in 1921, more doses of BCG have been administered to humans than any other vaccine. However, due to variable efficacy of BCG in clinical trials ranging from zero in USA, South India to approximately 80% in the UK, it usefulness against tuberculosis in humans is still a matter of debate (Hart *et al.*, 1977). It has been found that BCG does protect against

tuberculosis meningitis and against miliary tuberculosis, and thus WHO still recommends that all countries with high incidence of tuberculosis should immunise with BCG at or soon after birth.

Similar variable protection has also been reported in cattle against bovine tuberculosis. During the 1920's and 1930's, following its original preparation, BCG was investigated as a vaccine against *M.bovis* infection of cattle by several researchers including Calmette and Guerin (Hawgood., 1999). They reported that BCG vaccination did confer protection to vaccinated cattle during direct challenge experiments. They noted little or no pathology characteristics of tuberculosis infection in vaccinated cattle 3-18 months after challenge (Hawgood., 1999). A recent report in New Zealand have reported significant levels of protection in BCG vaccinated cattle against *M.bovis* infection, up to 70% in some experiments (Buddle *et al.*, 2002). However, regardless of the ranging protective efficacies of BCG vaccination of cattle, it is the only vaccine that could currently be applied in the field and remains the standard against which any new vaccines will be compared.

By the early 1990s, two separate genetic variants in BCG strains had been found due to differences in culturing in different laboratories (Talbot *et al.*, 1997). These genetic variations have had an effect on the potency of vaccines against tuberculosis. The main difference between the BCG Pasteur and BCG Tokyo strains is in culturing and laboratory conditions in which they were produced (Behr *et al.*, 2002). Initially BCG was cultured at the Pasteur institute in Paris. As freeze-drying for storage was not available, the BCG culture medium was changed every two weeks (Behr *et al.*, 2002). The strain was then distributed to laboratories across the world and cultured in much the same way. The main goal of culturing was to maintain the potency of the bacterium *M.bovis* from which BCG vaccine is derived while preventing it returning to its virulent form (Behr *et al.*, 2002). Over the years the BCG vaccine laboratories developed their own daughter strains of BCG, which was named after the city, laboratory director or country (hence the names Pasteur

and Tokyo). This practice ended in the 60s or 70s when seed lots of the daughter strains were prepared (Behr *et al.*, 2002).

For a live attenuated vaccine to provoke an immune response, the M.bovis bacteria has to be alive, survive long enough after inoculation to stimulate an immune response and express the antigenic components necessary to trigger the appropriate response (Bedwell *et al.*, 1999). The first and third of these aspects is known to vary among BCG strains (Behr *et al.*, 1999). Another factor affecting the immune response is that all BCG strains are prepared as lyophilised stocks that are resuspended before inoculation (Behr *et al.*, 1999). The proportion of live to dead bacteria varies greatly after reconstruction with BCG Tokyo having 25% live bacteria at vaccination (Orme *et al.*, 2001). It has also been noted that after 1931 the BCG strains do not possess myolic acid, a component of the cell wall, and thus their survival rate is reduced. This behaviour is manifested by reduced bacterial survival in macrophage cell culture (Behr *et al.*, 2002).

### 1.8.2.1  Morphology of BCG

BCG is of rounded cylinder shape approximately 2.36μm long and 0.47μm wide (Groves et al., 1997). BCG tends to clump and consequently when counting the colonies on media plates only shows as one colony. Zhang and Groves measured the aggregates of BCG to be between 4 to 7μm, which corresponds to about 500 cells in one aggregate (Zhang *et al.*, 1988). Surfactants have been used to disperse the microorganisms into individual cells. However they do not disperse completely due to the surface of the cell wall of the bacteria (Groves et al., 1997), which consists primarily of polysaccharides. The structure of the BCG cell wall is like that of other gram-positive mycobacteria, composed for the most part of complex peptidoglycan structures with an inner and outer cell wall (Zhang *et al.*, 1988).

### 1.8.2.2 Safety and efficacy of BCG

BCG vaccine is relatively safe but it does produce some non-life threatening side effects. A common minor adverse effect is the swelling incurred around the injection site. Most health authorities claim this to be advantageous as it leaves a scar that is visible evidence of vaccination. Other side effects include fever. It should be used with caution in patients with impaired immune system such as HIV.

Generally BCG is given at birth and certainly within the first three months of life in countries where there is a high risk. Giving BCG early in these countries reduces the chances of miliary TB and tubercular meningitis to the extent that they are now almost eliminated amongst vaccinated children. In the UK BCG is given when the child enters secondary school at the age of eleven to thirteen. In the USA there is controversy surrounding its use as it produces the same response to Tuberculin as TB infection, thereby confusing and obscuring the disease diagnosis (Groves *et al.*, 1997). In Western Europe there is a more favourable approach to its use. In the UK the TB rate has fallen from 250 per 100000 populations to 10.5 over a twenty-year period (Groves *et al.*, 1997). This may be due to the use of BCG together with better health services. However with HIV-infections and drug abuse contributing to an impaired immune system, BCG vaccination is expected to be maintained.

### 1.8.2.3 BCG and anti-cancer effects

BCG has been shown to offer protection against cancers by Rosenthal *et al* (1980) who performed an in depth study of the effect of BCG on citizens in Chicago (Groves *et al.*, 1997). He administered BCG to 85356 newborns from 1957 to 1969 and compared the data to the unvaccinated population of 534870 in Chicago. The incidence of TB dropped as expected from 4.39 per 100000 per year to 1.17, a protection rate of 74%. However he also noted a significant drop in deaths from malignancies, particularly leukaemia, lymphoma and soft tumours of the CNS and bone and connective tissues. Morales *et al* (1976) were the first to use BCG in clinical trials to show the benefits as a treatment for bladder cancer. BCG was

46

approved by the FDA for treatment of bladder cancer in 1990 and is now the preferred treatment. Klegerman *et al* (1991b) demonstrated both high dose tumour inhibition and low dose tumour stimulating activity for both viable and heat-killed BCG. This variability has led to the identification and further study on a series of heat-stable antineoplastic glycans in BCG (Lou et al., 1994a), which is providing a possible mode of action of BCG as an immunostimulant and particularly as an anticancer agent.

The mechanism of BCG bacteria targeting cancerous cells is considered to induce fibronectin either expressed by tumours or are associated at increased concentrations at the junction of the invading tumour and the healthy tissue (Kavouski *et al.*, 1990). It has been suggested that a major component in the reaction was the cell wall protein identified as antigen 85 (Klegerman *et al.*, 1993). These are a homologous group of three proteins that act as fibronectin antibodies.

**Figure 1.8**  Structure of BCG cell wall (Groves M.J, BCG: The past, present and future of a tuberculosis vaccine, Journal of Pharmacy and Pharmacology, 1997, 49(supp 1), 7-15)

Lou *et al* (1994) isolated an antineoplastic agent, PS1, from the Tice strain of BCG. This is a polysaccharide believed to be a bacterial glycan with antitumour activity in-vivo but not in-vitro suggesting immunostimulant properties (Lou *et al* 1994). BCG is effective in bladder cancer due to delivery of BCG directly to the site. However in lung cancer it is difficult to localise BCG at the tumour site. Kwok *et al* (1991) developed a method for delivering BCG directly to tumour cells in the lungs by encapsulation in alginate-polylysine microcapsules prepared using the Air-Atomisation technique. This involved suspending BCG in 0.5ml of 0.1% w/v bile salt and dispersion in 15ml of 1.2% sodium alginate in an air-atomising device (Turbotak, a hollow stainless-steel cylinder) at a rate of 2.2ml/min. The suspension was sprayed into a pan containing 500ml of 1.5% calcium chloride solution. This forms temporary cacium alginate microgel capsules, which are then cross-linked with polylysine to form microcapsules of 5-15µm. The microcapsules were then frozen at -70°C overnight and freeze-dried at -60°C at 10µm for 72hrs. The capsules were then stained with Rhodamine T TB fluorescent stain. The BCG organisms fluoresce under a Zeiss fluorescent microscope. The encapsulated BCG was also identified using the Fisher Diagnostic AFB stain kit, which stains BCG red.

## 1.9   Oral delivery of vaccines

Most of the currently available, licensed vaccines are administered parenterally with the exception of poliomyelitis and typhoid. The parenteral route ensures that adequate amounts of the vaccine can reach the systemic circulation, giving an effective systemic immune response to most invading pathogens. A systemic immune response is adequate for the prevention of infectious diseases, such as tetanus and malaria, where the infectious agent enters the host by the parenteral route. However it is limited when a protective immune response is required at the mucosal surfaces of the body, the sites where the majority of pathogens enter. Numerous studies have demonstrated the induction of mucosal immunity at the

48

initial site of infection to be the main protector against infection. A strong mucosal immune response is needed since systemic immunity only results in partial protection. The limitations of parenteral vaccines have prompted much research into non-parenteral vaccines, particularly oral delivery, to induce effective and protective local and systemic immunity against invading pathogens.

The most effective way to induce local immunity is to administer a vaccine directly to the site in question. Fortunately the mucosal immune sites are interconnected by a common mucosal system whereby stimulation of an inductive immune site results in migration of antigen specific lymphocytes to other mucosal sites in the body (Chen *et al.*, 2000). For this process to be effective the mucosal tissue must possess lymphoid tissues and not all sites do. The largest accumulation of lymphoid tissue is in the gastrointestinal tract (GIT) (Mcghee *et al.*, 1992). Therefore oral administration of vaccines results in an immune response not only in the GIT but other mucosal sites. This is one of the major advantages of oral vaccine delivery over parenteral delivery and others are shown in table 1.1.

Table 1.1 The advantages and disadvantages of oral delivery

| Advantages | Disadvantages |
| --- | --- |
| Increased patient compliance | Not acceptable to some patients, cows, badgers |
| Ease of administration to humans and animals | Short plasma half-life |
| The potential for almost unlimited frequency of boosting | Can be destroyed by low pH of stomach and enzymes |
| Does not require the presence of trained personnel | Low levels of macromolecular absorption |

Oral administration continues to be the most attractive route for the delivery of all medicinal agents due to the ease of administration; lack of associated pain and cost effectiveness. Oral dosage forms constitute the most commonly used products for drug administration with 60% of all available pharmaceutical products being orally delivered (Singh *et al.*, 1998). Although the oral route is extensively used for drug delivery it has yet to become a prominent route for vaccine administration. To date

only polio and live typhoid vaccine are Ty21a ('Vivotif') are licensed for oral delivery. A major problem, which needs to be overcome is the protection of the antigen against the harsh conditions of the gastrointestinal tract notably the low pH and the presence of enzymes. One approach to the development of new delivery systems for oral vaccines involves encapsulation of the antigen by a biodegradable polymeric matrix.

The objective of formulation of oral vaccines is to improve on one or more of the following parameters: -

- To reduce gastric and enzymatic degradation of the antigen
- To enhance antigen absorption
- To extend residence time in the gut
- To reduce the dose of antigen needed to induce a significant immune response
- Reduce non-specific interactions with food proteins
- To promote uptake of antigen by the gut-associated lymphoid tissue (GALT) - discrete nonencapsulated aggregates of lymphoid follicles known as Peyer's patches.

### 1.9.1 Mucosal immune system

The immune system of the mucosal surfaces differs in many ways from the systemic immune system. Mucosal immunisation results in the stimulation of both mucosal and systemic immune responses. The stimulation of mucosal immunity results in complex immunoregulatory mechanisms resulting in the production of secretory IgA antibody, which is not associated with parenteral immunisation. The principle cell types involved following mucosal immunity are B and T lymphocytes, mast cells, macrophages, dendritic cells, natural killer cells (NK) and

specialised follicle associated epithelial (FAE) or microfold (M) cells (Mcghee *et al.*, 1993).

Functionally the mucosal immune system can be divided into two separate but interconnected sites (Mcghee *et al.*, 1992).

- Inductive sites, where the antigen is encountered and initial B and T cells are triggered.
- Effector sites that comprise larger surface areas where IgA antibody results in local immune protection at local and distal mucosal sites.

**Table 1.2**   Comparison of the main characteristics of mucosal and parenteral immunisation (Compiled from Nugent et al, Journal of Clinical Pharmacy and Therapeutics, 1998, 23, pp258)

| Mucosal immunisation | Parental immunisation |
|---|---|
| Local and systemic immunity | Systemic immunity only |
| S-IgA and S-IgG produced | IgG and IgA produced |
| Enhanced parental priming | Boosts the mucosal response |
| No age-associated immune dysfunction | Age-associated immune dysfunction |
| More antigen needed to induce an immune response | Responds to small amounts of antigen |
| Mucosal adhesion is a critical property for the induction of a mucosal response | Sensitive to complex microbial antigens |
| Poor adsorption across mucosal barriers | |
| Injection not necessary | Administration by injection |
| Little training to administer vaccine | Training required to administer vaccine |

*1.9.1.1  Gut-Associated Lymphoid Tissue*

The GALT consists of specialised regions, which possess the capability of uptake of antigens or peptides and proteins. The most important region is known as the Peyer's patches. In this region stimulation of T helper cells and IgA precursor B cells with orally delivered antigens/peptides leads to stimulation and release of B

and T helper cells to mucosal effector sites, such as lamina propria in the GIT and secretory glands, leading to antigen specific secretory IgA antibody responses (Lavelle *et al.*, 1995). The two compartments are linked whereby cells induced by the antigen/peptide in the GALT migrate via the lymphatic and thoracic ducts into blood circulation and subsequently interact with specific receptor molecules on the endothelium of mucosal capillaries and enter the mucosae.

### *1.9.1.2 Specialisation of the GALT for antigen uptake*

Separating the organised lymphoid tissues from the intestinal lumen is the lympho-epithelium compromising a single layer of columnar epithelial cells with goblet and specialised M cells (Yeh *et al.*, 1998). The M cells uptake antigens/peptides from the lumen via endocytosis and transport them to MHC class II molecules. M cells are composed of short microvilli, small cytoplasmic vesicles, lysosomes and can endocytose and transport protein antigens, inert particles, microorganisms (including bacteria, viruses) into GALT (Lavelle *et al.*, 1995). The primary function is the transport of antigens/peptides from the surface luminal membrane of the Peyer's patches with little degradation or chemical change. It is thought that because the Peyer's patches have reduced mucus secreting goblet cells compared to the surrounding epithelium they are more accessible for microorganism binding and hence uptake (Yeh *et al.*, 1998).

Directly beneath the Peyer's patches are follicles, which contain germinal centres that are involved in the production and cell division of B cells. T cells are found directly adjacent to these follicles. The fact that T cells are present in Peyer's patches demonstrates their importance in inducing an immune response (Lavelle *et al.*, 1995). CD8 and cytotoxic T lymphocytes can also be induced in the Peyer's patches. Therefore it is apparent that all the necessary cells for induction of mucosal immune responses are present at the GALT inductive sites, particularly in the Peyer's patch region.

## 1.9.2 Peyer's patches

Peyer's patches are collections of lymphoid follicles, which are separated from the intestinal lumen by a single layer of specialised epithelium containing M cells and FAE. Peyer's patches play a central role in antigen uptake and induction of an immune response. The number and location of peyer's patches varies between species, but the basic morphological structure is very similar. The number and size of the Peyer's patches increases with age, body weight, and length of the small intestine (Yeh et al., 1998).

### 1.9.2.1 M cells

Uptake of macromolecules and particulates across healthy gastrointestinal epithelium occurs mainly by vesicular transport across epithelial cells i.e. transcytosis. Vesicular uptake by enterocytes is hindered by rigid, closely packed microvilli, which are coated with thick filamentous brush border glycocalyx (Holmgren et al., 1993). The major component of the glycocalyx is a transmembranous mucin with oligosaccharide chains. The glycocalyx forms both diffusion and an enzymatic barrier (Bowerstock et al., 1999).

M cells use multiple endocytic mechanisms for uptake of macromolecules, particulates and microorganisms. They can carry out fluid phase endocytosis, adsorptive endocytosis and phagocytosis (Kim et al., 1999). Each of these processes results in transport of material into endosomes and large multivesicular bodies, followed by exocytosis across the basolateral membrane. It also offers a pathway for invasion with the risk of infection and disease. Different pathogens have developed diverse strategies for invasion via mucosal surfaces (Kim et al., 1999).

**Figure 1.9** Diagrammatic representation of possible mechanisms of macromolecular transport (DA Norris et al Advanced Drug Delivery Reviews 34 (1998) 135-154

A= uptake into GALT and macrophages C=uptake into goblet cells
B= endocytosis into columnar epithelial cells D= paracellular transport

**Figure 1.10** Schematic diagram of particle absorption by M cells (H.chen et al Journal of controlled release 2000, 67: 117-128)

Viruses rely simply on binding to and transcytosis across M cells, while bacteria use more complex strategies, involving alteration of the M cell surface, initiation of signal transduction events and recruitment of inflammatory cells.

In both cases the end result can vary from simple transport across the M cell to M cell and enterocyte death and/or destruction of FAE.

### 1.9.3 Uptake of particulate materials across the Gastrointestinal Tract

In addition to its role in nutrient digestion and absorption the digestive tract occupies a vital defensive position, responding to immunological challenges via a number of specific and non-specific processes. However oral delivery of peptide/protein drugs and antigens is frequently compromised by poor uptake due to their size, hydrophilicity and because of lumenal, brush border and intracellular degradation (Lavelle *et al.*, 1995). There are also physiological barriers such as acidity of the stomach and enzymes. Three possible routes for gastrointestinal uptake of small particles exist: -

- Intracellular uptake by enterocytes
- Intercellular/paracellular transfer
- Uptake via the M cells of the Peyer's patches.

The most popular opinion appears to suggest that particulate uptake in mammals is principally via the M cells of the Peyer's patches. Although uptake by enterocytes in the villous part of the GIT and particle transport by paracellular (between cells) pathway has been demonstrated by microscopy, these probably play only a minor role in particle uptake. Either pathway could result in the delivery of particles to macrophages in the Peyer's patch.

### 1.9.3.1 Mechanisms of transepithelial transport

Paracellular pathway

Epithelial cells make up the majority of cells lining the gastrointestinal tract. Transport between cells (or paracellular transport) occurs via aqueous channels that vary in pore diameter between species (Norris *et al.*, 1998). A variety of approaches for enhancing absorption of peptides and proteins have focused on the junctional pathway. Recent evidence has shown that these junctional complexes can be altered by a variety of cellular mechanisms that may be responsible for an increase in pore diameter associated with an increase in transport of macromolecules with molecular weight ranging from the size of insulin to dextran (molecular weight ranging from 10 to 2000KD) (Yeh *et al.*, 1998).

Transcellular pathway

Transport of proteins and peptides across the intestinal epithelium can occur by a number of different transcellular pathways, including passive transcellular transport, carrier-mediated transport and endocytosis/transcytosis (Norris *et al.*, 1998). For particulates it appears that transport across the intestinal epithelium occurs by transcytosis (Chen *et al.*, 2000).

Transport of particulates

It is generally believed that the bulk of particle translocation across the GIT occurs in the follicle-associated epithelium (FAE) (Brayden *et al.*, 2001) of the Peyer's patches. This is a specialised epithelium covering mucosal lymphoid tissue and consists of M cells that are specialised for endocytosis/transcytosis of antigens and microorganisms to the organised lymphoid tissue within the mucosa. After translocation across the M cells particles still need to cross the underlying basement membrane, which contains pores 3μm or larger (Brayden *et al.*, 2001). M cells

contain a special pocket into which transcytosed particles are released after crossing the basement membrane. This pocket is filled with lymphocytes and macrophages. Antigens delivered via particulate carriers may be protected from degradation and will be presented directly to the mucosal immune system following transcytosis by the M cells.

### 1.9.4   Factors influencing the uptake of particulates from the GIT

Particulate uptake has been shown to depend on size, charge, hydrophobicity and polymeric composition of the particles.

__Particle size__ has been identified as a critical factor in determining both uptake and biological fate. In mice ploy(DL-lactide-co-glycolide) microspheres greater than 5μm in diameter are excluded from Peyer's patches whereas those between 2 and 5μm remained in the Peyer's patches; and those below 2μm migrated from Peter's patches to mesenteric lymph nodes (Holmgren *et al.*, 1993). Particle size is a critical determinant of the fate of orally delivered microparticles and possibly the immune response to the antigen. Larger particles may be retained for longer periods in the Peyer's patches while smaller particles are progressively transported to other major organs (Chen *et al.*, 2000). Particles of less than 10μm are absorbed by M cells and translocated to the Peyer patch region (Chen *et al.*, 2000). Studies with poly(lactide-co-glycolide) (PLG) microparticles demonstrated that particles less than 5μm in diameter were endocytosed and transported through efferent lymphatics to systemic lymphoid tissues where they stimulated serum antibody response. Particles greater than 5μm in diameter remained in the Peyer's patch leading to the sustained release of antigen (Chen *et al.*, 2000).

In general smaller microparticles are distributed more easily to distant sites and remain detectable for longer periods of times. Jani *et al* (1992) showed that three sizes of polystyrene (50nm, 500nm and 1μm) were distributed from Peyer's patches

to the mesentery, liver and spleen at different rates and extents. 50nm particles were taken up into Peyer's patches within 6 hours after gavage administration to rats. 500nm and 1μm particles were absorbed more slowly and to a lesser extent into all tissues examined (Peyer's patches, Mesentery lymph network nodes, Liver and Spleen) (Jani *et al.*, 1992)[58].

**The polymeric composition** of particles also appears to be important in determining the uptake of particles. Microparticles (50nm-1μm) administered by oral gavage to rats were found only in Peyer's patches regions of small intestine (Jani *et al.*, 1992). Examination of lymphoid and non-lymphoid tissues after the administration of particles by oral gavage revealed a total uptake of approximately 10% with 60% of the particles taken up into lymphoid tissue.

A comparative study investigating a range of particles demonstrated that the accumulation of orally administered biodegradable microspheres, including PLG, was less extensive than that of polystyrene and suggested that the extent of accumulation in Peyer's patches was dependent on the hydrophobicity of the polymeric material. It was suggested that this behaviour might have reflected a greater association of the PLG particles with mucus in the gut (Chen *et al.*, 2000).

**Particle charge** has been found to determine the extent of uptake from the gut. Uptake of carboxylated microspheres in rats was less than that of non-ionic microspheres (Brayden *et al.*, 2001).

**Species differences** are also important in the uptake of particulate materials. Studies have found the uptake of polystyrene microspheres was lower in murine Peyer's patches than in rabbits. The rabbit follicle-associated epithelium (FAE) is known to be rich in M cells so the uptake of particles may be greater.

In general particles with a relatively greater hydrophobicity have been found to be absorbed more readily onto Peyer's patches. Florence et al have shown that

decreasing **surface hydrophobicty** by the adsorption of poloxamers 235, 238, 407 or poloxamines 901, 904, and 908 may decrease the uptake of polystyrene microparticulates into cells of the immune system thereby avoiding elimination (Chen *et al.*, 2000). They also showed up to 10% cumulative absorption throughout the GI tract of uncoated polystyrene particles, while coating with poloxamer 188 and 407 decreased the cumulative absorption to less than 3% and 1.5% respectively.

### 1.9.5 Vaccine formulation

Systemic routes administer all widely used vaccines, except the polio vaccine. These vaccines are effective in inducing systemic cell mediated and antibody responses but are poor at inducing mucosal immunity in humans who have not had previous mucosal infection by the causative organism. Higher and more frequently administered antigen doses are generally required for oral vaccination. The poor immune responses to orally delivered antigens result from enzymic degradation and low absorption levels; consequently little antigen reaches the GALT (Kim *et al.*, 1999). When incorporating antigens/peptides and proteins into formulations for oral delivery, certain considerations apply: -

- Live microorganisms provide much better antigens than killed bacterial or viral antigens. This is possibly due to their capacity to adhere to the mucosal surfaces.
- Most soluble antigens are less effective in inducing mucosal responses than particulate antigens. This is due to the different cell types involved in antigen processing. Uptake of particulate antigens into Peyer's patches may lead to presentation to dendritic cells and macrophages. Not all soluble proteins are poor mucosal immunogens, some such as cholera toxin can effectively induce antibody responses in serum and secretions of orally immunised subjects.

Strategies are available to increase the efficacy of orally delivered molecules. Most approaches are concerned with avoidance or modification of gastric secretions by the use of gastric inhibitors, anti-proteases, acid resistant films or encapsulation (Holmgren *et al.*, 1993). An adjuvant activity has been demonstrated when liposomes and recombinant gram-negative bacteria are delivered orally (Holmgren *et al.*, 1993). Immune stimulatory complexes (ISCOMS) confer immunogenicity on proteins delivered by the oral route and very low amounts of antigen in such structures are immunogenic. The incorporation of antigens into liposomes or microparticles protects them from harmful digestive secretions and thus allows the use of lower doses than is the case when soluble antigen is administered. Oral live vaccines yield higher antibody titres in serum than do oral killed vaccines. Research is now focusing on the use of attenuated live organisms, both as oral vaccines and as carrier vehicles for enteric delivery of heterologous antigen.

**Aluminium salts** have been used as adjuvants in many human and veterinary vaccines, primarily due to their low cost and safety. The adjuvant activity is dependent on surface charge, surface area and morphology.

**Oil in water (o/w) or water in oil (w/o) emulsions** exhibit a wide range of properties (such as particle size) depending on emulsifying conditions and type and amount of surfactant used (Bowerstock *et al.*, 1999). The antigen is usually included during the emulsification stage and becomes incorporated within the droplets. The surfactant and also oils used have immunostimulatory effects. The formulations act as a depot either at the site of injection or within APC's. The immune responses produced by emulsions are varied due to differences in formulation conditions from laboratory to laboratory.

**ISCOMS** (Immunostimulatory complexes) are small particles of cholesterol and phospholipids. The particles produced are generally in the range of 30 – 40nm to which the vaccine antigen can be added without affecting the size or function (Bowerstock *et al.*, 1999). ISCOMS generally induce a very strong immune

response, which is partly due to their small size, and hydrophobicity, which enhance their phagocytosis by antigen presenting cells. Ponies vaccinated with inactivated equine influenza virus or purified haemagglutinin (Equip™) encapsulated in ISCOMS shed less virus, had fewer days of fever, less coughing, and greater serum antibodies than non-vaccinated ponies (Mumford et al., 1994). Also the immune response could be measured at 15 months, which is much longer than conventional adjuvants that normally require a booster dose after 3 months (Mumford et al., 1994).

Microparticles consisting of the vaccine antigen and a carrier formulation are generally in the size range of 1-200μm in diameter (Bowerstock et al., 1999). The materials and techniques used to produce these vehicles differ greatly from each other. Copolymers of polylactide and polyglycolide esters have been used extensively to produce small biodegradable microspheres for delivery of vaccine antigens (O'Hagan et al., 1993). These polymers have FDA approval for use in humans and are also biocompatible and biodegradable drug delivery vehicle (O'Hagan et al., 1993). Particles generally produced are within the range of 1-10μm so macrophages and other antigen presenting cells can phagocytose them. Particles are made by either coacervation or emulsion techniques. Long lasting immunity can be induced by parenteral administration of microparticles produced using different ratios of polymers of different molecular weights that hydrolyse at different rates and thus deliver antigen over a wide time range. In addition the use of particles of mixed sizes, especially larger particles that avoid uptake by macrophages and hence breakdown at a slower rate, can induce long lasting immunity (O'Hagan et al., 1994).

Liposomes are bilayered phospholipid particles made of phospholipids or sterols surrounding an aqueous core and can vary in size from 0.01 to 150μm (Bowerstock et al., 1999). Water-soluble materials can be encapsulated in the aqueous core and hydrophobic agents in the outer lipid layer. They are readily adsorbed onto mammalian cells where they can release antigen or can be phagacytosed by APC

and then release the antigen. The use of liposomes for vaccine delivery is limited due to their poor stability and the use of organic solvents in their preparation that could affect antigens (Bowerstock *et al.*, 1999).

### 1.9.6 Oral vaccination of animals

Most infections begin at a mucosal surface. The most effective way to induce mucosal immunity is to administer a vaccine directly to the site where a pathogen invades the host. However this is not always practical. For example intranasal administration of vaccines to prevent respiratory diseases in livestock where there are hundreds of animals that must be individually caught and treated is both time consuming and labour intensive. An alternate delivery to the parenteral route of vaccines to induce local immunity is needed in animals. As in humans, the mucosal immune sites are interconnected by a common mucosal immune system (CMIS) whereby stimulation of an inductive immune site results in migration of antigen specific lymphocytes to other effector mucosal sites in the body. The largest accumulation of lymphoid tissue is in the gastrointestinal tract (GIT) as in humans. Similar techniques have been used in humans and animals to protect antigens during oral delivery i.e. encapsulation of antigens in microparticles or microspheres. Bowerstock *et al* (1999) used sodium alginate to encapsulate antigens. Alginates are readily available, relatively inexpensive, and polymerise under very mild conditions thereby avoiding degradation or alteration of antigens during encapsulation (Bowerstock *et al.*, 1999). Ovalbumin was encapsulated in sodium alginate using an aerosolisation technique for delivery to rabbits and cattle or using an emulsification method for delivery to mice. The mean levels of IgA after vaccination with OVA encapsulated in sodium alginate microspheres was greater than in the unencapsulated group, suggesting that the OVA was protected from digestive enzymes and low pH. There was variation in immune response in cattle most probably due to the need for antigen transit through four stomachs before stimulation of the intestinal Peyer's patches. The results in mice also

indicated a greater immune response. The emulsion method produced repeatable sizes of particles compared with the spray drying technique. The loading was also higher than spray-dried particles.

Bowerstock *et al* (1994) loaded the bacterium *Pasteurella haemolytica*, (the primary organism responsible for pneumonia in cattle) using crosslinked poly(methacrylic acid) (PMMA) in hydrogels. This was selected as a durable non-biodegradable polymer that could be molded in a size and density that could bypass the rumen when administered to cattle. Cattle were administered the vaccine for five consecutive days with control calves given hydrogels without antigen. After three weeks the calves were challenged individually by intrabronchial instillation of live *P.haemolytica*. All surviving calves were killed after three days and lungs analysed. Orally vaccinated calves lived longer and had less severe pneumonia.

### 1.9.7 Enteric coating systems

Enteric coating is used in the pharmaceutical industry to mask the taste of drugs, to protect the incipients from the environment and to function as a controlled release system but mainly to prevent degradation of the active species by the harsh conditions of the stomach and deliver the drug to the small intestine. Commercially available polymers commonly employed for enteric coating consist of cellulose acetate phthalate, hydroxypropylmethylcellulose phthalate, copolymers of methacrylic acid and acrylic esters and polyvinylacetate phthalate (Crotts *et al.*, 2001). These substances are generally anionic polymers that are insoluble in acidic environment but are soluble in neutral to alkaline conditions due to the functional groups along the polymer chain.

An important factor to consider during tablet coating is the pH of the tablet core containing the drug, which can affect the disintegration time. Crotts *et al* (2001) noticed that a core pH adjusted to 3 had a longer in vitro disintegration time than

similar tablets with a core pH of 5. This result was repeated in experiments performed in vivo. They proposed that this difference in dissolution properties was due to ionisation of functional groups at low pH thus affecting the tablet break up. Consequently tablets with a high proportion of an acidic therapeutic agent or acidic excipients would probably exhibit retardation in dissolution if directly enteric coated. Crotts *et al* (2001) in their latest study attempted to overcome this problem by applying a polymer barrier film between the core and enteric coat. They chose an acidic tablet core (microcrystalline cellulose with citric acid 87%w/w) containing a marker drug separated into three groups and seal coated to levels of 0%, 1% and 3%. The tablets were then coated using Eudragit® L30D with different amounts of plasticizer (10-20 parts) and talc (10-50 parts). Dissolution studies revealed that all enteric coat formulations inhibited drug release for 2 hours in 0.1M HCl. Seal coated tablets exhibited a 1.5-5 fold greater drug release at most intermediate sample time points in phosphate buffer, pH 6.8, than tablets without a seal coat. This suggests that the dissolution of the tablets without the seal coat was delayed by the generation of an acidic environment at the interface of the enteric coat/acidic tablet core as discussed above.

Freichel *et al* (2000) have used enteric coating systems to modify the release profile of drugs incorporated into a hydrocolloid drug delivery system. Hydrocolloid matrices are limited in their use as drug delivery systems due to decreasing drug release over time, resulting in incomplete absorption. The aim was to accelerate the drug release of hydrocolloid matrices during the final release process. They incorporated methylhydroxyethylcellulose as the base polymer and hydroxypropyl methylcellulose acetate succinate as the release modifier with Pentoxifylline as the model drug. The greater amount of modifier in the formulation lead to a later and also more pronounced progression of release, it is also insoluble at acidic pH 1.1 and 4.5 used in this study. When the tablets were transferred to the dissolution media of pH 6.8 the modifier begins to dissolve from the gel layer and thus decreasing the stability of this layer. Consequently an accelerated erosion of the

base polymer gel occurs. This finally leads to loss of structural integrity of the gel layer and subsequent increased erosion occurs.

Aqueous polymeric dispersions have been used widely in the pharmaceutical industry for enteric coating of dosage forms such as tablets (Crotts *et al.*, 2001). However there is a problem of premature drug release in the stomach or under acidic conditions. Guo *et al* (2002) used amylopectin as a co-filler in the enteric coating to aid in gastric resistance and dissolution. They used riboflavin sodium phosphate as a model drug and microcrystalline cellulose (MCC) and lactose as fillers in pellet cores. The pellets were subcoated with 5% amylopectin solution or with 5% hydroxypropyl methylcellulose (HPMC) and film coated with cellulose acetetate phthalate (CAP). Guo et al found that pellets with amylopectin improved the acid resistance of the enteric coating compared with HPMC in 0.1M HCl (Guo *et al.*, 2002). Amylopectin in 4% concentration and CAP at 35% loading resisted 0.1M HCl for 1hr and dissolved in simulated intestinal fluid in 10 minutes.

Delgado A *et al* (1999) formulated PLG microparticles with enteric polymers Eudragit L100-55 and carboxymethylcellulose (CMEC) as stabilisers. These are water insoluble and dissolve at pH greater than 5.5. Thus they are capable of protecting the antigen from the harsh conditions of the stomach. A higher percentage of antigens were detected bound to the microparticles after incubation in pepsin (pH 1.2) and trypsin (pH 7.4) solutions. However in simulated gastric fluid the microparticles protected with CMEC exhibited a higher percentage of bound antigen compared with the Eudragit system. In vivo experiments in mice resulted in correspondingly higher levels of specific IgA. Enteric coating systems may therefore be useful for increasing the efficacy of microparticle based oral vaccines.

In 1921 Calmette chose the oral route for BCG vaccination for its simplicity of administration, the demonstration of BCG penetration through the intestinal epithelium in newborn animals and babies, and the capacity of BCG to induce specific mycobacterial immunity through this route (Lagranderie *et al.*, 2000).

65

Lagranderie *et al* (1997) has shown that BCG is able to cross the intestinal barrier through the M cells of Peyer's patches. They found BCG in Peyer's patches of mice 6 hours after oral ingestion (Lagranderie *et al.*, 1997). Despite the successful immunity achieved with oral BCG, this route of vaccination was abandoned due to it causing cervical adenitis, otitis, retropharyngeal abscesses and weak tuberculin reactions, due to the large dose required to give immunity (x10$^9$ cfu's). Large doses were primarily given due to BCG being degraded by the acidic environment of the G.I.T.

Mutwiri *et al* (2002) analysed whether porcine rotavirus antigens encapsulated in alginate microspheres could induce mucosal immune responses following oral immunisation. They immunised mice with either live porcine rotavirus (PRV) or its recombinant protein (VP6) encapsulated in alginate microspheres prepared by emulsion-cross linking technique and spray drying respectively. They compared the results to intraperitoneal immunisation with PRV and VR6 combined with incomplete Freund's adjuvant. Oral immunisation of VP6 encapsulated with alginate microspheres induced the highest level of IgA antibody, similar to oral immunisation with live PRV. Further experiments were performed in a sheep's intestinal loop model to evaluate uptake of microspheres by Peyer's patches. Microspheres were found in the follicle-associated epithelium of Peyer's patches and thus confirming that alginate microspheres have the potential as an effective delivery vehicle for oral immunisation.

Ming-Kung Yeh *et al* (2002) encapsulated *Vibrio cholerae* (VC) into poly(DL-lactide-co-glycolide)(PLG) microparticles prepared using water-in-oil-in-water emulsion/solvent extraction technique. They evaluated the immunogenic potential of VC-loaded microparticles in comparison to PLG microparticles or VC solution on its own by oral immunisation. VC-loaded microparticles induced IgG and IgM antibody thus concluding that PLG microparticles can be used as an oral delivery system.

## 1.10  Immunological approaches to the control of tuberculosis in the wild

National bovine tuberculosis programmes based on regular tuberculin testing and removal of infected animals has had a profound influence on reducing the incidence of bovine tuberculosis in cattle and farmed deer herds (Buddle *et al.*, 2000). Since the 1970s, accumulating evidence suggests that badgers in the UK act as maintenance hosts for *M bovis* infection and as important sources of infection for cattle and farmed deer (Buddle *et al.*, 2000). The role of badgers as a source of *M.bovis* infection for cattle has been difficult to ascertain since badgers are protected species. Early control strategies, and more recent studies, revealed that the removal of badgers from areas, which had persistent problems of bovine tuberculosis in cattle, resulted in reductions in numbers of cattle reactors. Although *M.bovis* control strategies for badgers based on culling may prove effective in reducing cattle herd breakdowns, they are non-discriminatory and large numbers of uninfected badgers are likely to be killed needlessly. A more attractive option from an animal welfare standpoint would be vaccination of badgers against *M.bovis* (Buddle *et al.*, 2000). Development of an effective tuberculosis vaccine coupled with an appropriate delivery system would be of great assistance in controlling bovine tuberculosis in this situation (Krebs., 1997).

Recent advances in immunology and the molecular biology of mycobacteria have greatly increased the options for candidate vaccines. The two major groupings are live attenuated vaccines such as BCG and other attenuated mycobacteria of the *M.tuberculosis* complex, and subunit vaccines based on either mycobacterial protein or DNA. Live attenuated vaccines are advantageous as they promote strong cellular immune responses required for protective immunity against tuberculosis and may only need a single delivery to induce life-long immunity. The latter feature is particularly important for vaccinating wildlife where animals may only take up vaccine bait on one occasion, or be trapped once for vaccine administration (Buddle *et al.*, 2000). The existing BCG vaccine, derived from *M.bovis*, is inexpensive to produce, can be administered via a number of routes, is safe and relatively stable.

However cattle exposed to BCG vaccine may become sensitised for a positive tuberculin reaction and on this basis would be scored positive for bovine tuberculosis and be slaughtered. Therefore the development of new vaccines that would not compromise the use of the tuberculin test in this way is a major research goal. Alternatively, vaccines engineered with an immunological 'tag' or the use of diagnostic tests with refined antigens could permit the differential diagnosis of bovine tuberculosis and exposure to vaccine (Buddle *et al.*, 2000).

There are a number of strategies for delivering vaccines to wildlife species. Usually the target species is trapped and the vaccine delivered to the restrained animal, or the vaccine is delivered in some form of bait or food (Buddle *et al.*, 2000). However administration of BCG by the oral route has not induced high levels of protection, possibly due to inactivation of the BCG in the stomach (Buddle *et al.*, 2000). Thus protection of BCG from inactivation in the stomach would be the first step towards an effective oral BCG vaccine. For delivery in bait form, the stability of a live vaccine such as BCG under diverse environmental conditions, for prolonged intervals will also have to be addressed.

## 1.11 Development of diagnostic reagents to differentiate between BCG vaccination and *M.bovis* infection in cattle

Bovine tuberculosis is caused by *M. bovis* and forms a significant economic loss to agricultural communities (Neill *et al.*, 1994b) and its control requires the implementation of test and slaughter programmes. In Great Britain the test and slaughter programme relies on the tuberculin skin test to detect cattle infected with *M. bovis* (Stenius *et al.*, 1938). The skin test uses bovine purified protein filtrate (PPD) prepared from culture filtrate of a laboratory strain of *M. bovis* (Whipple *et al.*, 1995). However, false positive reactions can occur with a range of microbial infections including *Mycobacterium avium*, *Mycobacterium paratuberculosis* and the agent of skin tuberculosis. To overcome this problem, the comparative

intradermal tuberculin skin test (inoculation with both bovine and avian tuberculin) has been used in this country and others (Whipple *et al.*, 1995).

A compulsory eradication program for bovine tuberculosis (TB) in cattle based on tuberculin testing and slaughter of reactor animals began in the UK in the 1950s Pritchard *et al.*, 1988). By 1960 the program had been successfully implemented throughout the whole of the country. However attempts to eradicate the disease have not been totally successful due to wildlife reservoirs, such as deer and badgers (Krebs *et* al., 1997). The tuberculin skin test lacks specificity, does not detect infection early on in animals and the results can be inconclusive. The retest can be expensive and time-consuming due to the de-sensitisation caused by the initial tuberculin skin test. The animals can only be re-tested after a period of 60 days followed by another 60-day waiting period if the retest remains inconclusive (Radnuz *et al.*, 1985). Consequently this delay and inconclusive results can result in spread of infection between cattle. The test is also compromised by the attenuated vaccine strain BCG and may be similarly affected by novel vaccines comprising *M. tuberculosis* or *M.* bovis (Vordermeier *et al.*, 1999).

More recently novel cellular diagnostic methods have been developed to overcome some of the problems associated with the skin test. The most commonly used assay is the BOVIGAM™ assay, based on a sandwich enzyme immunoassay for the measurement of bovine interferon-γ (IFN-γ) (Rothel *et al.*, 1990; Wood *et al.*, 1991). The BOVIGAM™ interferon-gamma (IFN-γ) has shown promise in New Zealand to resolve doubtful skin-test results earlier, since a retest using the BOVIGAM™ assay is possible as early as 7 days after the initial skin-test. Using defined mycobacterial proteins, which are present in M. bovis but not in environmental Mycobacteria species, has also increased the specificity of this assay. ESAT-6,p45 has been identified as an important T-cell target for *M. bovis*-infected cattle and a dominant antigen for stimulating IFN-γ responses (Pollock *et al.*, 1997a). It is absent from environmental species of Mycobacteria (Pollock *et al.*, 1997b), which can induce non-specific skin test responses and in *M. bovis* BCG,

69

thus IFN-γ responses to ESAT-6,p45 can differentiate between vaccinated and non-vaccinated cattle (Buddle *et al.*, 1999).

The number of cases of bovine tuberculosis (TB) in cattle is once again on the increase and has been increasing since 1988 (Vordermeier *et al.*, 1999). A recent independent scientific panel set up to review the control of bovine TB in UK, concluded that the development of a cattle vaccine against *M.bovis* holds the best long term prospect for TB control in British herds.

Along with vaccination, the development of complementary diagnostic tests to differentiate between vaccinated animals and those infected with *M.bovis* is required so that the test and slaughter control strategies can continue alongside vaccination.

The comparative intradermal tuberculin test is compromised by the attenuated vaccine strain BCG and may be similarly affected by novel vaccines comprising *M. tuberculosis* or *M. bovis* (Vordermeier *et al.*, 1999). Researchers at the Veterinary Laboratories Agency (VLA) Addlestone are pursuing an alternative diagnostic approach involving target antigens expressed by *M. bovis* but absent from or present at only low levels in BCG (i.e. ESAT-6,p45). Use of these antigens in an in vitro blood-based assay (BOVIGAM) is envisaged in which blood lymphocytes will be stimulated with target antigens followed by analysis of the culture supernatants for lymphokine production (IFN-γ, Il-2). The specificity of response to a cocktail of the above target antigens has already been demonstrated in that *M.bovis* infected cattle produced lymphokines after stimulation while vaccinated did not. Proof of principle has thus been provided for the intended diagnostic approach. In addition, research at VLA has identified several peptides based on the above target proteins that were recognized by the majority of the infected cattle. Sensitivity levels to a cocktail of these peptides compared favourably with those induced to the recombinant proteins.

70

Progress in developing diagnostic assays for tuberculosis (TB) in cattle would be achieved by improving the sensitivity of the peptide-based assay described above. The addition of higher concentrations (>10-15µg/ml) of soluble peptides to an in vitro assay to improve sensitivity is undesirable in view of increased cost. Furthermore, it is felt that the mechanism of displacement of peptides already associated with MHC molecules expressed on the cell surface by the added peptides would not be very effective. In contrast, peptide association with MHC molecules via a natural mechanism within an endosomal compartment is considered more effective and would require lower peptide concentrations.

### 1.11.1 Bovine Gamma Interferon Test (BOVIGAMTM)

The (Bovine Gamma Interferon Test) BOVIGAM$^{TM}$ was developed and patented by CSL Limited in Australia. The BOVIGAM$^{TM}$ test is a rapid, blood-based assay of cell-mediated immunity (CMI) for the diagnosis of bovine tuberculosis (TB) infection in cattle (CSL manual). In general, animals infected with *M.bovis* have T lymphocytes in their blood that recognise specific mycobacterial antigens present in bovine tuberculin purified protein derivative (PPD) (CSL manual). Tuberculin PPD antigens are presented to T lymphocytes in whole blood cultures and the production of IFN-γ from stimulated T cells is detected using a monoclonal antibody-based sandwich enzyme immunoassay (EIA). Lymphocytes from uninfected cattle do not produce IFN-γ and hence IFN-γ detection correlates with infection.

Unlike the skin test, where detection is based on inflammation and swelling, the BOVIGAM$^{TM}$ is a non-invasive, in vitro test that is performed in two stages. The test in its original form was a comparative test where the IFN-γ responses against bovine tuberculin were compared to those after stimulation with avian PPD. Avian PPD is included as a measurement of the response of cattle to environmental, *M.bovis*, mycobacteria.

71

Small aliquots of heparinised whole blood are first incubated simultaneously with either PBS (nil antigen), avian or bovine PPD. After overnight incubation the blood plasma is removed and the amount of IFN-$\gamma$ in each plasma sample is estimated as detailed below. The relative levels of IFN-$\gamma$ detected in the plasma samples indicate whether an animal is infected with *M.bovis*. Infection is indicated when bovine PPD stimulates more IFN-$\gamma$ than both avian PPD and the nil antigen control. Exposure to environmental mycobacteria is indicated when avian PPD stimulates most IFN-$\gamma$, compared to bovine PPD and nil antigens.

Field trials in Australia on over 13000 cattle have shown BOVIGAM$^{TM}$ to be significantly more sensitive than the intradermal tuberculin skin test for the diagnosis of bovine tuberculosis (CSL manual). This has been shown to be true by trials conducted in Republic of Ireland, Northern Ireland, New Zealand, Italy, Spain, USA and South Africa (CSL manual ). The test does not affect the immune status of animals and immediate repeat testing of suspect animals is possible without having to wait 60 days as for the skin test (Wood *et al.*, 1994). Another major advantage of the BOVIGAM$^{TM}$ test is that it is relatively unaffected by recent skin tests, and thus making confirmatory comparative testing of the initial skin test positive reactors possible within 7 days (Doherty *et al.*, 1995).

## 1.12 Research aims and objectives

### 1.12.1 Improving the sensitivity of peptide assays for diagnosis of bovine tuberculosis

**Aims**

To significantly reduce the amounts of peptide necessary to achieve satisfactory responses in the current blood lymphocyte- based diagnostic assay for TB by using microparticulate delivery systems.

**Objectives**

- To identify candidate particles for peptide adsorption and the size ranges of particles for promoting particle uptake and stimulation of blood lymphocytes. Investigations involved microparticles and nanoparticles produced from: -

Bioceramics e.g. Hydoxyapatite
Glass
Polystyrene
Calcium carbonate
Poly(L-lactide)

- To investigate peptide adsorption (ESAT-6,p45 and P107) and release characteristics in vitro using the selected carrier particles shown above. ESAT-6,p45 is not expressed in BCG Pasteur and thus is currently used in the BOVIGAM assay to differentiate TB infection in cattle, which have or have not been vaccinated. ESAT-6 refers to 6KDa early secreted antigenic target from a short-term culture filtrate of *M.bovis* (SDS-PAGE). ESAT-6,p45 has been

shown to induce strong proliferative and IFN-γ responses from PBMC's (Peripheral Blood Mononuclear Cells) isolated from *M.bovis* infected cattle.

- To assess the level of IFN-γ produced by lymphocytes in whole blood using the in vitro bovine gamma interferon test – an assay of cell mediated immunity for the diagnosis of bovine tuberculosis infection in cattle.

### 1.12.2 Improvement of oral tuberculosis vaccination strategies

**Aims**

Production of an oral BCG vaccine formulation that delivers dispersed active BCG to the small intestine.

**Objectives**

- To assess the dispersion properties of BCG in various aqueous media. Experiments were performed using various surfactants and suspending agents (see section 4.2.2) to prevent aggregation of BCG. This is important since aggregates of size greater than 2μm are not expected to be taken up by the Peyer's patches in the small intestine (Bowerstock et al., 1999).

- Selection of enteric coating polymers (eg Eudragit methacrylic acid copolymers, cellulose acetate phthalate (CAP)) for formulating oral BCG vaccines. Eudragit is a common enteric coating used in tablet formulations to protect drugs, such as ibuprofen (Perumal., 2001; Nykanen *et al.*, 2001; Nikolakakis *et al.*, 2000) against the harsh acidic environment of the stomach while completely undergoing dissolution in the small intestine.

- To establish optimum formulation conditions for BCG-loaded oral dosage forms. This study was performed to determine the characteristics of BCG disintegration, if any in HCl 0.1M pH 1.2 at 37°C, and dissolution in PBS solution pH 7 at 37°C. The aim is to protect BCG using enteric coating systems in HCl and allow controlled release of BCG in the small intestine to induce an immune response at the mucosal sites.

- Optimisation of enteric-coating matrices to establish no or minimum loss in 0.1M HCl pH 1.2 at 37°C for 2 hours and complete dissolution within 4 hours in PBS, pH 7 at 37°C.

# CHAPTER 2

# MATERIALS AND METHODS

# 2  MATERIALS

## 2.1  Improving the sensitivity of peptide assays for diagnosis of bovine tuberculosis

**Microparticles**

- Glass microspheres were obtained from Omya UK Ltd, Dorking, Surrey, UK.

  Surface modified glass microspheres (CPO3 5000) comprised soda lime glass beads coated with nylon having a median diameter of 3.5 – 7.0 μm.

  Unmodified glass microspheres (CPOO 5000) comprised soda lime glass beads having a median diameter of 3.5 – 7.0 μm.

- Sub-micron microcrystalline hydroxyapatite (MHA) nanoparticles (100nm) (P120 and P201) were obtained from Plasma-biotal, Tideswell, UK.

- Carbonate hydroxyapatite (CHA) nanoparticles (6nm) were provided by Dr J.E. Barralet, Biomaterials Unit, Dental School, St. Chad's Queensway, Birmingham, UK.

- Calcium carbonate microparticles having a median diameter of 3μm was obtained from Omya UK Ltd, Dorking, Surrey, UK.

- Polystyrene microspheres (2μm) and nanospheres (500nm) were obtained from Polysciences, Inc. Warrington, Pennsylvania, USA

77

**Protein and peptides**

- Ovalbumin (Grade V) was obtained from Sigma UK Ltd.

- Peptides (ESAT-6, p45) and P107 were supplied by VLA

- The peptide p45 is derived from the N-terminal sequence of the protein ESAT-6 and the sequence is MTEQQWNFAGIEAAAS. Peptide p45 was chosen because it is the ESAT-6 derived peptide that is most frequently recognised in cattle (and in man, mice or guinea pigs) (Vordermeier *et al.*, 1999). The peptide P107 is derived from the 38kDa protein PstS-1. The peptides were synthesised by using standard solid-phase peptide synthesis. Sequence fidelity and purity was checked by mass spectrometry and analytical HPLC. The peptide (P107) is a model peptide similar to that used for detection of TB in cattle. The sequence of the peptide is PKPATSPAAPVTTAAMADPA. Peptide P107 is derived from 38kDa protein PstS-1 and was synthesised by standard solid-phase peptide synthesis. The sequence fidelity and purity was checked by mass spectrometry and analytical HPLC.

**Reagents**

The Bovigam assay reagents were supplied by Veterinary Laboratory Agency, Weybridge, Surrey, UK. (See section 2.3.6).

PBS tablets were obtained from Sigma, UK Ltd. Borate and Saline buffers were prepared in the laboratory. (Appendix 1).

## 2.2　Improvement of oral tuberculosis vaccination strategies

**Bacteria**

VLA provided two strains of Bacillus Calmette-Guerin (BCG) (Pasteur and Tokyo) as potential candidates for formulation of BCG vaccine for oral delivery. Both

strains of BCG are stored frozen at -80°C and have to be reconstituted by allowing the vials to thaw naturally at room temperature.

Two samples of BCG Pasteur were provided, one having a concentration of $10^8$ and the other $10^{10}$ colony forming units (cfu) per ml.

**Surfactants**

Surfactants are characterised by the possession of two distinct regions in their chemical structure. These regions are hydrophilic (water liking) and hydrophobic (water hating). The hydrophobic portions are usually saturated/unsaturated hydrocarbon chains, and the hydrophilic regions are anionic, cationic or non-ionic. They are usually classified according to the nature of the hydrophilic group (Florence et al., Ch6, 2000).

The following surfactants were investigated as dispersing agents: -

- Polyvinylpyrrolidone (PVP) (SIGMA, UK, MW 10000)
- Sodium dodecyl sulphate (SDS) (SIGMA, UK, MW 288.4)
- Polyoxyethylene-sorbitan monolaurate (Tween 20) (SIGMA, UK, MW 1228)
- Polyoxyethylene-sorbitan monooleate (Tween 80) (SIGMA, UK, MW 1310)
- Poloxamer (PEO-PPO copolymer) F-68

The following reagents were also evaluated as dispersing agents for BCG: -

- Dextran (SIGMA, UK, MW 60000-90000)
- Polyethyleneglycol (PEG) (SIGMA, UK, MW 10000)
- Glucose (SIGMA, UK, MW 180.6)

**Enteric coating systems**

Rohm, Pharma-Polymers, Germany provided Eudragit S100, L100, L30 D-55 polymers. These enteric coating systems are based on methacrylic acid and methyl methacrylate and are freely soluble above pH 7, pH 6 and pH 5.5 respectively. Eudragit S100 and L100 are both in powder form. In order to deliver BCG, a solid oral dosage form was to be produced.

Eudragit L30 D-55 was selected because it is already supplied in aqueous dispersion. Thus incorporating BCG and other excipients is potentially easier than with the powdered Eudragits. (See appendix 2 for summary and comparison of Eudragit copolymers)

$$-CH_2-\underset{\underset{OH}{\overset{|}{\underset{|}{C=O}}}}{\overset{\overset{CH_3}{|}}{C}}-CH_2-\underset{\underset{OCH_3}{\overset{|}{\underset{|}{C=O}}}}{\overset{\overset{CH_3}{|}}{C}}-$$
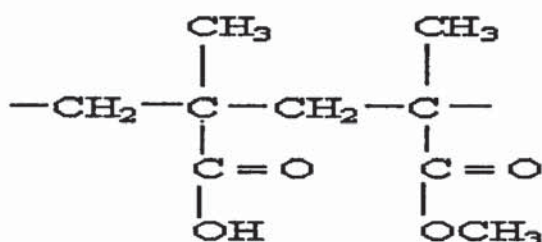
Chemical structure of Eudragit L100 and S100 copolymers. The ratio of free carboxyl groups to ester groups is approximately 1:1 in Eudragit L100 and approximately 1:2 in Eudragit S100. The molecular weight is 135 000

$$-CH_2-\underset{\underset{OH}{\overset{|}{\underset{|}{C=O}}}}{\overset{\overset{CH_3}{|}}{C}}-CH_2-\underset{\underset{OC_2H_5}{\overset{|}{\underset{|}{C=O}}}}{CH}-$$
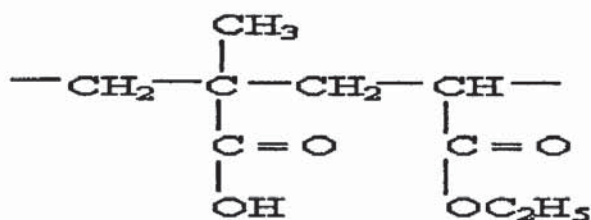
Chemical structure of Eudragit L 30D copolymer The ratio of free carboxyl groups to ester groups is approximately 1:1 in Eudragit L100-55 and approximately 1:2 in Eudragit L30D-55. The molecular weight is 250 000
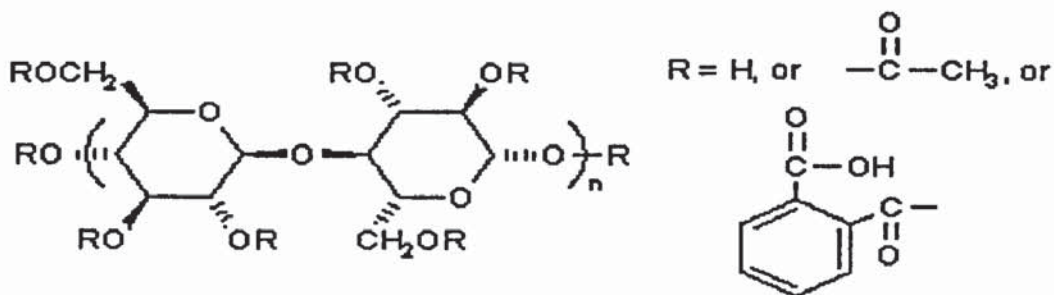
Above is the chemical structure for Cellulose Acetate Phthalate. Cellulose acetate phthalate (CAP) was provided by Aldrich, UK.

**Culturing BCG**

The media used to culture BCG was prepared using Middlebrook OADC enrichment, Middlebrook 7H10 powder (Beckton & Dickinson) and glycerol. The Middlebrook 7H10 powder provides the base for producing the agar gel used to grow live BCG. It contains Malachite green which acts as an antibacterial and prevents other bacteria growing in the media, which may be airborne at the time of transferring BCG from the Eudragit matrices to the agar. The OADC enrichment contains essential fatty acids (oleic acid) required for BCG to grow. The glycerol provides a carbon source for BCG growth. (See section 2.4.5 for detailed description).

**Tablet compressor**

Eudragit matrices were prepared in tablet form using a Specac KBr disc compressor. The 41mm cavity depth was just sufficient to hold 200mg of Eudragit matrix powder, which is then compressed to produce a 13mm diameter tablet.
Specac KBr disc compressor with a cavity depth of 15mm was used to produce 25mg tablets with a diameter of 5mm.

81

## 2.3   Improving the sensitivity of peptide based assays for the diagnosis of bovine tuberculosis

## METHODS

### 2.3.1   Preparation of Poly(L-lactide) particles (PLA)

PLA (MW 2000, 100mg) was dissolved in 5ml acetone to give a 2%w/v solution. To assist polymer dissolution the glass beaker was placed on a magnetic stirrer and heated gently. Once dissolved 10ml of distilled water was added to the PLA solution using a Pasteur pipette drop by drop to give a cloudy/milky suspension. This was then left overnight on the magnetic stirrer without heating to allow solvent evaporation. The resulting suspension of PLA particles was made up to 10ml with distilled water. An aliquot of this suspension (1ml) was placed in a pre-weighed eppendorf, frozen for 4 to 6 hours and then placed in a freeze-dryer overnight. The eppendorf containing lyophilised particles was then re-weighed and the empty eppendorf weight subtracted to give the concentration of the particle suspension (mg/ml).

### 2.3.2   Determination of particle size and charge of particles

**Particle size analysis by laser diffraction – Background**

Laser diffraction method, correctly termed Low Angle Laser Light Scattering (LALLS), measures particle volume and relies on the fact that the diffraction angle is inversely proportional to particle size. It consists of a coherent intense light, which acts as a laser (helium-neon gas laser with wavelength of 633nm in Mastersizer E), a photosensitive silicon detector and a means of passing the sample through the laser beam. The particles pass through a laser beam in front of a lens where a photosensitive detector is positioned consisting of a series of annular rings.

The unscattered portion of the laser beam is removed from the system by bringing it to focus in the detector plane and allowing it to pass through an aperture at the centre of the detector and eventually out the system. The annular rings record the intensity of the light scattered over a range of angles and the distribution of scattered intensity is analysed by computer size distribution software.

**Procedure**

2 to 3mg Polystyrene microparticles MHA P201 and P120, CP03, CP00 and calcium carbonate were suspended in 20ml distilled water. The suspension was ultrasonicated for 30-60 seconds from which 1ml was then added to a cuvette. Particle size was analysed using the Mastersizer/E™ (Malvern Instruments Ltd, Malver, Worcs., UK). Results were expressed as volume (±SD) and before size measurement the instrument was checked using polystyrene microspheres dispersed in aqueous solution (Malvern Instruments Ltd, Malvern, Worcs., UK).

Polystyrene nanoparticles and CHA particles were prepared as noted above with the other particles and analysed using the Brookhaven Zeta Plus, Zeta Potential Analyser Instrument. The Brookhaven instrument works on the same principle, as the Mastersizer/E™ except it is able to detect particles in the range 3 to 3000nm.

**Surface charge determination using zeta potential analysis - Background**

A net electrical charge exists at the surface of a particle in liquid medium. These include the ionisation of groups (acidic or basic) on the particle surface or the adsorption of specific ions from the medium. An electrical double layer forms around each particle comprising of two layers (inner and outer) in the liquid layer surrounding the particle. The inner region (Stern layer) contains strongly bound concentrations of ions where as the outer (diffuse) region consists of loosely bound ions. The ions and particles form a stable entity within the diffuse layer. During movement of particles (e.g. due to gravity), the ions move with it, as opposed to

83

those ions outside this boundary, which remain dissociated from the particle. The potential at this boundary is the zeta potential. The magnitude of the zeta potential gives an indication of the potential stability of particles within a given medium. Systems that retain particles in an unaggregated state are termed "colloidally stable", whereas unstable systems form aggregates or clumps. Colloidal particles generally have an electronegative surface charge with zeta potentials in the range of −14 to −30mV. Particles with highly negative zeta potentials (i.e. more electronegative than −30mV) exhibit electrostatic repulsion between the discrete charged units, preventing flocculation and stabilising the dispersion. Between −14 and −30mV there is a plateau region, marking the threshold of either coagulation or dispersion. Flocculation is often seen when the colloidal particles are more positive than −14mV.

**Procedure**

The zeta potential determinations were carried out using a Brookhaven Zeta Plus, Zeta Potential Analyser Instrument consisting of two coherent laser beams derived from the output of a low power laser. These beams form a crossover pattern of interference fringes. Particles moving across the fringes in response to the applied electric field scatter light with intensity, which fluctuates at a frequency, related to their velocity. The signal from individual photons of scattered light are detected by a photomultiplier and analysed by a digital correlator to give a frequency spectrum from which the particle mobility and the zeta potential are calculated.

2 to 3mg Polystyrene micro-nanoparticles, CHA, MHA P201 and P120, CP03, CP00 and calcium carbonate were suspended in 20ml 0.01M PBS solution at pH 7. The suspension was ultrasonicated for 30-60 seconds from which 1ml was then added to a cuvette. Particle charge was analysed using the Brookhaven instrument.

### 2.3.3 Adsorption of peptide and OVA

#### 2.3.3.1 Adsorption of peptide (P107 and ESAT-6, p45) and protein (OVA) onto microparticles and nanoparticles

Glass microspheres (CP00), surface modified glass microspheres (CP03) calcium carbonate, microcrystalline hydroxyapatite (MHA, P120 and P201), (10mg respectively), were added to 16mm diameter Pyrex glass culture tubes. Peptide and protein solution in water (P107 and OVA respectively) or PBS (ESAT-6, p45) (1ml, 1mg/ml) was added to each tube. The tubes were placed in a sonication bath for 10–15 seconds to help disperse the particles and placed on a rotating mixer for 2, 4, 6 and 8 hours incubation. Calibration samples of the peptide solution (1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9 0 μg/1000μl) were prepared using a series dilution of the stock peptide solution (1mg/ml). Phosphate buffer saline (PBS) was used to dilute the samples to the required concentrations. After incubation the microparticles with adsorbed peptide were centrifuged for 4 minutes at 3500rpm using a centrifuge (MSE Mistral 2000) and the nanoparticles at 1500rpm for 4 minutes using a centrifuge (MSE Mistral 2000) to ensure the supernatant was free of microparticles. The supernatant was removed and stored in 1.5ml eppendorfs. The particles were then washed with 1ml PBS resuspended and centrifuged as above. The supernatant was removed and stored in eppendorfs. Triplicate 20μl samples of each of the supernatants, wash media and calibration samples were taken and added to microtitre plates (flat bottomed 96 well microtitre plates, Fisher, Leics, UK). Bicinchoninic acid (BCA) reagent was prepared just prior to the BCA assay by mixing BCA solution (Pierce, Rockford, Illinois, USA) (50 parts by volume) with (1 part) copper sulphate solution (Sigma Ltd, UK) (cupric sulphate 4% w/v). In this assay the reaction of protein with an alkaline copper II ($Cu^{2+}$) reagent (Reagent A) produces copper I ($Cu^{+}$). This copper I ion then reacts with two molecules of reagent B containing 4,4-dicarboxy-2,2-biquinolone (bicinchoninic acid) to form a copper/peptide chelate. The product of the reaction is water-soluble with an intense purple colour, which can be quantified by

spectrophotometric measurements at a wavelength of 562nm. BCA reagent (200µl) was added to each sample of supernatant, wash media and calibration samples in the microtitre plates and incubated at 60°C for 30 minutes for colour development. A calibration curve was constructed of absorbance against peptide concentration. The peptide content of the supernatant and wash media obtained from the microparticle samples was determined by comparison with the calibration curve. The quantity of adsorbed peptide was obtained by subtraction of the peptide content in the supernatant (1ml) and washes (1ml) from the peptide content of the starting incubation medium. The percentage (%w/w) peptide adsorbed on the microparticles was calculated by dividing the weight of the adsorbed peptide by the weight of the microparticles.

### 2.3.3.2  *The adsorption of peptide (ESAT-6,p45 and P107) onto CHA nanoparticles*

CHA nanoparticles were provided suspended in water. The concentration was calculated by placing 1ml of this suspension into a preweighed 1.5ml eppendorf. This was then frozen and freeze-dried. The weight of the eppendorf and CHA nanoparticles were weighed and the difference gave the concentration of the suspension in 1ml.

The CHA nanoparticles were difficult to work with. After centrifuging the nanoparticles (3500rpm for 4mins) to remove unbound protein or peptide, redispersion was difficult and required use of a glass pipette to break up the aggregated particles as well as sonication. A reduction in the speed of centrifugation to 1000rpm for 4minutes overcame the problem of sedimentation and redispersion of CHA nanoparticles and was applied during adsorption experiments and during release studies.

0.35 ml (µg/ml) CHA nanoparticles was added to 16mm Pyrex test tubes and 1ml (1mg/ml) peptide solution added. Samples were incubated on a rotating mixer for 2 hours. The remaining stages were as described in section 2.3.3.1.

### 2.3.3.3 The adsorption of peptide (ESAT-6, p45 and P107) onto polystyrene microspheres and nanospheres

A 2.5% suspension of polystyrene beads 0.5ml was added to an eppendorf tube (1.5ml capacity). The tube was filled with either 0.1M borate buffer pH 8.5, PBS or normal saline and mixed using a vortex mixer. The beads were then centrifuged for 5 minutes. The supernatant was removed using a Pasteur pipette and the tube was refilled with the respective buffers. The beads were resuspended using a vortex mixer and centrifuged as above. The step was repeated a further time and the pellet resuspended in 1ml of the respective buffers. Peptide solution (400µl, 1mg/ml) was added to the polystyrene bead preparations in the respective buffers and incubated on a rotating mixer for 2 hours. The remaining stages are as described above in section 2.3.3.1.

### 2.3.3.3.1 Adsorption of OVA and peptide (ESAT-6,p45) onto PLA particles

PLA particle suspension of the required volume to give a particle concentration of 10mg/ml was placed in a test-tube. OVA solution (1ml, 1mg/ml) or peptide solution (1ml, 1mg/ml) was added and shaken using a vortex and placed on a test-tube shaker for 2 hours. Adsorption was performed as described in section 2.3.3.1.

### 2.3.4 Release of peptide and OVA from particles

### 2.3.4.1 Peptide (ESAT-6, p45 and P107) and protein (OVA) release from microparticles and nanoparticles

PBS (1ml) was added to the washed particles and the sample tubes were held in a water bath at 37°C. The samples were shaken manually at regular intervals to ensure the microparticles remained in suspension. At intervals of 2, 4, 6, 8, 16 and 24 hours the release medium was removed completely and fresh medium was added. The peptide content of the release medium was measured using the BCA assay. Peptide release was calculated in terms of cumulative (%w/w) release.

87

### 2.3.4.2 OVA and peptide (ESAT-6, p45) release from PLA particles

PBS release medium (1ml) (pH 7.2 and 5.5) respectively were added to washed particles and these were incubated in a water bath at 37°C. The pH of PBS solution was adjusted to 5.5 by adding 0.1M NaOH solution and measured using a pH meter. The sample tubes were shaken manually at regular intervals to ensure the particles remained in suspension. At intervals of 2, 4, 6, 8, 16 and 24 hours the release medium was removed completely and fresh medium was added. The peptide content of the release medium was measured using the BCA assay. Peptide release was calculated in terms of cumulative (%w/w) release.

### 2.3.4.3 Release of peptide (ESAT-6, p45) from particles in different pH media

Peptide was adsorbed on the micro- and nanoparticles by incubation for 2 hours as described in section 2.2.3, one set of particles will have either 1ml Hepes solution (pH 5.5) or PBS solution adjusted to pH 5.5 added and a second of particles will have PBS solution added (pH 7.2). The particles were removed and centrifuged and the supernatants stored in eppendorfs. 1ml Hepes or PBS solution (pH 5.5 adjusted) or PBS solution (pH 7.2) were added to the particles and the sample tubes were placed in a water bath for 24hrs. The release medium was replaced with fresh medium at 2, 4, 6, 8 and 24hrs. A calibration curve was set up as described above and the supernatants are added to 96 well plates and read using a plate reader at 570nm as mentioned above.

### 2.3.5 Performing scanning electron microscopy (S.E.M) and transmission electron microscopy (T.E.M)

Metal stubs coated with carbonated paper were covered uniformly by very small amounts of glass microspheres (CP03), calcium carbonate and microcrystalline hydroxyapatite (MHA, P120 and P201) respectively. Two to three drops of carbonate hydroxyapatite (CHA); polystyrene microparticles and nanoparticles

were also applied to carbonated paper using a Pasteur pipette and allowed to dry. The metal stubs were coated with gold for 15 minutes under argon atmosphere (Emsope SC500). Specimens were analysed using a Cambridge Instruments Stereoscan 90B. Photomicrographs were taken using a Nikon 35mm camera.

Glass microspheres (CP03), calcium carbonate, microcrystalline hydroxyapatite (MHA, P120 and P201), carbonate hydroxyapatite (CHA), polystyrene microparticles and nanoparticles were suspended in ethanol and a drop of suspension was allowed to evaporate on a carbon film coated copper grid (~ 3mm diameter) for T.E.M analysis. Micrographs were recorded using a CM20 Philips electron microscope at an accelerating voltage of 200KV.

### 2.3.6   Bovine Gamma Interferon Test (BOVIGAM™ ASSAY)

**Stage one – whole blood culture method**

Whole blood cultures were performed in 96 well plates in 0.2ml aliquots by mixing 100µl of heparinised blood with 100µl antigen-containing solutions prepared in RPMI 1640. Avian and bovine tuberculin were used at a concentration of 10µl/ml. 10mg Particles with adsorbed peptide antigen (1mg/ml) (polystyrene 8.9-9.2 %w/w, hydroxyapatite 3-4.9 %w/w, calcium carbonate 1.8 %w/w and 1.2 glass) and free peptide (ESAT-6,p45) were added over a 3-log concentration range (25 – 0.003 µg/ml). The particle-adsorbed antigens and free peptides in solution were mixed thoroughly in the aliquoted blood using a microplate shaker. The tissue culture trays were incubated for 24 hours at 37°C in a humidified atmosphere in air containing 5% $CO_2$. After 24 hours incubation, 100µl aliquots of plasma supernatant were transferred to a 96 well plate using a sterile pipette and stored at -20°C prior to testing.

**Stage two – bovine IFN-γ measurement using enzyme immunoassay (EIA)**

The plasma supernatants were analysed for IFN-γ content by EIA as follows. Green diluent (50μl) was added to the required wells in a 96 well plate and 50μl of the test and control samples were added (the green diluent is used as a plasma diluent buffer and a visible colour marker to ensure the wells containing the blood plasma have been diluted). The positive control consisted of IFN-γ and the negative control did not contain IFN-γ. The plate was mixed thoroughly by vortexing for 1 minute on a microplate shaker, covered with a lid and incubated at room temperature for one hour. The plates were then washed four times with Bovigam wash solution ensuring that on the last wash all the wash media was removed. The next step was to add 100μl of freshly prepared conjugate reagent to the wells. The conjugate watery solution was prepared by diluting the conjugate (horse–radish peroxidase labelled anti-bovine IFN-γ) 1:100 in blue diluent (the blue diluent is used as a conjugate diluent buffer and a visible colour marker to ensure the conjugate has been diluted). The plates were incubated as above for one hour and, then washed five times. The enzyme substrate buffer (horse–radish peroxidase) solution was prepared by diluting chromogen solution 1:100 with enzyme substrate buffer. 100μl of freshly prepared enzyme substrate solution was added then to each well. The plates were covered and incubated at room temperature for 30 minutes. Enzyme stopping solution (50μl) was then added to each well, being careful not to transfer chromogen from well to well, and mixed by gentle agitation. The colour changes from blue to yellow, indicating the reaction is terminated. The plates were then read using a microplate reader at 450nm.

### 2.3.7 Investigation of the influence of 'free' versus 'particle-bound peptide'

This experiment was performed to determine whether the immune response (IFN-γ) produced was due to free peptide released from the microparticles and nanoparticles over 24 hour period or due to peptide bound to the particles.

After the adsorption stage using microparticles, nanoparticles and polystyrene beads, the particle-adsorbed peptide samples were incubated for 8 hours minimum in 1ml PBS at 37°C in a water bath. The incubation medium was removed and analysed for peptide content using the BCA assay. The amount of peptide remaining bound to the particles was calculated by subtraction. The volume of particle suspension used in the Bovigam assay was adjusted for each microparticles system to obtain a constant dose of bound peptide and the Bovigam assay was performed as described in section 2.3.6.

### 2.3.8 Animal study work to determine particles as vaccines

**Mouse lymphocyte proliferation experiment**

The particles chosen (Polystyrene micro- and nanoparticles, MHA P201 and MHA P120, CHA and PLA particles) for this study were adsorbed with peptide ESAT-6,p45 as described in section 2.3.3. Freunds adjuvant (IFA) was incorporated with ESAT-6,p45 or PBS.

8 female C57BL/10 mice 6 to 10 weeks old were sensitised by subcutaneous injection in the footpads with 50µg of IFA/p45, IFA/PBS, polystyrene micro- and nano, MHA P201, MHA P120, CHA and PLA all loaded with ESAT-6,p45. Popiliteal LN cells were removed after 7 days post-vaccination and single cell suspensions prepared for lymphocyte proliferation assay. LN cells ($4 \times 10^5$ cells/well of flat-bottomed 96-well microtitre plates) were kept in culture medium (RPMI 1640) supplemented with 10% heat inactivated foetal calf serum, mm L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin and $5 \times 10^{-5}$ M 2-mercaptoethanol). Plates were incubated for 3 days at 37°C in 5% $CO_2$, then pulsed with 18.5 kBq of $^3$H-thymidine and the incorporated radioactivity determined 6 hours later. The results are expressed as stimulation indices (cpm with peptide/cpm without

peptide). The experiment and analysis of data was performed by Dr. Martin Vordermeier at Veterinary Laboratory Agency, Weybridge, Surrey.

**Skin test in cattle**

MHA P201 was chosen and adsorbed with ESAT-6,p45 and polymeric peptide (TBC-p45-S-EAK) as described in section 2.3.3. The polymeric peptide was obtained from Dr Ferenc Hudecz at Eotvos L. University, Budapest, Hungary. This is peptide ESAT-6,p45 with polypeptide $poly[Lys(GLU_i-DL-Ala_m)]$ (EAK) attached to it by thioeter bond through the N-terminal position.

3 female cows were injected with $100\mu g$ of MHA P201 (containing ESAT-6,p45 or polymeric peptide), PPD-B and ESAT-6,p45. Initially the hair around the site of injection was clipped to a radius of two centimetres and measurements recorded from folds of skin. After 72 hours the sites were measured and the difference in readings were recorded. Measurements were recorded millimetres and an average of readings from the sites on each cow were calculated and represented as a graph.

## 2.4 Improvement of oral tuberculosis vaccination strategies

**METHODS**

### 2.4.1 Methods for assessing the redispersion characteristics of BCG using different suspending agents

BCG has a tendency to aggregate (Section 1.8.4). The average size of BCG is $2\mu m$ (Zhang *et al.*, 1988), which corresponds with the size of particles taken up effectively by the Peyer's patches (Chen *et al.*, 2000; Holmgren *et al.*, 1993). If aggregation occurs during incorporation of BCG into Eudragit matrices then uptake

by the Peyer's may not occur or may be reduced. Consequently experiments were performed to identify surfactants, which would help maintain BCG dispersion during matrix formulation.

The following reagents were investigated for redispersion of BCG.

- Water
- Phosphate buffered saline (PBS)
- Polyvinylpyrrolidine (PVP)
- Dextran
- Polyethylene-glycol (PEG)
- Glucose
- Sodium dodecylsulphate (SDS)
- Polyoxyethylene-sorbitan monolaurate (Tween 20)
- Polyoxyethylene-sorbitan monooleate (Tween 80)
- Poloxamer (PEO-PPO copolymer) F-68

The dispersion characteristics of BCG in the above suspending agents were studied using UV spectroscopy. An LKB Biochrom, Ultrospec K was used to analyse dispersions of BCG in the surfactants and dispersing agents at a wavelength of 470nm. If the suspension containing BCG is well dispersed the UV absorbance value will be high, whereas if aggregation the UV absorbance value is reduced.

The BCG stock suspension was thawed at room temperature and 100µl was transferred into a 1ml cuvette containing 900µl of suspending agent. This initial UV absorbance reading (i) was used as a benchmark for comparison of the various suspending agents. The suspension was transferred to an eppendorf and centrifuged at 13000rpm for 10min using a bench centrifuge. The supernatant removed (ii). The BCG sediment in the eppendorf was resuspended by adding 1ml of the suspending agents, shaking manually for 2min and transferring into a cuvette. The absorbance was measured (iii). The supernatant from (i) was added to the eppendorf to pickup

residual BCG if any and the absorbance re-measured (iv). To further gauge the effectiveness of the suspending agent 100μl of BCG suspension was added to 900μl of water and centrifuged at 13000 rpm for 10min. The supernatant was removed and 1ml of the suspending agent added. This was then transferred to a 1ml cuvette and the absorbance measured at 470nm. (v)

### 2.4.2 Assessing the redispersion characteristics of BCG after freeze drying

Formulation of BCG into a solid oral dosage form was envisaged to involve a freeze-drying stage. BCG viability and thus cfu counts are affected by freezing and freeze-drying[72] possibly due to aggregation of BCG. Consequently the dispersion characteristics of BCG after freeze-drying were analysed using the method described in section 2.4.1.

BCG suspension (100μl) (either BCG-Tokyo or BCG-Pasteur) was added to 900μl suspending agent (0.1%w/v) and the absorbance was measured at 470nm. The supernatant was removed and 1ml of the suspending agent was added. This was transferred to a cuvette and the UV absorbance measured at 470nm to give the value before freeze-drying. The BCG suspension containing the suspending agent was transferred to a glass vial and sealed with parafilm. The samples were frozen at -20°C for 4 hours and then freeze-dried using a Modulyo, Edwards UK freeze-drier at 60mbar (6000Pa) pressure, -50°C for 6 hours. Distilled water (1ml) was added to each glass vial to disperse the freeze-dried BCG preparation and the sample absorbance was measured at 470nm.

### 2.4.3 Formulation of BCG-loaded matrices for oral vaccine delivery

Vaccination of animals by the parenteral route is time-consuming, expensive and requires a qualified vet to administer the vaccine (Bowerstock *et al.*, 1999; Buddle

94

*et al.*, 2000). Consequently the development of an oral dosage form is desirable for use with domesticated animals and in the wild.

The oral BCG formulations are designed for animal use, with the badger as a potential target. The gastric and intestinal residence time were taken to be similar to humans. Therefore the gastric time was taken to be 2 hours and the intestinal transit time to be 4 hours. Clarke *et al* (1993) showed that 0.4 to 2 hours was typical for gastric-intestinal transit times with a mean of 3 hours.

Initially two formulations were investigated, one based on organic solvents and the second aqueous systems.

### 2.4.3.1   Tablet formulation using organic solvents

Two enteric coating polymers (CAP and Eudragit S100) were investigated for production of matrix-type oral delivery systems for live BCG in preliminary experiments. CAP and Eudragit are both freely soluble in acetone but insoluble in water. The effect of polymer weight on tablet properties was evaluated by adding 5ml, 10ml and 20ml of a 1% polymer solution in acetone to glass vials, followed by addition of 0.9ml of 0.1%w/v surfactant solution. (The surfactant concentration equated to that used in pre-formulation studies of BCG aggregation). The acetone was allowed to evaporate using a magnetic stirrer at room temperature. When the acetone had evaporated the samples were placed in a freezer at -20°C for 8 hours. The samples were freeze-dried overnight and compressed using a Specac tablet compressor (5 tons applied force for 4 minutes). Visual comparisons of the discs were made according to the three weights of the enteric coating polymers used (i.e. 50mg, 100mg and 200mg respectively).

### 2.4.3.2   Tablet formulation using aqueous systems

Aqueous systems were investigated to avoid the use of organic solvents, which denature live BCG. Hence Eudragit was suspended in distilled water and PBS

solution. PBS was chosen as the media for dispersing Eudragit as it gave the better dispersion characteristics.

200mg of Eudragit S 100, L 100 and L 100-55 respectively were suspended in 2ml PBS solution in a glass vial and the surfactant Tween 80 (0.9ml, 0.1%w/v) was added. The pH was adjusted to 7 from 4 using a 1M NaOH solution, to protect BCG from denaturation in the acidic environment, and 100µl of BCG Pasteur or BCG Tokyo were added to respective vials. The glass vials were placed in a freezer (-20°C) for 6 hours and subsequently freeze-dried for 24 hours. Eudragit L30 D55 suspension (2ml) was added to glass vials. The pH was again adjusted to pH 7 and the above steps were repeated. After freeze-drying the samples were compressed using a Specac KBr disc compressor (4 tons applied force for 3 minutes) to produce 13mm diameter discs.

### 2.4.3.3  Tablet formulation using different PBS concentrations

The above process of tablet formation was carried out using PBS of different concentrations to suspend the Eudragit powder. The PBS concentration was adjusted with distilled water to give concentrations of 100% PBS (2ml PBS solution), 50% PBS (1ml PBS and 1ml water), 25% PBS (0.5ml PBS and 1.5ml water). This study was performed to determine if PBS concentration affected the disintegration and dissolution properties of Eudragit matrices. The formulations prepared using 2ml PBS solution take longer than 4 hours to dissolve in PBS at 37°C, which may be due to salt concentration. Analyses of disintegration properties in HCl were also analysed.

### 2.4.4  Disintegration of BCG-containing matrices in simulated Gastric Intestinal Fluid

Disintegration studies were performed to test matrix stability in simulated gastric fluid (SGF) (hydrochloric acid 0.1M pH 1.2) and simulated intestinal fluid (SIF)

(PBS pH 7.4). Once the tablets had been compressed they were weighed and placed in a 50ml polypropylene tube containing 10ml of either HCl or PBS and retained for 2 hours in a water bath at 37°C. They were then left to dry to constant weight (48-72 hours) at room temperature and re-weighed.

### 2.4.5    Preparation of sterile formulations containing BCG

All equipment and solution specified were sterilised by autoclaving at 15psi, 121°C for 15 minutes.

200mg of Eudragit S100 were weighed out using a spatula and placed in a glass vial. 1ml PBS and 1ml water were added using tips, followed by 0.9ml of Tween 80 (0.1% w/v). The pH was adjusted to 7 using 0.1M NaOH (The pH meter was cleaned using 70% ethanol). A 1ml vial of BCG Pasteur was allowed to thaw and 100μl was added to the Eudragit suspension using sterile tips. The glass vial containing the suspension was placed in a freezer at -20°C for 6 hours, after which it was placed in a freeze-dryer overnight. The powder was pressed into a tablet. The KBr discs used to press the tablet and the mount to hold the powder were cleaned with 70% ethanol. The weight of the tablet was recorded before disintegration studies. BCG-containing matrices were retained in 10ml sterilised 0.1M HCl for 2 hours at 37°C in 100ml polypropylene tubes. After this time the media were removed and the tablets washed with sterilised water 5 times before the addition of sterilised 10ml HEPES solution at pH 7.

Eudragit L100 tablets containing BCG were produced using the same procedure as above except that 2ml PBS solution was added instead of 1ml PBS and 1ml water. The concentration of live bacteria in matrix formulations before and after exposure to SGF and SIF was analysed as described in section 2.4.5.1.

### 2.4.5.1  Analysis of live BCG in matrix formulations

In order to determine if BCG remains viable during all stages of formulation, (freezing, freeze-drying, matrix compression), disintegration and dissolution studies, samples were grown on Middlebrook media (see section 2.4.5.2).

### 2.4.5.2  Preparation of 7H10 Middlebrook medium – 50 plates

19g of 7H10 Middlebrook medium was weighed and dissolved in 900ml of water and added with 5ml glycerol to a 1-litre bottle. The preparation was autoclaved at 15 psi, 121°C for 15 minutes. The flask was allowed to cool to approximately 55°C before the addition of 5x20ml Middlebrook OADC supplement and the contents were poured into 50 petri-dishes. When the media were set the petri-dishes were stored in a fridge.

500µl of Eudragit matrix containing BCG from each stage of formulation, (freezing, freeze-drying, matrix compression), dissolution and disintegration was added to test-tubes containing 0.05% Tween 80 to provide serial dilutions of $x10^3$, $x10^4$, $x10^5$ and $x10^6$. 100µl of each dilution was added to Middlebrook media in petri-dishes in triplicate samples. The petri-dishes were covered with foil to prevent the plates from drying over the incubation period. The petri-dishes containing BCG were incubated inverted at 37°C for three weeks after which colony forming units (cfu's) at each dilution on each of the petri-dishes were counted and the average cfu's recorded. This figure was then multiplied by 10 to give a final reading of cfu's per ml so that a comparison of loss in viability could be made between the stock sample (control) and each formulation stage.

### 2.4.5.3  Dilution of release medium-containing BCG for viability testing

Figure 2.1 shows the stages of series dilution of 10ml BCG-containing release medium prior to plating. Initially 500µl of medium (HEPES or PBS) in which the tablet formulation containing BCG was dissolved was added to 4.5ml water and

98

0.05% Tween 80 (tube A) which had been sterilised by autoclaving (15 psi, 121°C for 15 minutes). 500µl of tube A was taken and added to tube (B). This process was repeated for the remaining tubes. The series dilution was performed using sterile tips and in a clean room using a flow cabinet. 100µl aliquots were taken from tubes A, B, C and D and added to the Middlebrook agar media prepared as described above (section 2.4.5.2). Each tube A, B, C and D were plated in triplicate.

**Figure 2.1**   Series dilution of BCG prior to plating on Middlebrook medium
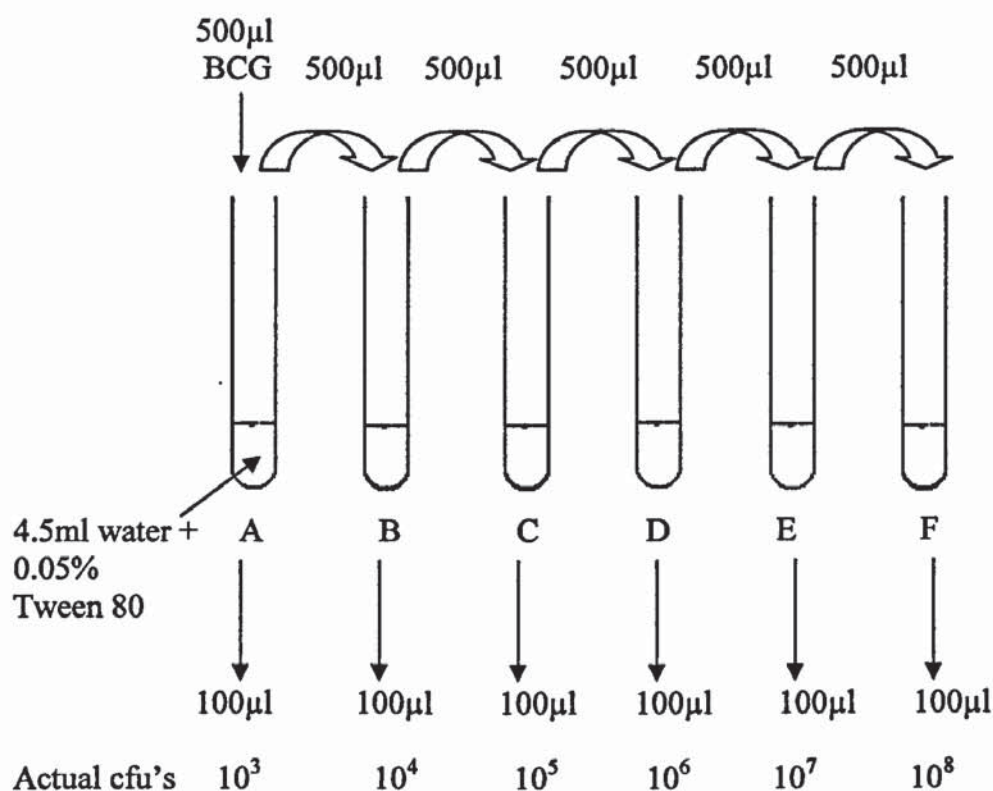


2.4.5.4  *Preparation of control samples for BCG viability testing*

BCG viability was measured at each stage of formulation (adjusting pH, freezing, freeze-drying, compression), disintegration in HCl and dissolution in PBS/HEPES). The control for each stage was the stock BCG supplied by VLA treated as described below.

**Adjustment of pH**

100μl of BCG stock suspension was added to 10ml PBS solution. From this preparation 500μl was added to tube A. Series dilution and plating was performed as described in section 2.4.5.4 and figure 2.1.

**Freezing**

100μl of BCG stock suspension was added to 2ml PBS solution and 0.9ml Tween 80 and frozen at the required temperature (-20°C, -80°C or liquid nitrogen) for 6 hours. The preparation was made up to 10ml with PBS solution. From this preparation 500μl was added to tube A. Series dilution and plating was performed as described in section 2.4.5.4 and figure 2.1.

**Freeze-drying**

100μl of BCG stock suspension was added to 2ml PBS solution and 0.9ml Tween 80 and frozen at the required temperature (-20°C, -80°C or liquid nitrogen) for 6 hours. The preparation was freeze-dried using a Modulyo, Edwards freeze-drier at 60mbar (6000Pa) pressure, -50°C for 24 hours. The sample was then redispersed in 10ml PBS solution and 500μl was added to tube A. A series dilution and plating was performed as described in section 2.4.5.4 and figure 2.1.

Controls were performed with BCG in HCl, but this denatured the bacteria and no colony forming units were detected.

### 2.4.5.5 Preparation of microscope slides of BCG at various stages of matrix formulation

Samples of BCG containing formulations were examined using an optical microscope (Axioscope, zeiss, Germany) for bacteria clumping (see Table 2.1).

**Table 2.1**  Samples examined for BCG clumping using optical microscopy

| Eudragit Matrix/BCG Stock | Formulation Stage |
|---|---|
| 100µl BCG | Direct from sample vial |
| 100µl BCG | Sample taken from vial, frozen at -20°C for 6 hours and made up to 10ml in PBS solution |
| 100µl BCG | Sample taken from vial, frozen at –20°C for 6 hours and freeze-dried overnight. Redispersed in 10ml PBS solution |
| 200mg Eudragit S100 + 1ml PBS + 1ml water + 0.9ml Tween 80 + 100µl BCG pH 7 | 1. Suspension<br>2. Frozen at -20°C for 6 hours and made up to 10ml PBS solution<br>3. Frozen at -20°C for 6 hours and freeze-dried overnight. Redispersed in 10ml PBS solution<br>4. Disintegration and dissolution studies, following compaction stage in HCl 0.1M pH 1.2 37°C and PBS/HEPES solution 0.1M pH 7 37°C |
| 200mg Eudragit L100 + 2ml PBS+ 0.9ml Tween 80 + 100µl BCG pH 7 | 1. Suspension<br>2. Frozen at -20°C for 6 hours and made up to 10ml PBS solution<br>3. Frozen at -20°C for 6 hours and freeze-dried overnight and redispersed in 10ml PBS solution<br>4. Disintegration and dissolution studies |

A metal loop was sterilised by passing through the flame of a Bunsen burner. The loop was placed in the vials containing the formulations shown in Table 2.1 and smeared onto a glass microscope slide. The sample slide was then passed through a flame to fix the smear. Methylene blue stain was added to the smear and after 3-4 minutes the stain was removed by washing with water under a tap. The slide was subsequently viewed under an optical microscope at various magnifications.

### 2.4.6  Preparation of BCG-loaded Eudragit L100 matrices using cryoprotectants

These experiments were performed to maximise the viability of BCG after freeze-drying and compression. Freeze-drying is known (Gheorghiu *et al.*, 1988) to cause

101

loss in viability of cells and activity of proteins. Crystal formation during freezing for example could cause rupture of the cell wall of BCG during compression.

Mannitol and trehalose were selected as cryoprotectants as they are widely used for stabilising biopharmaceuticals. 20mg was added to the formulation (containing 180mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100µl BCG pH adjusted to 7). The remaining stages were performed as described in section 2.4.3.2.

### 2.4.7 Preparation of BCG-loaded Eudragit L100 matrices using concentrated BCG suspension ($10^{10}$ c.f.u's/ml)

BCG was grown at VLA at a concentration of $10^{10}$ cfu/ml to maximise the number of viable bacteria following formulation and matrix dissolution in SGF and SIF.

The protocol for preparation of matrices and powders (200mg) containing high loadings of BCG are shown in Figure 2.2.

The same protocol was applied for production of 25mg tablets or powders. Note the preparation of tablets or powders using the concentrated BCG suspension was carried out using sterile equipment which was autoclaved at 15psi 121°C for 15 minutes.

### 2.4.8 Scanning electron microscopy (SEM)

Small fragments of Eudragit L100/S100 matrices (without BCG loading) were placed on metal stubs coated with carbonated paper for analysis by S.E.M. The metal stubs were coated with gold for 15 minutes under argon atmosphere (Emsope SC500) and envisaged using a Cambridge Instruments Stereoscan 90B, the surface of particles was studied. Photomicrographs were taken using a Nikon 35mm camera.

**Figure 2.2** Preparation of high BCG loading matrices and powders using Eudragit L100 and BCG suspensions (x10$^{10}$ c.f.u's/ml)
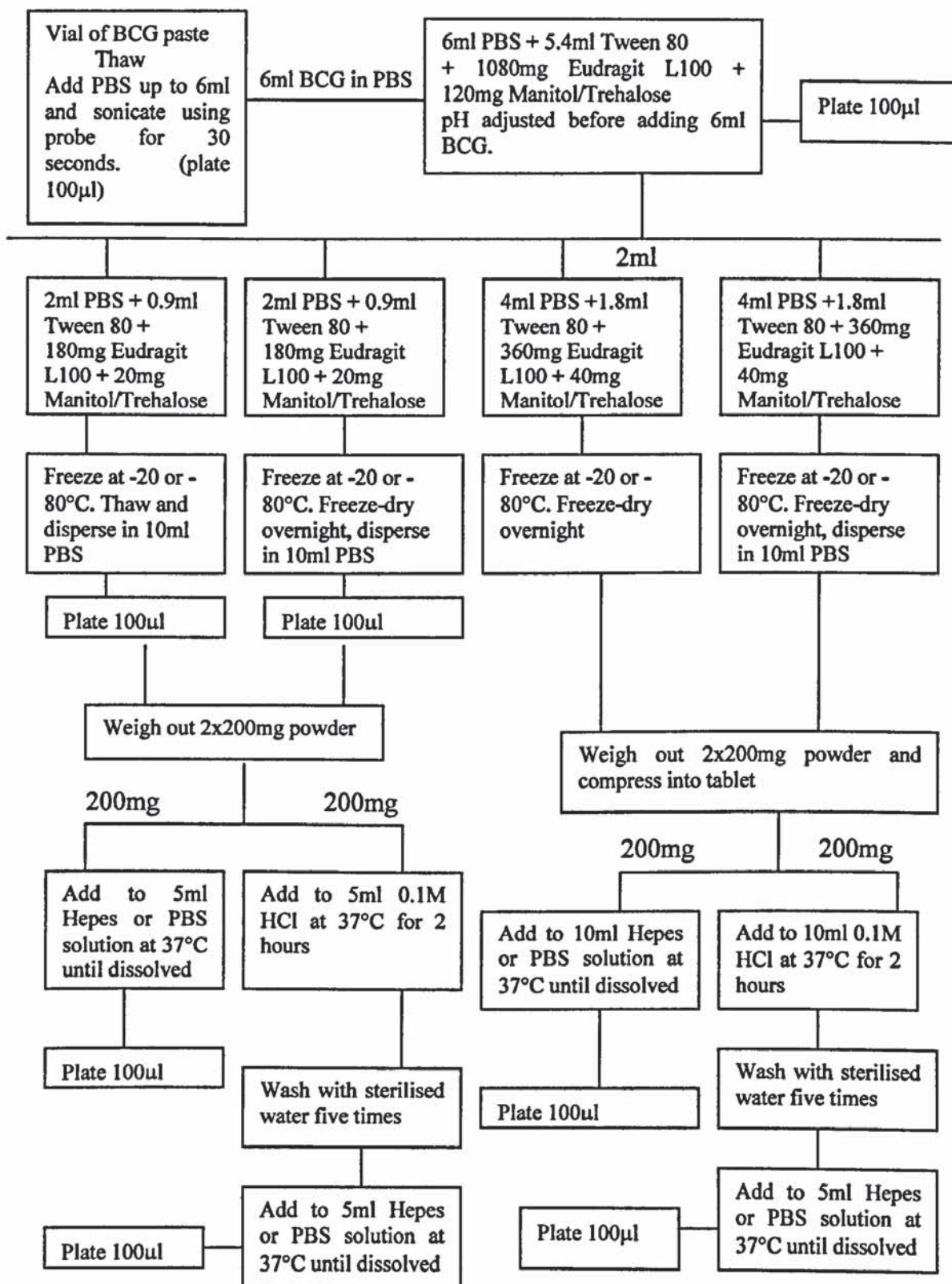
# CHAPTER 3

# RESULTS AND DISCUSSION

# IMPROVING THE SENSITIVITY OF
# PEPTIDE BASED ASSAYS FOR THE
# DIAGNOSIS OF TUBERCULOSIS

# 3 IMPROVING THE SENSITIVITY OF PEPTIDE BASED ASSAYS FOR THE DIAGNOSIS OF TUBERCULOSIS

## INTRODUCTION

The comparative intradermal tuberculin test is compromised by the attenuated vaccine strain BCG and may be similarly affected by novel vaccines comprising *M. tuberculosis* or *M. bovis* (Vordermeier *et al.*, 1999). This part of the project was involved with developing alternative diagnostic approaches involving target antigens expressed by *M. bovis* but absent from or present at only low levels in BCG (i.e. ESAT-6). Use of these antigens in an in vitro blood-based assay (BOVIGAM) is envisaged in which blood lymphocytes will be stimulated with target antigens followed by analysis of the culture supernatants for lymphokine production (IFN-$\gamma$, IL-2).

Progress in developing diagnostic assays for tuberculosis (TB) in cattle would be achieved by improving the sensitivity of the peptide-based assay. The addition of higher concentrations (>10-15µg/ml) of soluble peptides to an in vitro assay to improve sensitivity is undesirable in view of increased cost. Furthermore, it is felt that the mechanism of displacement of peptides already associated with MHC molecules expressed on the cell surface by the added peptides would not be very effective. In contrast, peptide association with MHC molecules via a natural mechanism within an endosomal compartment is considered more effective and would require lower peptide concentrations.

The aim of formulation is to achieve peptide release from the delivery system in a mildly acidic compartment or enhanced surface presentation of peptides and detachment triggered by association with lymphocytes.

## Aims

To significantly reduce the amounts of peptide necessary to achieve satisfactory responses in the current blood lymphocyte- based diagnostic assay for TB by using microparticulate delivery systems.

## Objectives

- To identify candidate particles for peptide adsorption and the size ranges of particles for promoting particle uptake and stimulation of blood lymphocytes. Investigations involved microparticles and nanoparticles produced from: -

- To investigate peptide adsorption (ESAT-6,p45 and P107) and release characteristics in vitro using the selected carrier particles shown above. ESAT-6,p45 is not expressed in BCG Pasteur and thus is currently used in the BOVIGAM assay to differentiate TB infection in cattle, which have or have not been vaccinated. ESAT-6 refers to 6KDa early secreted antigenic target from a short-term culture filtrate of *M.bovis* (SDS-PAGE). ESAT-6,p45 has been shown to induce strong proliferative and IFN-γ responses from PBMC's (Peripheral Blood Mononuclear Cells) isolated from *M.bovis* infected cattle.

- To assess the level of IFN-γ produced by lymphocytes in whole blood using the in vitro bovine gamma interferon test – an assay of cell mediated immunity for the diagnosis of bovine tuberculosis infection in cattle.

## 3.1 Microparticle size, morphology and surface charge

Glass microspheres have been utilised for repairing bone following surgical procedures and for covering metal prostheses (Krajewski *et al.*, 1996). They have also been used to protect metal surfaces from chemical attack by biological fluids

and to stimulate bone growth. Many glass microspheres are inert and the biological interaction with living bone occurs when proteins from blood or lymph cover the surface. Studies have been performed to increase this interaction with proteins by coating the glass microspheres.

Two types of glass microspheres were selected for studies of protein and peptide adsorption and release, unmodified (CP00) and microsphere surface modified by nylon to improve interfacial bonding with polymers (CP03). The glass microspheres are spherical in nature, but the size is inconsistent, as can be seen from figure 3.1. Some of the microspheres have irregular shapes. The size range determined from T.E.M was approximately between 1 and 3μm. This falls within the range given by the manufacturer and that achieved using Mastersizer/E™. However the average size determined using the Mastersizer is approximately 2μm, yet the T.E.M micrograph shows a wide distribution range of sizes.

Hydroxyapatite (MHA) is used extensively in dental restoration and binds protein efficiently and thus is a prime candidate for peptide adsorption to improve the sensitivity of peptide-based diagnosis of TB. MHA is a major inorganic component of biological hard tissues and possesses a high affinity for biomolecules, such as proteins, enzymes etc. MHA due to its excellent biocompatibility and nontoxicity has also been used to separate proteins using high-performance liquid chromatography (Kandori et al., 2002). Figure 3.2 shows MHA P201 particles, which are thin needle-shaped crystalline structures. The structures are of irregular size and shape. MHA P120, also consists of crystalline needle structures similar to MHA P201 (Fig 3.3). The main difference in physical characteristics between the two samples is that MHA P120 seems to be more compacted and the needle structures are not as well defined as MHA P201.

Kandori et al (2002) studied the adsorption of bovine serum albumin and lysozyme onto oleyl phosphate-grafted hydroxyapatite with different degrees of hydrophobicity. The hydrophobicity was adjusted by varying the number of oleyl

107

groups per unit nm$^2$. They found that the amount of adsorbed OVA increased with increase in hydrophobicity (up to 1 group per 0.6 nm$^2$) due to an increase of hydrophobic interaction between hydrophobic particles and proteins. However OVA adsorption decreased when the oleyl groups $\geq$ 1 per 1.3nm$^2$ due to an increase in electrostatic repulsive forces between the negatively charged OVA and MHA particles.

Polystyrene (PS) was selected as a candidate substrate for peptide adsorption due to the good adsorption qualities reported in vaccine research (Revilla et al., 1996). In much the same way as for MHA particles many studies using polystyrene particles have concentrated on the effect of surface charge on adsorption of protein (OVA). Revilla et al (1996) studied the adsorption of BSA as a function of pH and ionic strength compared with adsorption on negatively charged polystyrene particles. The functionalised latexes were produced by seeded copolymerisation of (0.3μm) liposaccharide monomer onto polystyrene particles obtained by soap-free emulsion polymerisation of styrene using potassium persulaphate as an initiator. The adsorption onto modified polystyrene particles (containing sugar residues) and non-modified was explained by protein-surface interaction (electrostatic and hydrophobic interactions). They suggested adsorption of BSA onto charged latex particles is governed by conformational changes in BSA, which depends on pH of medium as well as electrostatic interactions between proteins and latex particles. The pH dependence of the adsorption plateau of BSA was found to be related to the surface properties of the particles. A maximum was achieved at the isoelectric point of BSA (pH 4.5). This follows the general tendency that protein molecules become more compact near the isoelectric point, which in turn favours more numerous molecule adsorption. The authors also reported that BSA displayed a higher affinity for hydrophobic polystyrene particles than hydrophilic. Furthermore those containing sugar residual groups adsorbed less BSA than plain polystyrene particles.

The spherical nature and uniform size distribution of PS microspheres and nanospheres are indicated in Figure 3.4 and 3.5 respectively. The particle size measured from T.E.M's were 2μm and 500nm for PS microspheres and nanospheres respectively. These fall within the range found by measuring the size of the particles using Mastersizer and Brookhaven respectively (see section 2.3.2).

Carbonate hydroxyapatite (CHA) and polystyrene nanoparticles were selected as potential candidates for peptide adsorption because of the high surface area, which will allow greater adsorption of antigens. The roughly spherical form of the CHA nanoparticles is revealed in figure 3.6. The irregular size and shape of calcium carbonate particulates used in this study are revealed in figure 3.7.

**Figure 3.1** T.E.M of surface modified glass microspheres (CP03) (2μm manufacturers size) x 11500
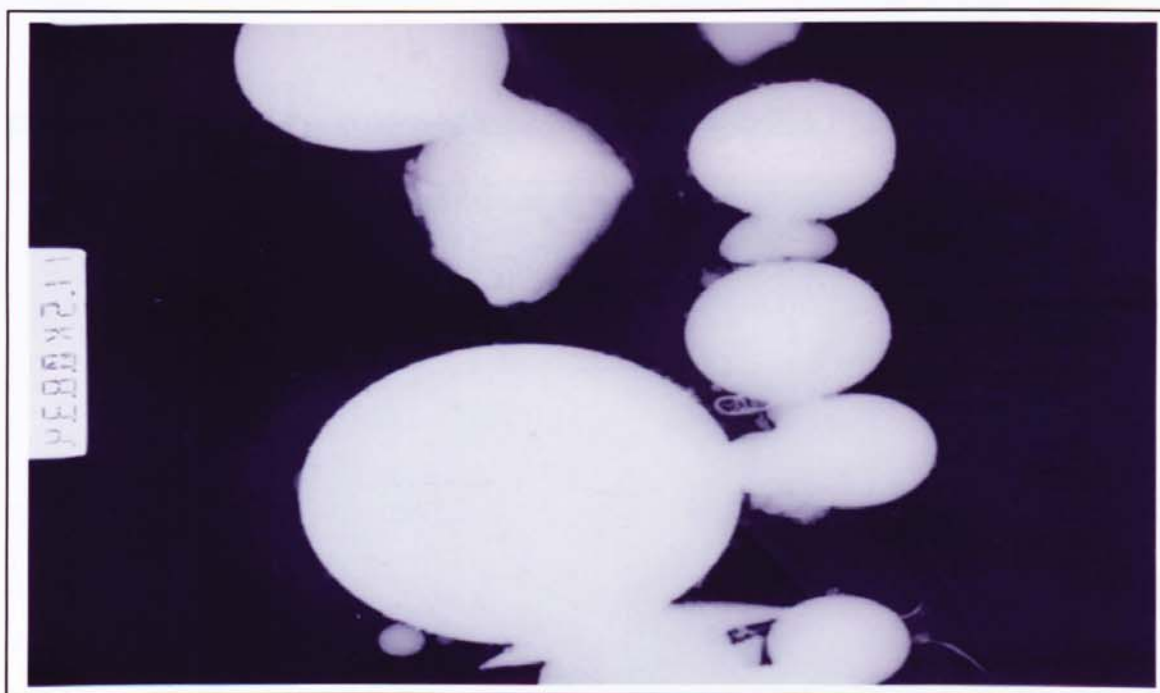


1μm

**Figure 3.2**  T.E.M of microcrystalline hydroxyapatite (MHA P201) x 115000



100nm

**Figure 3.3**  T.E.M of microcrystalline hydroxyapatite (MHA P120) x 115000



100nm

**Figure 3.4** <u>T.E.M of polystyrene microparticles (2µm manufactures size)</u> <u>magnification x 11500</u>
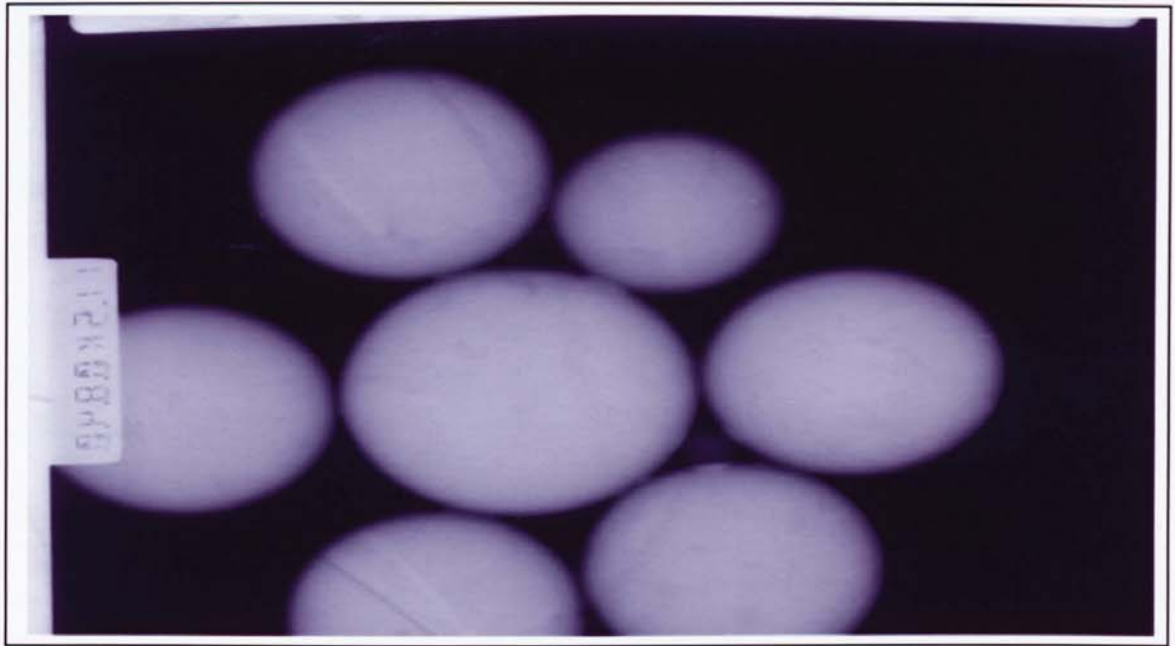


1µm

**Figure 3.5** <u>T.E.M of polystyrene nanoparticles (500nm manufactures size) at x</u> <u>11500</u>



1µm

**Figure 3.6** <u>T.E.M of carbonate hydroxyapatite (6nm manufactures size)</u>
<u>magnification x 115000</u>



100nm

**Figure 3.7** <u>T.E.M of Calcium carbonate (3µm manufactures size) magnification x</u>
<u>11500</u>



1µm

## Surface charge and particle size

All the particles under investigation were found to be negatively charged (table 3.1) and thus binding of OVA will most likely be by hydrophobic interactions or van der waals forces. The magnitude of charge on CHA particles is smaller than the other particles. The particle size data for PS provided by the manufacturers are fairly close to the measurements obtained using the Brookhaven and Malvern Instruments (table 3.1). The size of the CHA and MHA particles are much larger than the sub-micron value expected indicating aggregation of the particles. Surfactants (PVP, PEG, Tween 80) were used in attempts to separate the particles in water but had no effect on the measurements.

**Table 3.1**   Particle size and charge determined using the Brookhaven Instrument - Zeta Potential Analyzer and Mastersizer

| Type of particle used | Size of particles (nm) | Surface charge (Zeta potential) |
|---|---|---|
| Modified glass microspheres (CP03) | 2245.8 ± 288.2 | -11.63 ± 0.26 |
| Unmodified glass microspheres (CP00) | 2504.4 ± 328.5 | -12.08 ± 0.34 |
| Calcium carbonate | 2070.6 ± 20.3 | -15.04 ± 0.28 |
| Hydroxyapatite (MHA P201) | 1416.8 ± 84.1 | -22.16 ± 0.32 |
| Hydroxyapatite (MHA P120) | 1844.3 ± 52.5 | -18.46 ± 0.28 |
| Carbonate Hydroxyapatite (CHA) | 594.5 ± 69.1 | -5.21 ± 1.28 |
| Polystyrene microparticles | 1667.0 ± 13.9 | -35.47 ± 1.57 |
| Polystyrene nanoparticles | 398.7 ± 19.0 | -28.25 ± 1.14 |

Particles smaller than 2.5µm in diameter favour interaction with lymphocytes and more importantly uptake (Wilkinson *et al.*, 2000). Wilkinson *et al* (2000) demonstrated that for interaction and uptake to occur between lymphocytes and polystyrene particles the size of the particles must be around 2µm in size. This is

the approximate size of *M.*tuberculosis bacteria (Wilkinson *et al.*, 2000). The mean particle size of the glass microspheres was measured at approximately 2.5μm, which is therefore based towards the population of smaller particles shown in figure 3.1.

*M.tuberculosis* interacts and invades lymphocytes. Wilkinson *et al* (2000) found that culture filtrate fractions (CFF) of actively replicating *M.tuberculosis* strain H37Rv adsorbed onto polystyrene microspheres significantly enhanced IFN-γ production compared to soluble antigens. Hence for the production of IFN-γ efficient antigen interaction and uptake must first occur with lymphocytes. The authors noted that only particles of approximately 2μm and smaller with adsorbed antigen gave an enhanced IFN-γ response. The particles chosen for the present study are around this size range and could be expected to interact effectively with blood lymphocytes.

Yin *et al* (2002) studied the surface physicochemical properties of MHA and their impact on adsorption of OVA. The zeta-potential of MHA was measured as function of the liquid composition in terms of concentration of cooperative anions and cations ($PO_4^{3-}$ and $Ca^{2+}$). They analysed the zeta-potential of MHA in pH 7 sodium phosphate buffer at different concentrations and found that the magnitude of the negative zeta-potential increased in response to an increase in phosphate concentration. The results displayed in table 3.1 were achieved using suspensions of particles in 0.01M PBS solution at pH 7. Yin *et al* (2002) achieved a zeta-potential of -30mV compared to -18mV and -22mV for MHA in the present study (table 3.1). The investigations by Yin *et al* (2002) showed $PO_4^{3-}$ and $Ca^{2+}$ ions to be the potential determining ions that lead to significant change in both polarity and magnitude of the zeta-potential in MHA.

Other studies have also been performed using MHA to assess the protein adsorption characteristics in terms of the zeta-potential. Zhu *et al* (2001) attempted to clarify the mechanism of site-selective adsorption via the $NH_2$ and $OH^-$ group of OVA

onto MHA. The quantitative analysis of adhesion was performed using a quartz crystal microbalance. They found the MHA particles to be negatively charged (-11.5mV) as indicated in this present study. The values achieved in the present study are greater than that achieved by Zhu *et al* (2001), probably due to the different conditions used to analyse the microparticle charge. Zhu *et al* (2001) used simulated body fluid (SBF) buffered at pH 7.7 with 1M tris(hydroxymethyl)aminomethane $(CH_2OH)_3CNH_2$ and 1M hydrochloric acid (HCl) at 50°C. In the present study 0.01M PBS solution was used at pH7.4 at room temperature.

Makino *et al* (2002) studied the interaction and uptake of polystyrene microspheres by macrophages determining the amount of superoxide generated. They found polystyrene microspheres of 1μm generated larger amounts of superoxide and hence were taken up by macrophages more than particles less than or greater than 1μm. They then studied the functional groups located onto the surface, amine, sulphate, hydroxyl and carboxyl groups. They found that macrophages most effectively trapped polystyrene microspheres with primary amine groups then carboxyl groups. They also found the charge to be negative (-20mV) on all the polystyrene microparticles independent of the surface group. This correlates with the findings for PS microspheres in table 3.1 although slightly more than those reported by Makino *et al* (2002), should interact with macrophages and induce an immune response when adsorbed with peptide.

## 3.2 OVA adsorption and release characteristics from candidate particles for improving the BOVIGAM assay

Ovalbumin (OVA) was used as a model antigen in preliminary experiments to establish adsorption techniques and the protocol for release experiments. Ovalbumin has been used extensively as a model antigen in encapsulated and adsorbed formulations. Thus useful data was expected concerning the influence of

alternative substrates on protein adsorption. The first experiments were performed to study the effect of adsorption time (2-8 hours) on adsorption of OVA onto the glass microspheres. Release profiles were generated over 8 hours.

### 3.2.1 Protein adsorption and release from glass microspheres

Both modified (CP03) and unmodified (CP00) glass microspheres were difficult to keep dispersed in the OVA solution so as to achieve efficient adsorption of the protein. The glass microspheres tended to clump and sediment rapidly, therefore presenting less surface area for adsorption. Sonicating the suspension of glass microspheres for 30 seconds did not improve the dispersion characteristics. Sonication was not performed for longer periods due to the possibility of denaturing the proteins and interfering with particle-protein binding.

Electrostatic forces and hydrophobic interactions play an important role in the binding of proteins to microspheres. Protein adsorption would be expected to plateau or increase over time.

Krajewski *et al* (1996) evaluated the adsorption of OVA onto biologically active and inactive glass microspheres at pH 7.4 and 4.5. The pH 4.5 was chosen as it mimics the environmental conditions of damaged tissues. They found protein adsorption to be greater at pH 4.5 ($130\mu g\ m^{-2}$ to $1000\mu g\ m^{-2}$) as this is closer to the isoelectric point than pH 7.4 ($2\mu g\ m^{-2}$.to $270\mu g\ m^{-2}$). They also found the adsorption to be greater for inert glass molecules than biologically active molecules. Protein adsorption was influenced by the zeta potential values of glass microspheres, the pH of the solution and the isoelectric point of OVA and the glass microspheres. The greater the difference between the zeta potential of active and inactive glass microspheres and pH or isoelectric point of OVA, the more OVA was adsorbed onto the glass microspheres.

The results of protein adsorption (Table 3.2) onto the glass microspheres over time periods of 2 – 8 hours showed that OVA adsorption tended to decrease slightly with incubation time beyond 2 hours, which may indicate time dependent changes in protein conformation in solution or in the adsorbed protein. Norde *et al* (2000) studied the structural changes of OVA that occur during exchange between the adsorbed and dissolved states in 10mM concentration of phosphate buffer at pH7. The particles used were silica and polystyrene microparticles. They found that the structure of OVA is reversible when adsorbed onto hydrophilic silica. However when adsorbed onto hydrophobic polystyrene microparticles irreversible changes occurred in the stability and secondary structure of OVA. They concluded that the irreversible, surface-induced, conformational change might be related to aggregation of OVA molecules after being exposed to hydrophobic surfaces.

**Table 3.2** Protein adsorption on surface modified and unmodified glass microspheres

| Time of adsorption (hours) | OVA adsorbed (% w/w) | |
| :---: | :---: | :---: |
| | Unmodifed (CP00) | Modified (CP03) |
| 2 | 0.26 ± 0.03 | 0.21 ± 0.03 |
| 4 | 0.20 ± 0.02 | 0.20 ± 0.02 |
| 6 | 0.17 ± 0.02 | 0.17 ± 0.02 |
| 8 | 0.16 ± 0.01 | 0.18 ± 0.02 |

For example Norde *et al* (2000) found that adsorption and desorption from polystyrene particles caused irreversible changes in the stability and structure of bovine serum albumin. Initially at 2 hours there appears to be more protein adsorbed on the unmodified glass surface than modified surfaces. Comparing modified and unmodified glass microspheres; there is no statistical difference in the percentage OVA adsorbed over time (Anova single factor > 0.05).

Dispersion of the glass microspheres with adsorbed OVA in the PBS release medium was more effective than uncoated microspheres, probably due to a surfactant effect after protein adsorption (Norde *et al.*, 2000). The results for

protein release from the glass microspheres (Table 3.3) reveal a gradual increase in the amount of protein released over time. The high values obtained for the amount of protein released over time show that the protein retention properties of the glass microspheres are poor and thus they are probably not ideal for formulation of TB diagnostic reagents unless a rapid release is required.

The surface modified glass microspheres tended to release less OVA than the unmodified microspheres (for adsorption times up to 6 hours). The improved protein binding properties may result from electrostatic interactions involving the positively charged amide groups in the nylon coating (see appendix 2) and the negatively charged OVA. Hence a combination of electrostatic interactions, hydrophobic interactions and van der waals forces, may contribute to protein-glass binding in the case of modified glass microspheres.

**Table 3.3**  OVA release from surface modified and unmodified glass microspheres

| Adsorption time (hours) | Time of release (hours) | % OVA released | |
|---|---|---|---|
| | | Unmodified (CP00) | Modified (CP03) |
| 2 | 2 | $19.3 \pm 2.6$ | $13.4 \pm 1.1$ |
| | 4 | $42.7 \pm 2.7$ | $26.5 \pm 3.6$ |
| | 6 | $57.6 \pm 5.0$ | $49.9 \pm 5.7$ |
| | 8 | $76.0 \pm 1.8$ | $66.9 \pm 3.1$ |
| 4 | 2 | $18.9 \pm 2.0$ | $14.7 \pm 2.5$ |
| | 4 | $49.1 \pm 5.6$ | $45.0 \pm 2.6$ |
| | 6 | $63.5 \pm 0.7$ | $53.4 \pm 2.4$ |
| | 8 | $77.0 \pm 1.5$ | $62.5 \pm 2.6$ |
| 6 | 2 | $62.8 \pm 2.5$ | $52.7 \pm 1.9$ |
| | 4 | 100 | $90.9 \pm 2.4$ |
| | 6 | 100 | 100 |
| | 8 | 100 | 100 |
| 8 | 2 | $63.3 \pm 2.1$ | $59.8 \pm 3.9$ |
| | 4 | $96.7 \pm 1.0$ | $89.7 \pm 4.5$ |
| | 6 | 100 | 100 |
| | 8 | 100 | 100 |

The results (Table 3.3) also show that the longer the adsorption period the more rapidly protein is released from the glass microspheres. An adsorption time of 6 hours resulted in approximately 50-60% protein release after 2 hours incubation in PBS at 37°C, whereas an adsorption time of 4 hours and below resulted in less than 20% protein release over the same time period in PBS. These observations suggest that protein conformational changes are occurring over time during the adsorption stage. Whether this occurs in the adsorbed protein or in the protein in solution is unclear.

### 3.2.2 OVA adsorption onto microcrystalline hydroxyapatite (MHA)

An initial study was carried out using MHA P120 to gain experience of OVA dispersion adsorption and release characteristics. MHA particles were easier to use than the glass microspheres although sonication was still required to disperse the particles in suspension. Sonication was performed for no longer than thirty seconds. The particles with weights 50mg and 100mg did tend to sediment out and settle at the bottom of the glass test tubes. This was overcome by manual shaking to redisperse the particles. A weight of 10mg of MHA particles was subsequently used for adsorption and release experiments.

Table 3.4  Protein adsorption on MHA P120 (2 hours adsorption) and release after 2 hours in PBS at 37°C

| Weight of MHA (mg) | % (w/w) OVA adsorbed | % (w/w) released in 2hrs in PBS at 37°C |
|---|---|---|
| 10 | 2.7 ± 0.2 | 13.4 ± 4.8 |
| 50 | 1.2 ± 0.4 | 29.0 ± 2.7 |
| 100 | 0.9 ± 0.2 | 39.1 ± 3.4 |

MHA is known to exhibit good protein and peptide binding properties and this property was supported by the results shown in table 3.4 (Revilla *et al.*, 1996; Norde *et al.*, 2000). An increase in weight of MHA particles resulted in a decrease in protein adsorption on a percentage weight per weight (%w/w) basis due to

inefficient dispersion of the particles during adsorption. Preliminary studies (Table 3.4) revealed an OVA adsorption value of 2.7% w/w. Approximately 13% of the protein load was released after 2 hours incubation at 37°C in PBS.

### 3.2.3 The effect of incubation medium on OVA adsorption onto MHA particles

Experiments were also performed to investigate the effect of adsorption medium (PBS or distilled water) on OVA adsorption to MHA particles (Table 3.5). Protein adsorption on materials is influenced by the pH, ionic strength and composition of the adsorption medium. However the use of OVA solution in PBS and OVA solution in water resulted in similar protein adsorption. In the study of Yin *et al* (2002) bovine serum albumin (BSA) was buffered with pH 6.8 phosphate of different concentrations and adsorbed onto MHA particles. When the BSA was dissolved in PBS more $PO_4^{3-}$ ions are present than in water resulting in more $PO_4^{3-}$ at the surface of MHA and thus altering the surface charge (Yin *et al.*, 2002). This enhances the electrostatic repulsion forces between MHA and BSA, reducing the amount of BSA adsorbed. In the present study, the phosphate ions appear to have little influence on OVA adsorption.

**Table 3.5**   OVA adsorption on MHA P120 and P201 (10mg MHA, 2 hours adsorption)

| Adsorption medium | % OVA adsorbed | |
|---|---|---|
| | MHA (P201) | MHA (P120) |
| Water | $1.8 \pm 0.1$ | $1.2 \pm 0.1$ |
| PBS | $1.7 \pm 0.1$ | $1.0 \pm 0.1$ |

There was a statistical difference between OVA adsorption onto MHA P201 and P120 (t-test: Non-paired two sample means, <0.05). MHA P201 adsorbed more protein than MHA P120 whether the OVA was in solution in water or PBS. MHA P201 particles were observed to disperse well whereas MHA P120 particles tended

to clump together. Thus less adsorption occurs due to the presentation of a lower surface area in the case of MHA P120.

## 3.3 Adsorption and release of peptide from candidate particles for improving the BOVIGAM assay

### 3.3.1 The effect of adsorption medium on peptide (p148 83/5) adsorption onto MHA

This experiment was performed as a preliminary experiment in the study of peptide adsorption on particulates. The peptide (P148 83/5) was used to study the adsorption characteristics of MHA and also the effect of adsorption medium. The sequence of the peptide is PKPATSPAAPVTTAAMADPA.

The results in Table 3.6 were obtained by incubating MHA (10mg) in 1ml PBS or water containing 2mg peptide (p148 83/5) at room temperature for 2 hours.

Table 3.6    Peptide (P148 83/5) adsorption onto MHA P120 and P201 (10mg MHA, 2 hours adsorption)

| Adsorption medium | % Peptide adsorbed (w/w) | |
| --- | --- | --- |
| | MHA (P201) | MHA (P120) |
| Water | $15.5 \pm 2.8$ | $14.9 \pm 1.9$ |
| PBS | $15.3 \pm 2.0$ | $14.1 \pm 2.6$ |

High levels of peptide adsorption were measured on MHA of approximately 15 % w/w (Table 3.6). Peptide adsorption on MHA was much improved relative to OVA (approximately 2% for MHA P201) and there was no significant effect of solution composition (i.e. water vs. PBS) (t-test: Non-paired two sample mean >0.05). Overall, the present findings indicated that MHA efficiently adsorbed the P148 83/5 peptide. Again MHA P201 gave the higher adsorption of the two-hydroxyapatite samples.

The results presented in figure 3.8 show that OVA release from MHA particles is more pronounced in PBS than water. The results also indicate that MHA P201 retained the protein more effectively than MHA P120. The difference in ability to bind protein between the two types of MHA may be due to the relative proportion of C and P binding sites present (Kandori *et al.*, 2002). Hydroxyapatite molecules possess positively charged adsorption sites on their crystal surfaces. (C-sites which are rich in calcium ions) and thus bind effectively to negatively charged or acidic groups of proteins (Kandori *et al.*, 2002). They also possess P-sites, which are negative in charge and thus bind to positive or basic groups of protein. Ionic species in the medium in which the proteins are dissolved, can alter the binding sites making them less accessible for binding.

**Figure 3.8**  OVA release from MHA P201 and P120 (10mg, 2 hours adsorption)
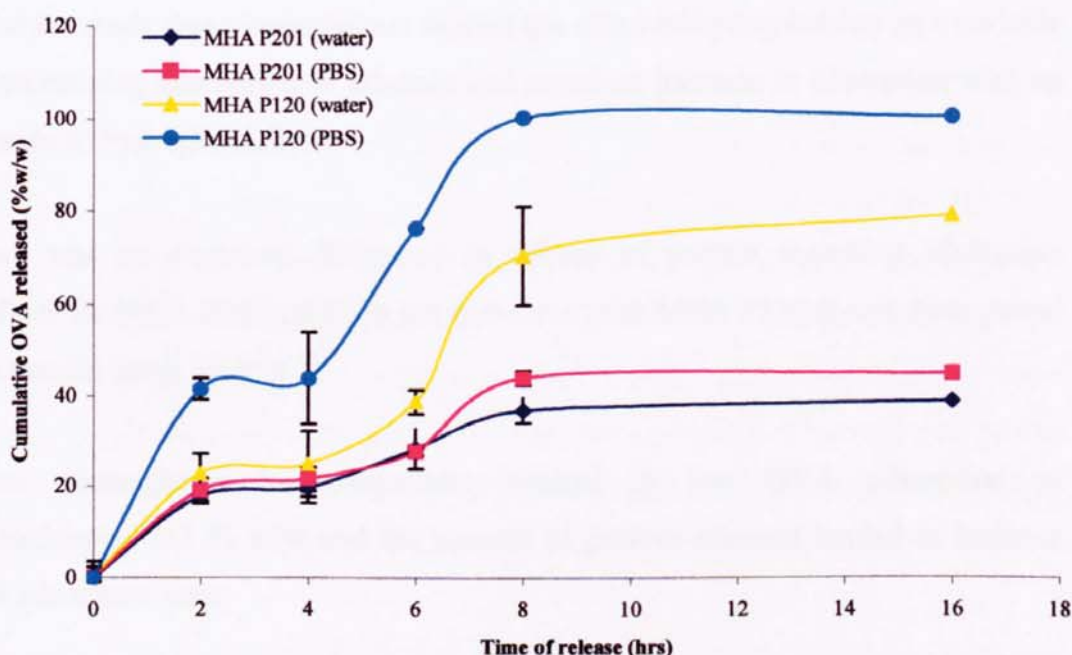


A reduction in C-sites may have occurred for MHA P120 in PBS giving rise to weaker electrostatic forces and thus explaining the larger release of protein. The higher protein adsorption and retention on MHA P201 may also be related to the particle morphology. Kandori *et al* (2002) studied the effects of acetonitrile on the adsorption behaviour of OVA onto MHA particles (30-100nm). They found that

122

acetonitrile decreases the α-helical content of OVA at concentrations above 30% while the β-helical content is increased. This resulted in structural changes to the secondary and tertiary structure. When OVA in acetonitrile (10-20% concentration) was adsorbed onto MHA (30nm), large amounts of OVA were adsorbed due to the high surface area of the nanoparticles. They also noted that despite aggregation of OVA at high concentrations in acetonitrile (>40%), the adsorption was still efficient on larger MHA particles (108nm). This was explained in terms of retained accessibility of binding sites.

Much work has been carried out regarding the effects of surface charge, particle size and particle texture of MHA on protein adsorption. Kandori *et al* (1999) investigated the effect of particle length on adsorption. They found that the amount of albumin adsorbed onto MHA is dependent on particle length, which has a linear relationship in that protein adsorption increased linearly with increase in length. In an earlier study these investigators studied the effect of hydrophobicty as a variable in determining adsorption of albumin and noted an increase in adsorption with an increase in hydrophobicity.

There was no statistical difference in release of protein regarding adsorption medium for MHA P201 particles but there was with MHA P120 (t-test: Non-paired two sample mean <0.05).

Glass microspheres in comparison resulted in low OVA adsorption of approximately 0.2 % w/w and the amount of protein released tended to increase with adsorption time.

MHA provides an effective substrate for protein adsorption (1.2 – 2.7 % w/w). The good OVA retention properties exhibited by MHA P201 over 16 hours in PBS at 37°C (57%) suggested that MHA P201 may be useful for improving the sensitivity of a peptide based assay for TB.

### 3.3.2   Adsorption and release of peptide P107 from candidate particles

Initially five microparticle systems where selected to study peptide P107 adsorption and release. Initial studies with polystyrene microparticles required washing the particles with saline solution, borate solution or PBS solution. The manufacturers information recommended washing the particles with borate solution. However the pH of borate is alkaline (pH 8.5) and as mentioned above the pH of the medium plays an important part in the adsorption properties of particles. As well as the polystyrene particles being pre-washed with saline, borate or PBS solution, the peptide was also dissolved in the respective solutions prior to adsorption.

Experiments were performed to determine the effect of the adsorption medium on adsorption. Polysciences suggest the use of borate buffer at pH 8.5. However adsorption of peptides is affected by pH and salt concentration (Florence et al., 2000). Therefore the borate buffer was compared to PBS and normal saline both at pH 7. One might expect adsorption to be largely affected by use of different buffers due to conformational changes in the peptide. However this proved not to be the case with borate buffer tending to give the best adsorption followed by PBS and saline last (Figure 3.9). However there was no statistical difference in adsorption of the model peptide P107 between the polystyrene particles (Anova: Single factor >0.05) washed with different buffers. A relatively low peptide adsorption below 1.5% w/w was achieved using the polystyrene microparticles. This was unexpected as previous work suggests polystyrene to be a very good substrate for binding proteins and peptides (Sallberg *et al.*, 1995).

Polystyrene microspheres were used by Kondo *et al* (1991) to investigate the adsorption of γ-globulin, (a model protein for antibody) as a function of pH and ionic strength. They found polystyrene to show high binding affinity for the protein. Revilla *et al* (1996) investigated the effect of pH and ionic strength of the aqueous phase on protein adsorption on polystyrene particles modified with sugar residues to change the surface characteristics and untreated polystyrene particles.

They found that the highest adsorption of BSA occurred at pH 5. However the BSA is likely to exhibit different adsorption characteristics to the model peptide used here. For example protein adsorption tends to be a maximum at the isoelectric point. For albumin this is around pH 5.

**Figure 3.9** Comparison of peptide P107 adsorption onto microparticles (2 hours incubation at room temperature)
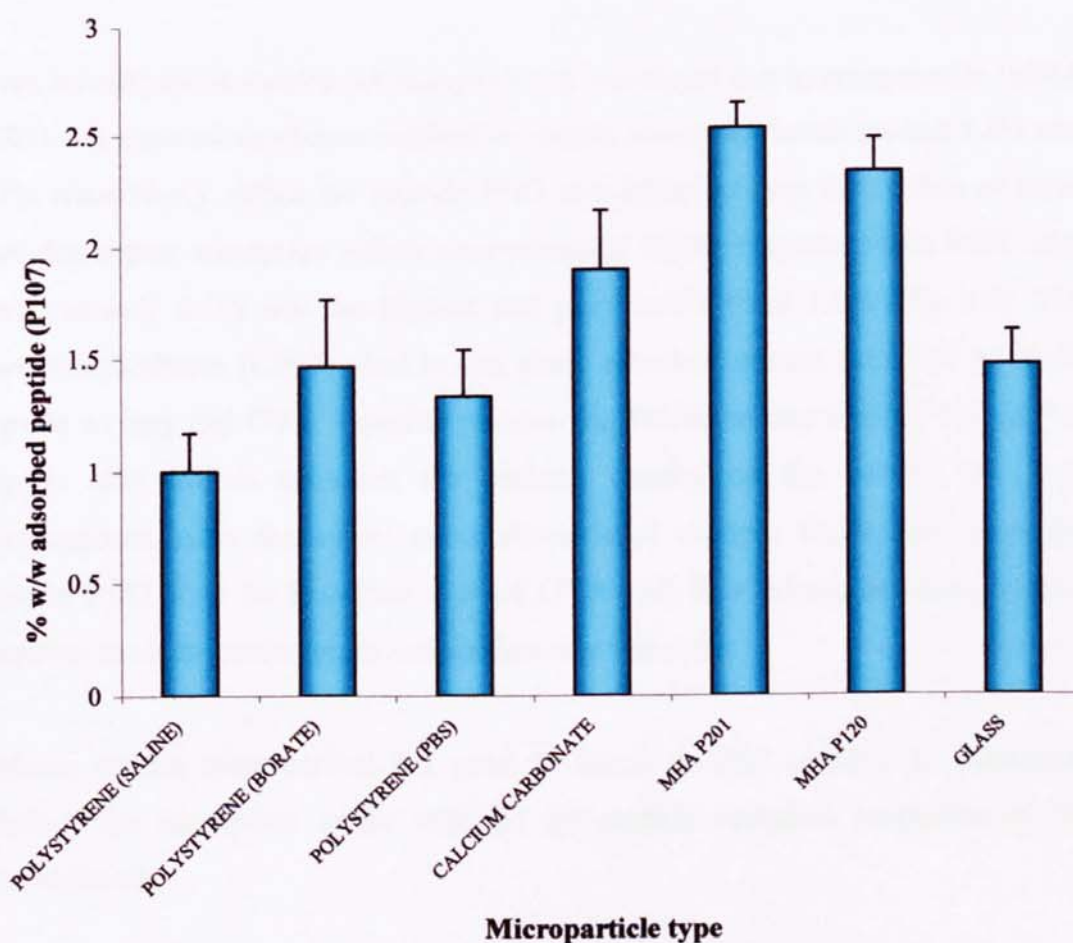


The conditioning of polystyrene particles with borate solution is likely to have altered the surface charge, therefore influencing peptide adsorption. Cassidy *et al* (1999) modified polystyrene microparticles by the addition of poloxomer (F77, F87 and F127 respectively) to the surface. They noted a reduction in electronegativity of

the particles, hence making the surface more positive and increasing the electrostatic forces between the particles and peptide P107.

Peptide adsorption was performed over 2 hours because experiments using glass spheres (CP03 and CP00), MHA and calcium carbonate over time revealed similar adsorption levels for OVA adsorption onto these particles. It was also important to achieve maximum adsorption in as short a time as possible to maximise speed of testing in the Bovigam assay.

From investigations carried out using OVA it was found that hydroxyapatite (MHA P201) and glass microspheres resulted in protein adsorption levels around 1.8% and 0.2% respectively. When the peptide P107 was adsorbed onto the surface of these particles higher adsorption values were obtained. Hydroxyapatite MHA P201 with approximately 2.5% was the highest and glass was around 1.5% (Fig 3.9). The glass microspheres (CP03) used in this study adsorbed around six times as much peptide as they did OVA. Possible reasons for this behaviour include the smaller peptide size, which enhances the packing density on the surface of CP03 microspheres. Also the extent of conformational changes that occurs with the peptide P107 may be less than that of OVA and thus adsorption may be less sensitive to electrostatic forces and surface characteristics.

Release studies were carried out over 24 hours in PBS at 37°C to determine whether the adsorption media affected the peptide retention properties of PS microspheres.

Figure 3.10 reveals that peptide adsorption onto polystyrene microspheres using borate buffer as the adsorption medium results in very poor peptide retention properties compared with the use of PBS or saline as the adsorption medium. The peptide retention is the lowest of all the microparticle systems investigated. The borate buffer is markedly affecting electrostatic forces, Van Der Waals forces and hydrophobic interactions which control binding properties between the peptide and

the polystyrene microspheres. Using the univariate analysis of variance there is a significant difference between the three types of polystyrene particles (<0.05) concerning release of peptide over time. The results in figure 3.10 also reveal that MHA P201 and calcium carbonate are the best of the microparticles for retaining the peptide. Calcium carbonate exhibited similar retention properties to the MHA P201 sample. Modified glass microspheres (CP03) also displayed good retention of peptide P107 compared with OVA (Table 3.3).

**Figure 3.10** Comparison of peptide P107 release from microparticles over 24 hours (PBS release medium, 37°C)
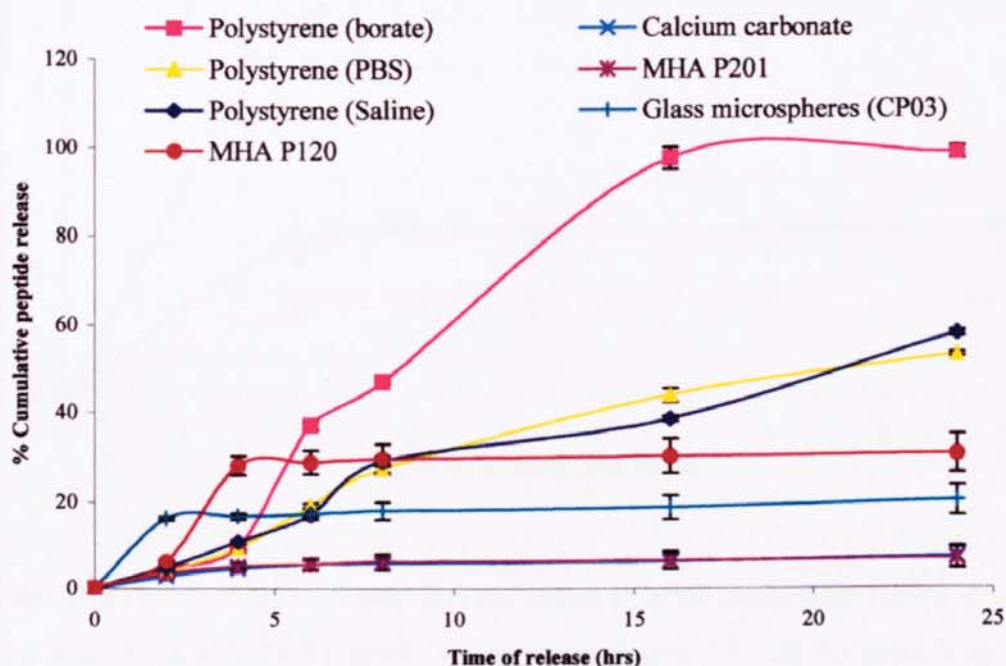


### 3.3.3 Adsorption and release of peptide (ESAT-6,p45) from candidate particles for improving Bovigam Assay

The sequence for the peptide (ESAT-6,p45) is MTEQQWNFAGIEAAAS. The peptide is slightly soluble in PBS requiring the addition of a few drops of ammonium chloride 0.1M to help dissolution.

Polystyrene microparticles conditioned with PBS were selected for adsorption of the actual peptide (ESAT-6,p45) to be used in the Bovigam assay. Although the amount of peptide P107 adsorbed was less using PBS than using borate buffer as a conditioning agent, the peptide retention properties were improved (Fig 3.10).

**Figure 3.11** Comparison of peptide (ESAT-6,p45) adsorption on microparticles over 8 hours
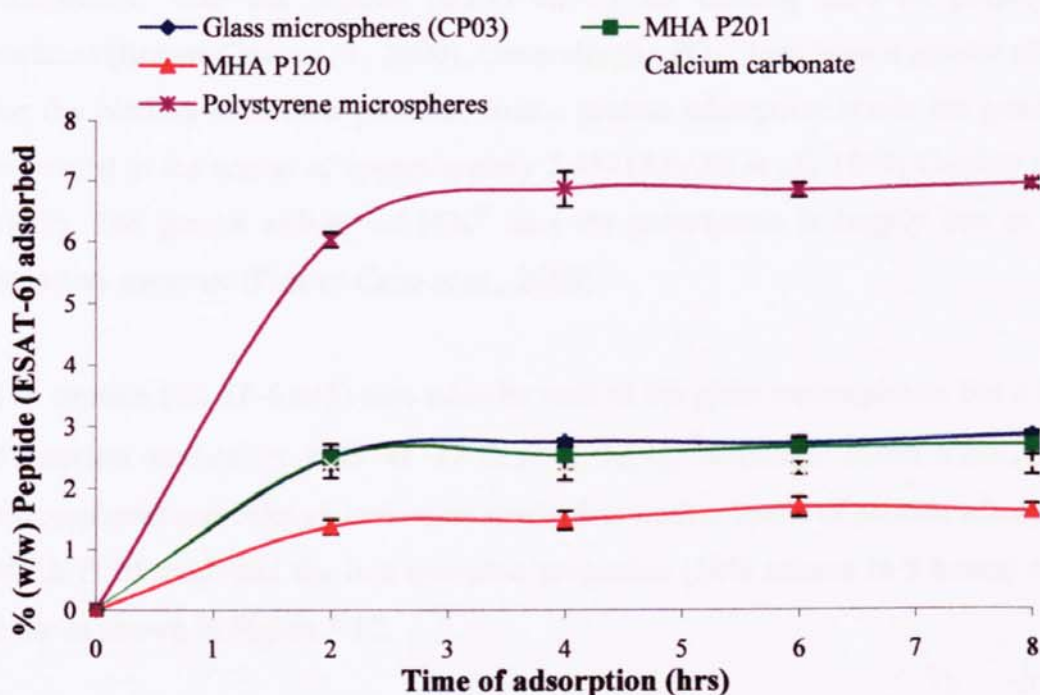


Studies were performed to determine the effect of adsorption time (up to 8 hours) on the amount of adsorbed peptide. As shown in figure 3.11 all the particle systems investigated resulted in a plateau in the adsorption versus time curve after 2 hours adsorption, indicating saturation of binding sites on the particles. The maximum peptide adsorption occurred on polystyrene microparticles to a level of approximately 7%. Comparing all the particle systems, according to univariate analysis of variance there is a significant difference between the amounts of peptide adsorbed over time ($<0.05$).

The polystyrene microspheres resulted in the highest (ESAT-6,p45) adsorption level of 7%. This is a large increase in adsorption compared with peptide (P107), which resulted in approximately 1.3% adsorption over the same time period.

The peptide ESAT-6,p45 differs structurally from model peptide P107 as indicated by the amino acid sequences, which seems to enhance peptide/polystyrene binding possibly via hydrophobic interactions. $PO_3^{4-}$ ions from PBS are in direct competition with the peptide (ESAT-6,p45) for binding sites on polystyrene surfaces (Romer-Cano et al., 2000). Generally the $PO_3^{4-}$ ions have a greater affinity for the binding sites than proteins; hence protein adsorption levels are generally measured in the region of approximately 2-4% (Revilla et al., 1996; Cassidy et al., 1999). The greater affinity of $PO_3^{4-}$ ions for polystyrene is largely due to their chemical structure (Romer-Cano et al., 2000).

The peptide (ESAT-6,p45) also adsorbs well to the glass microspheres but a rapid desorption occurs in PBS at 37°C (Fig 3.12). Although MHA P201, glass microspheres and calcium carbonate resulted in similar levels of peptide adsorption. MHA P201 exhibited the best retention properties (10% release in 8 hours) of the three as shown in Figure 3.12.

Polystyrene microspheres and MHA P201 provided good adsorption and retention of peptide (ESAT-6,p45) suggesting they may be potential candidates for improving the sensitivity of peptide-based diagnostic assay for TB.

The release profiles in figure 3.12 all tend to exhibit a plateau after 8 hours with only slight increases in %(w/w) peptide release beyond that time. For MHA P201, calcium carbonate and polystyrene microparticles, peptide desorption occurs after 2 hours whereas for glass microspheres and MHA P120 desorption occurs after 4 hours. This release characteristic for peptide (ESAT-6,p45) differs from that of the model peptide P107 where desorption occurred from the start of the release studies (Fig 3.10). Hence peptide substrate binding is more effective for ESAT-6,p45 than

P107. This behaviour may be related to charge differences between the peptides in influencing electrostatic interaction.

**Figure 3.12**   Comparison of peptide (ESAT-6,p45) release from microparticles over 24 hours at pH 7
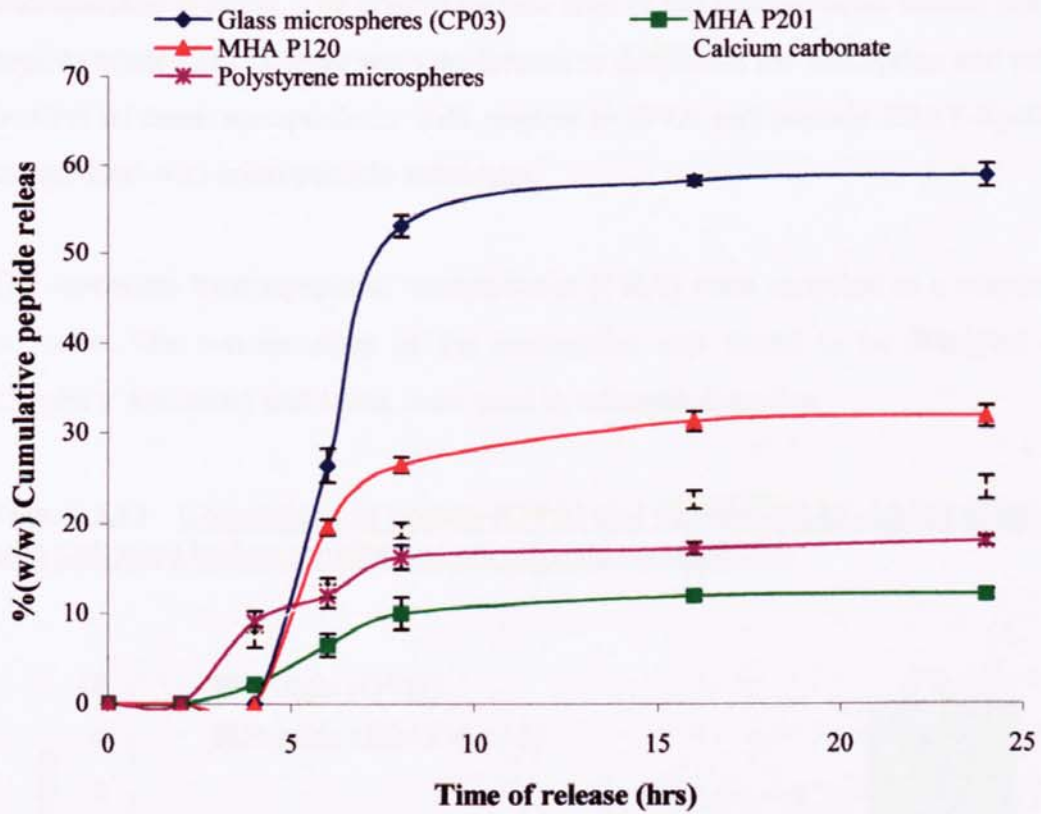
**3.3.4  Adsorption of OVA and peptide (ESAT-6,p45) onto nanoparticles of polystyrene and carbonate hydroxyapatite (CHA) and release properties**

Nanoparticles of carbonate hydroxyapatite (CHA) and polystyrene with adsorbed peptide were formulated for application in the Bovigam assay along with selected microparticle systems. The greater surface area of the nanoparticles should enhance peptide adsorption. Studies were performed to determine the adsorption and release profiles of these nanoparticles with respect to OVA and peptide ESAT-6,p45 for comparison with microparticle substrates.

The carbonate hydroxyapatite nanoparticles (CHA) were supplied as a suspension in water. The concentration of the suspension was found to be 30mg/ml (See Chapter 2 Methods) and 10mg were used in adsorption studies.

**Figure 3.13**  Comparison of protein (OVA) and peptide (ESAT-6,p45) adsorption onto carbonate hydroxyapatite and polystyrene nanoparticles
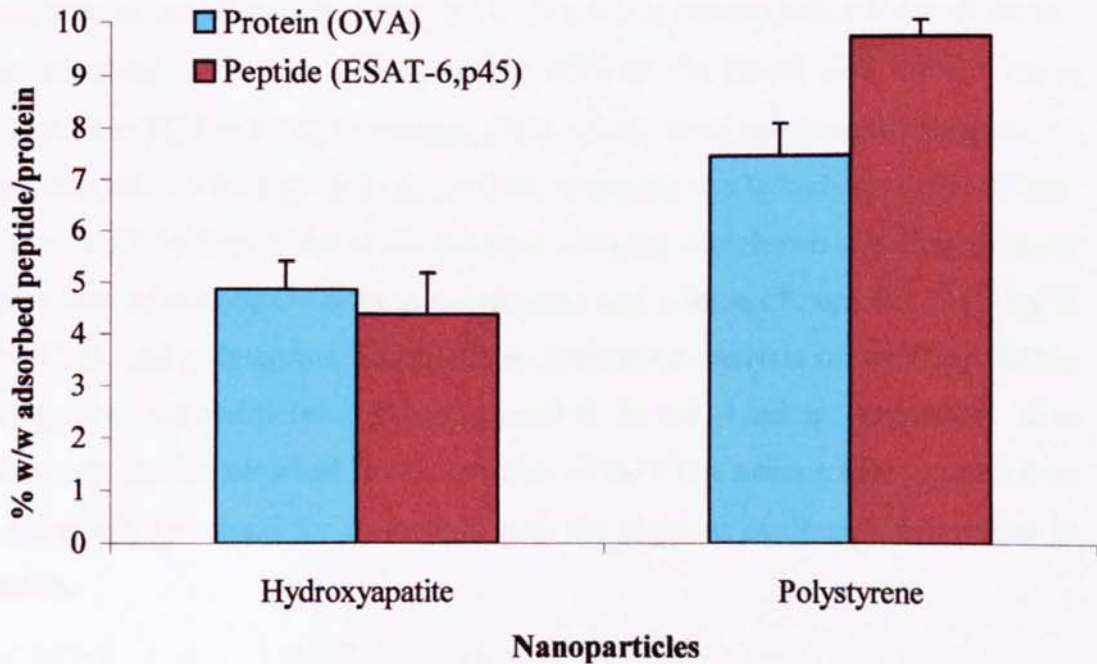
Comparing the two types of nanoparticles it is apparent from figure 3.13 that polystyrene nanoparticles adsorb more protein (7.5%) and peptide (9%) than CHA nanoparticles (4.5%) (ANOVA: single factor <0.05). The mean percentage adsorption of peptide onto CHA nanoparticles was lower than that of protein. However there is no significant difference between the adsorption of peptide ESAT-6,p45 and OVA on CHA nanoparticles (ANOVA: single factor >0.05). During the previous investigations with MHA microparticles, peptide (ESAT-6,p45) adsorption (2.5%) was generally higher than OVA (1.8%) (Table 3.6 and Figure 3.11). This change in behaviour underlines the complex interaction between surface chemistry, adsorption medium and surface area for adsorption (Table 3.6).

The adsorption of OVA and ESAT-6,p45 on both CHA and polystyrene nanoparticles is much higher than that obtained using glass microspheres CP03, calcium carbonate, polystyrene microparticles and MHA nanoparticles (100nm) (see Table 3.6 & Fig 3.11). This behaviour is most probably due to the larger surface area available for adsorption on the CHA and polystyrene nanoparticles.

OVA and peptide ESAT-6,p45 release profiles from CHA and polystyrene nanoparticles are shown in figure 3.14. The CHA nanoparticles (6nm diameter) with adsorbed OVA lose approximately 40% of the initial load after 5 hours incubation in PBS at 37°C. In contrast OVA release from polystyrene nanoparticles or peptide release from CHA or polystyrene nanoparticles is limited to 10% or less. Release of OVA from CHA at the 6-hour time point was shown to be significantly higher than release of OVA from polystyrene and release of peptide ESAT-6,p45 from CHA and polystyrene nanoparticles (univariate analysis of variance <0.05). Polystyrene nanoparticles (200nm) exhibit better binding properties than hydroxyapatite for adsorbed OVA. The size of the CHA nanoparticles (6mm) may be physically too small for interaction with the globular ovalbumin formations in solution.

**Figure 3.14**  Protein (OVA) and peptide (ESAT-6) release from CHA and polystyrene nanoparticles at pH 7
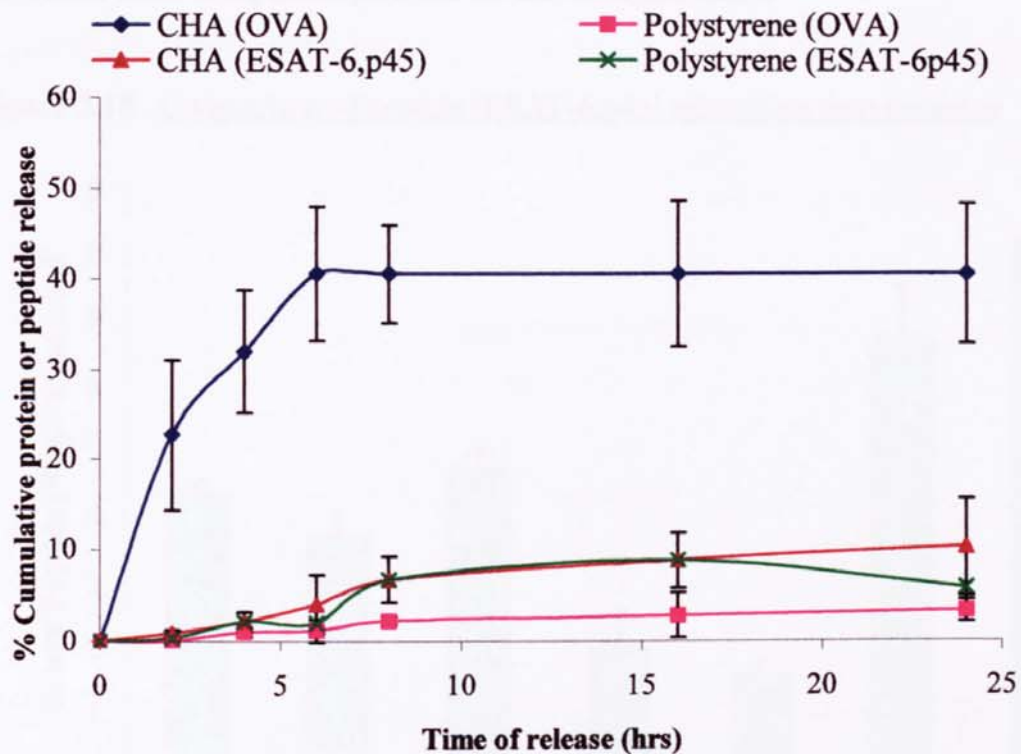


3.3.5  **Release of peptide (ESAT-6,p45) from particle carriers at pH 5.5**

The pH of the environment inside a lymphocyte is 5.5. Assuming that particles with adsorbed peptide are taken up by lymphocytes, release studies were performed at pH 5.5 to assess the effect on peptide retention by the particle carriers.

The peptide (ESAT-6,p45) was adsorbed onto MHA P201, MHA P120, CHA, polystyrene micro and nanoparticles, calcium carbonate and glass microspheres as described in section 2.3.2. The release study at pH 5.5 was performed as described in section 2.3.4.3.

The peptide loading for each particle used in the release study is shown in Figure 3.15. The results show similar adsorption levels of peptide on the particles as in

previous studies (Fig 3.11 & 3.13) with exception of MHA P201 and MHA P120. Hence this shows good reproducibility and consistency of adsorption, which is important when using these particles for the Bovigam assay.

**Figure 3.15** Comparison of peptide (ESAT-6,p45) adsorption onto particles
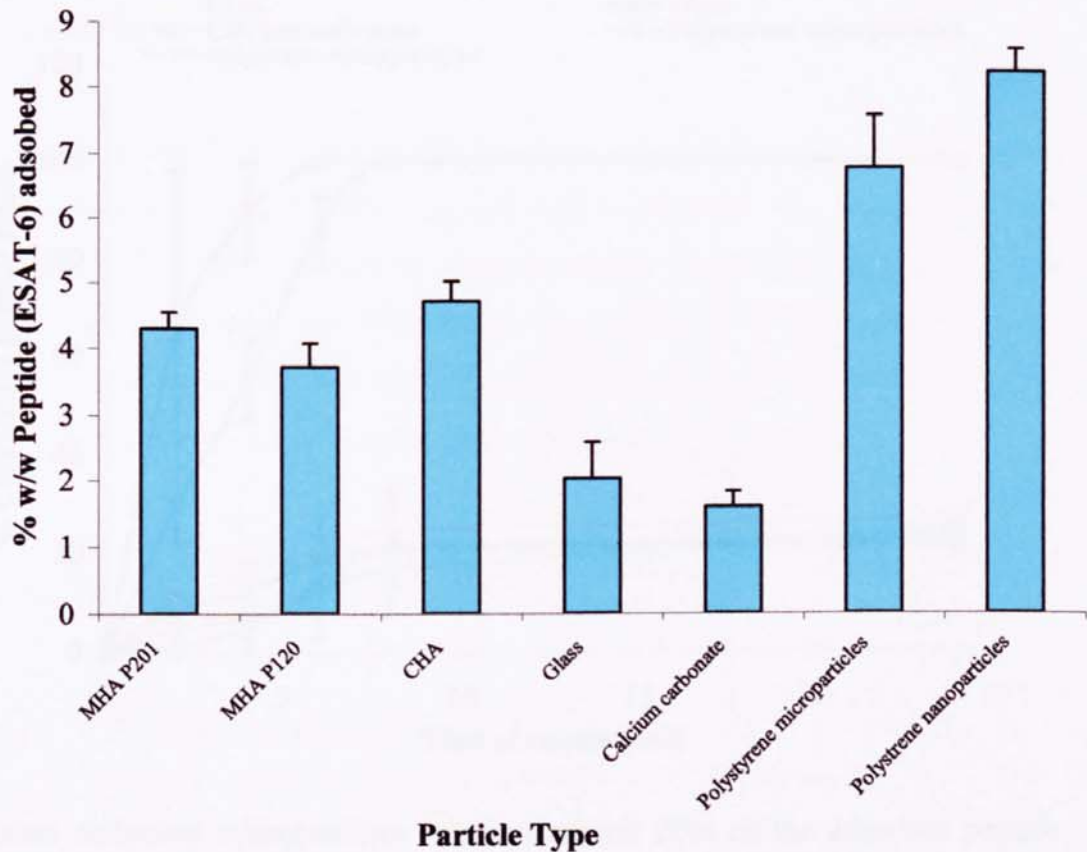


Release studies were performed at pH 5.5 using PBS solution adjusted using 0.1M NaCl. PBS was selected rather than HEPES solution (which has a pH of 5.5) to reduce variables in the experiment and to maintain previous experimental conditions as far as possible.

Figure 3.16 shows peptide (ESAT-6,p45) release from particles at pH 5.5. The only notable change in release characteristics at pH 5.5 compared to the release profiles of particles at pH 7.4 (Figure 3.12) is that calcium carbonate and glass

microspheres release all the peptide after 8 hours whereas at pH 7.4 only the glass microspheres lose more than 50% of the initial peptide (ESAT-6,p45) load.

**Figure 3.16**  Peptide (ESAT-6,p45) release from particles at pH 5.5 over 24 hours
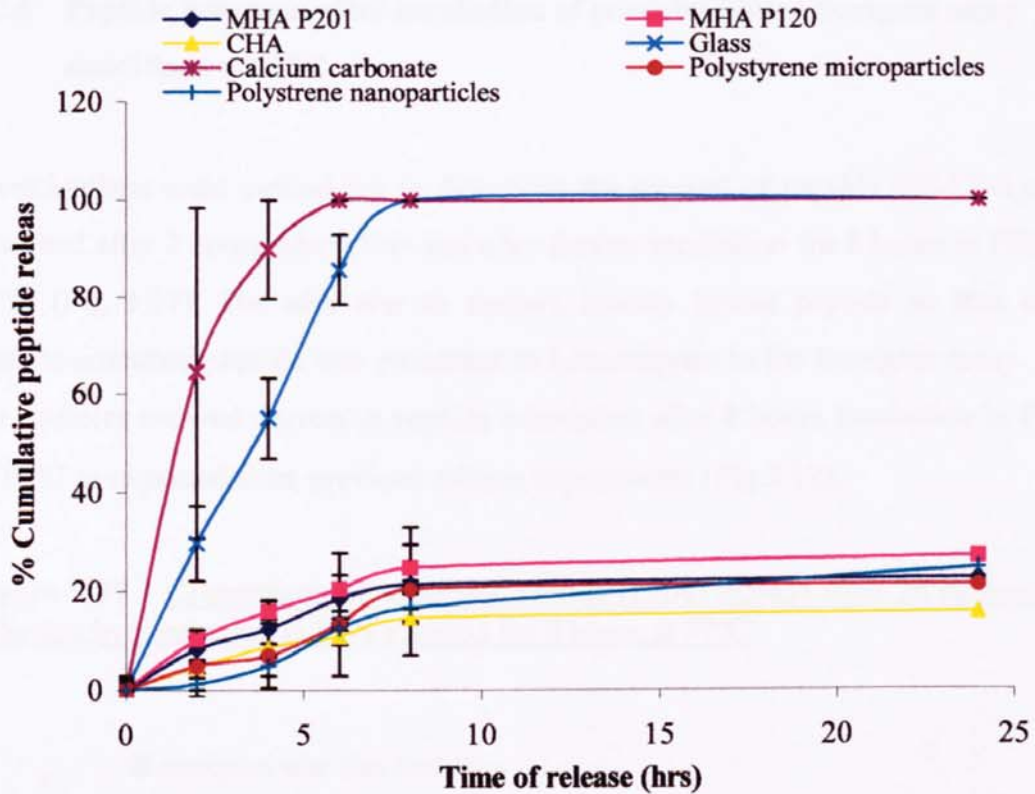


Calcium carbonate microparticles released around 20% of the adsorbed peptide (ESAT-6,p45) in 24 hours at pH 7.4 (Fig 3.12), whereas total release of adsorbed peptide (ESAT-6,p45) occurred in 6 hours at pH 5.5 (Fig 3.16). This major change may be due to dissolution of the microparticle carrier surface at the lower pH. Polystyrene micro and nanoparticles, MHA P201, MHA P120 and CHA all exhibit similar peptide retention properties at pH 5.5 and pH 7.4. CHA demonstrated the best retention properties overall at pH 5.5. Using univariate analysis of variance there is no statistical difference in release of peptide from these particles at pH 5.5 over time (>0.05).

Good peptide retention by the particulate carriers at pH 5.5 suggests that a particle-peptide complex would be presented for antigen processing within lymphocytes if particle uptake occurred.

### 3.3.6 Peptide retention after incubation of particles under Bovigam assay conditions at 37°C

Investigations were carried out to determine the amount of peptide (ESAT-6,p45) adsorbed after 2 hours adsorption and after further incubation for 8 hours in PBS at 37°C (Fig 3.17). The aim was to remove loosely bound peptide so that only particle-adsorbed peptide was presented to lymphocytes in the Bovigam assay. All the particles showed decreased peptide adsorption after 8 hours incubation in PBS at 37°C as expected from previous release experiments (Fig 3.12).

**Figure 3.17** Comparison of adsorbed peptide (ESAT-6,p45) after 2h adsorption followed by incubation in PBS solution for 8 hours at 37°C
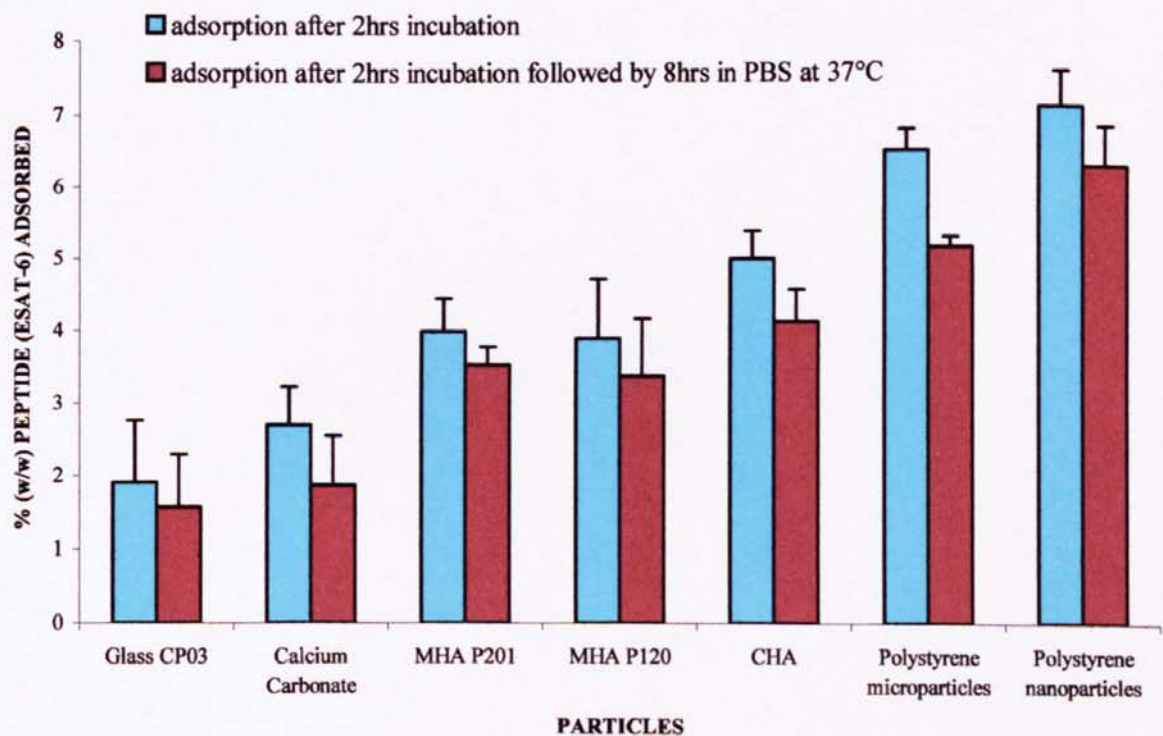
The reduction in peptide load after 8 hours in PBS is broadly in line with the results of the release experiments presented in Figure 3.12. Of the particles tested, only calcium carbonate, CHA and polystyrene microspheres showed a significant difference in adsorbed peptide (ESAT-6,p45) after 8 hours incubation in PBS (ANOVA: single factor <0.05).

# CHAPTER 4


# IMMUNE RESPONSE TO PARTICLE-ADSORBED PEPTIDE (ESAT-6,p45)

# 4 BOVINE GAMMA INTERFERON (IFN-γ) PRODUCTION FOLLOWING PRESENTATION OF FREE AND PARTICLE BOUND PEPTIDE TO LYMPHOCYTES IN A BLOOD BASED ASSAY

## INTRODUCTION

In the Bovigam assay the immune response to the presented peptide (mycobacterial antigens) is measured in terms of the production of gamma interferon (IFN-γ) by lymphocytes. If cattle are infected with *M.bovis* T-lymphocytes in the blood recognise specific mycobacterial antigens present in bovine TB.

In stage 1 of the Bovigam assay tuberculin-derived peptide (antigen or particle bound peptide) is captured and processed by monocytes. These cells phagocytose the antigen and particle bound peptide, which after modification in the endocytic vacuoles in the cytoplasm undergoes proteolysis. The fragments of the antigen and particle bound peptide become associated with class II molecules and the complex is transported to the surface of the cell, where it is accessible by T cells. The processed form is presented to CD4+ T cells in association with MHC class II molecules leading to the production of IFN-γ (Figure 4.1).

The supernatants of the plasma are harvested after 24 hours incubation at 37°C, 5% carbon dioxide for analysis in stage 2. Here the amount of bovine IFN-γ is measured by ELISA. Antibodies to bovine IFN-γ are coated onto the surface of microplates and these react with the plasma samples, binding to any bovine IFN-γ present. Horseradish peroxidase conjugate reacts with IFN-γ bound to the antibodies and an enzyme substrate is then added. The rate of conversion of substrate is proportional to the quantity of bound IFN-γ. See Figure 4.2.

**Figure 4.1** Stage 1 Capture and process of tuberculin-derived peptide (antigen or particle bound peptide)



Tuberculin-derived peptide

IFN-γ    CD4+ T cell    Monocyte

Stage 1:
Blood culture
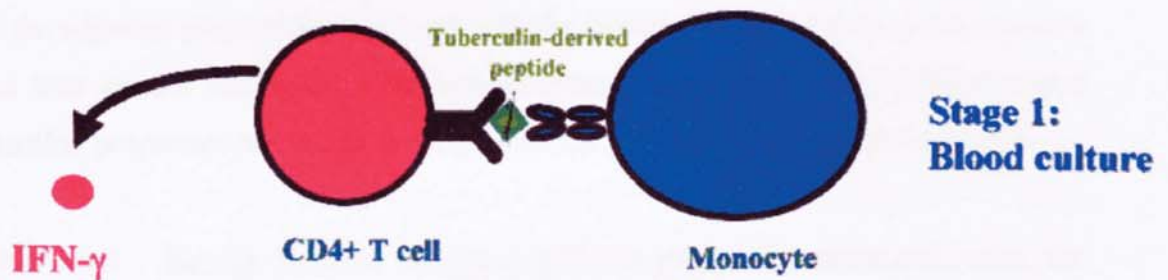
**Figure 4.2** Stage 2 Measurement of bovine IFN-γ using ELISA



Enzyme sustrate

Horseradish peroxidase conjugate

Antibodies coated to surface of microplate

Stage 2:
IFN-γ specific ELISA
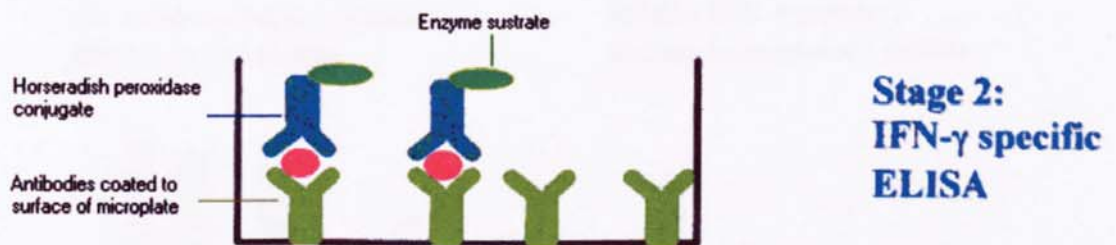
## 4.1 BOVIGAM™ assay 1

The results presented in Figure 4.3 show the amount of IFN-γ produced following presentation of free and particle bound peptide (ESAT-6,p45) to blood taken from a cow infected with TB. The results represent a measure of cell-mediated immunity, used for the diagnosis of bovine tuberculosis infection in cattle. Due to the limited amount of peptide available a single experiment was performed and thus no error bars included. If the experiment worked well then further testing would have been performed.

The response was comparable for the free peptide, polystyrene-bound, calcium carbonate and MHA-bound systems at high peptide concentrations (25µg/ml). The free peptide was more efficient than the peptide-bound systems for stimulating

lymphocyte production at lower peptide exposure. At lower concentrations 0.3, 0.1 and 0.03μg/ml the IFN-γ response is higher than calcium carbonate and glass with MHA P201, P120 and polystyrene microparticles (Fig 4.3). This can be explained by the adjuvant properties associated with the binding of the peptide to the particles and thus greater interaction with lymphocytes. Also with MHA having the best retention properties represents the high immune response produced in figure 4.3.

**Figure 4.3** <u>Bovine Gamma Interferon (IFN-γ) production measured using the BOVIGAM assay</u>
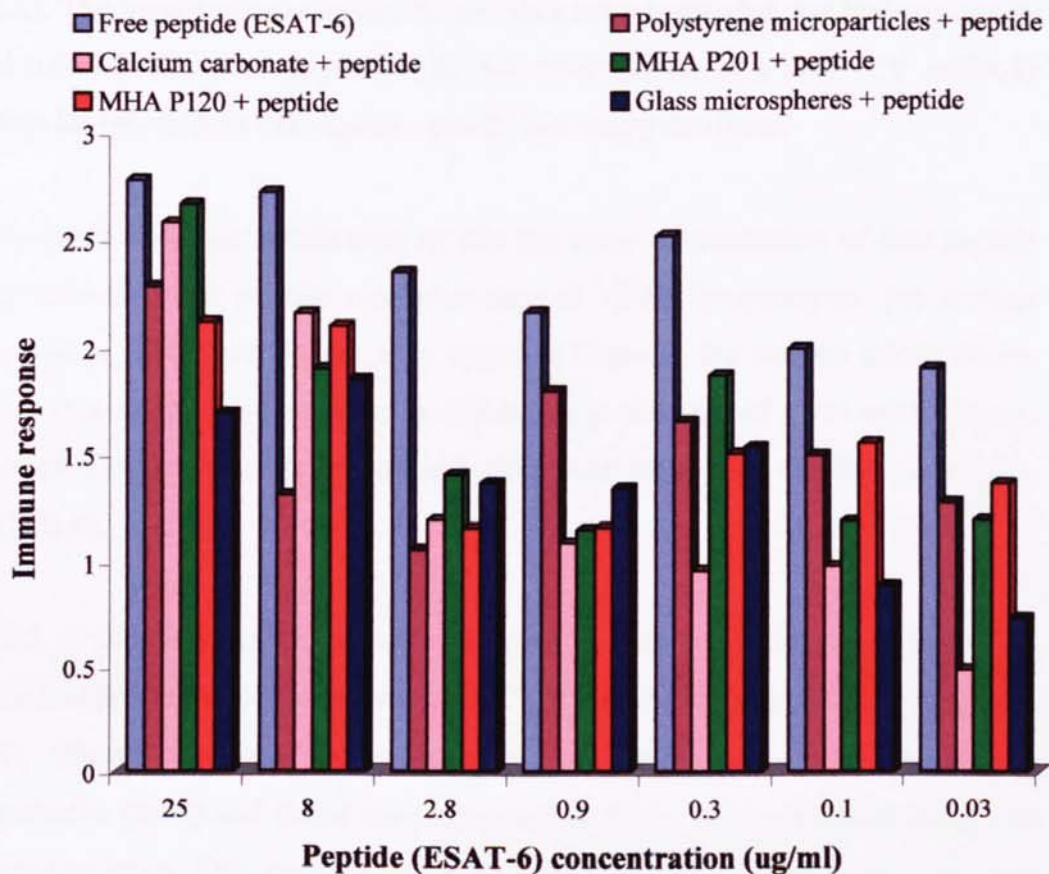


Since a high immune response was achieved with the free peptide at low concentrations, there is uncertainty as to whether the peptide bound on the microparticles is inducing the response or the free peptide released from the surface of the microparticles is inducing the response (Table 4.1). The results do show

however that the microparticles are not toxic to the cells and an immune response is produced.

Microparticle-adsorbed antigens are finding increasing usage in diagnostic kits. Yamamto *et al* (2000) have used hydroxyapatite-coated nylon beads as a new reagent for detecting Japanese encephalitis virus (JEV)-specific human antibodies. In their study, IgG concentrations were measured using ELISA microplates. They compared IgG production using hydroxyapatite coated nylon and JEV antibodies adsorbed with the conventional method using hemagglutination-inhibition assay (HIA), neutralisation assay (NTA) and enzyme-linked immunosorbent assay (ELISA). The results were comparable and thus they concluded that hydroxyapatite coated nylon beads were applicable in the development of a new JEV antibody detection kit which does not require specific laboratory facilities.

The Bovigam assay was performed so that the same concentration of free peptide and particle-adsorbed peptide was presented to blood lymphocytes. No distinct differences in IFN-γ production were apparent between the various microparticle systems. One might have expected a difference in the case of glass microspheres because of the high peptide release expected over stage 1 of the Bovigam assay (See Fig 4.3).

The size of the polystyrene particles (1.7μm) is similar to that of *M.bovis* and *M.tuberculosis* (2μm) (Groves *et al.*, 1997). Wilkinson *et al* (2000) adsorbed cultures filtrate fractions from replicating *M.tuberculosis* onto polystyrene microparticles (2μm) and found the production of IFN-γ to be enhanced compared to soluble antigens. This positive result could be due to the different antigen used and the adsorption conditions. In the study by Wilkinson *et* al (2000) the culture filtrate fraction was taken from *M.tuberculosis* and not *M.bovis*. Also the polystyrene particles were washed with distilled water and resuspended in coating buffer containing 0.01M $NaH_2PO_4$, 0.9% NaCl, at pH 7.8. In the present study the conditioning and adsorption media consisted of PBS at pH 7.2.

Further work was performed to elucidate the relative contribution of free and bound peptide in stimulating lymphokine production in vitro in the Bovigam assay and to apply the findings to vaccine formulation. Table 4.1 shows the expected peptide release from the particle systems during stage 1 of the Bovigam assay (24h) using the data presented Figure 4.3.

**Table 4.1**  Expected peptide release (µg/ml) at the end of stage 1 of the BOVIGAM assay (24 hours)

| Microparticle | Diameter (µm) | Initial conc. of adsorbed peptide (µg/ml) | % (w/w) release during Bovigam assay | Peptide conc. released during assay (µg/ml) |
|---|---|---|---|---|
| Glass (CP03) | 2.2 | 25 | 60 | 15.0 |
| Calcium carbonate | 2.1 | 25 | 20 | 5.0 |
| MHA P201 | Submicron | 25 | 10 | 2.5 |
| MHA P120 | Submicron | 25 | 30 | 7.5 |
| Polystyrene | 1.7 | 25 | 15 | 3.8 |

## 4.2  BOVIGAM™ assay 2 IFN-γ production following presentation of free and polystyrene, CHA, MHA particle-bound peptide (ESAT-6,p45) to lymphocytes in a blood based assay

The % w/w peptide ESAT-6,p45 adsorbed on the different particle systems used in the second Bovigam assay are shown in table 4.2.

The polystyrene and CHA nanoparticles were included in the Bovigam assay to assess the influence of particle size on the interaction with blood lymphocytes and the immune response.

Following peptide adsorption for 2 hours, and washing, the particles were placed in PBS at 37°C for 8 hours to remove any loosely bound peptide. The adsorbed

peptide amounts were recalculated and the particles were incubated with blood samples taken from a naturally infected cow (CK556). This animal had been shown in previous work at VLA to respond well to Tuberculin derived peptide in the Bovigam assay. The volume of RPMI 1640 used to dilute the microparticle samples was adjusted to achieve equivalent doses of peptide.

**Table 4.2** Comparison of peptide (ESAT-6) adsorption onto microparticles and nanoparticles used in the BOVIGAM assay

| Particle system | % Peptide adsorption (w/w) | Particle size (nm) |
|---|---|---|
| **Microparticles** | | |
| Polystyrene | $8.9 \pm 0.15$ | 1667 |
| **Nanoparticles** | | |
| Carbonate Hydroxyapatite (CHA) | $4.9 \pm 0.12$ | 6 |
| Polystyrene | $9.2 \pm 0.07$ | 399 |
| Hydroxyapatite (MHA P201) | $3.0 \pm 0.12$ | Submicron |
| Hydroxyapatite (MHA P120) | $3.5 \pm 0.30$ | Submicron |

Figure 4.4 shows the production of IFN-γ following exposure of blood-based lymphocytes to particle-bound peptide (ESAT-6,p45). At high peptide concentrations (>10μg/ml) all the particle systems gave a higher immune response than the free peptide. At low peptide concentrations (<1μg/ml), which is where higher immune responses are required, the particles MHA P201, MHA P120 and CHA gave higher immune responses than the free peptide, with MHA P201 giving the overall best results. With the hydroxyapatite particles there is a large drop in IFN-γ production when the concentration of adsorbed peptide falls below 2.8μg/ml. With all the other particles the IFN-γ response tends to show a gradual decrease with decrease in peptide concentration. Although both polystyrene microparticles and nanoparticles displayed higher peptide adsorption (9%w/w) than the hydroxyapatite particles (3%w/w) they do not interact as effectively with the lymphocytes. According to Wilkinson *et al* (2000) particles less than 1/10th of the size of *M.tuberculosis* (approximately 200nm) do not induce an enhanced IFN-γ response compared to free peptide. In the investigations reported here the carbonate

hydroxyapatite particles (6nm) are less than 1/10th of the size of *M.bovis* and yet they give a better immune response than the free peptide. The favourable interaction between MHA and CHA nanoparticles and lymphocytes could recommend them for use as vaccine adjuvants for peptide antigens. Figure 4.4 also shows the MHA particles to be non-toxic to the blood cells and being biodegradable provide further advantages for vaccine formulation.

**Figure 4.4** Production of Bovine Gamma Interferon (IFN-γ) following exposure of blood-based lymphocytes to particle free peptide (ESAT-6,p45) and particle bound peptide
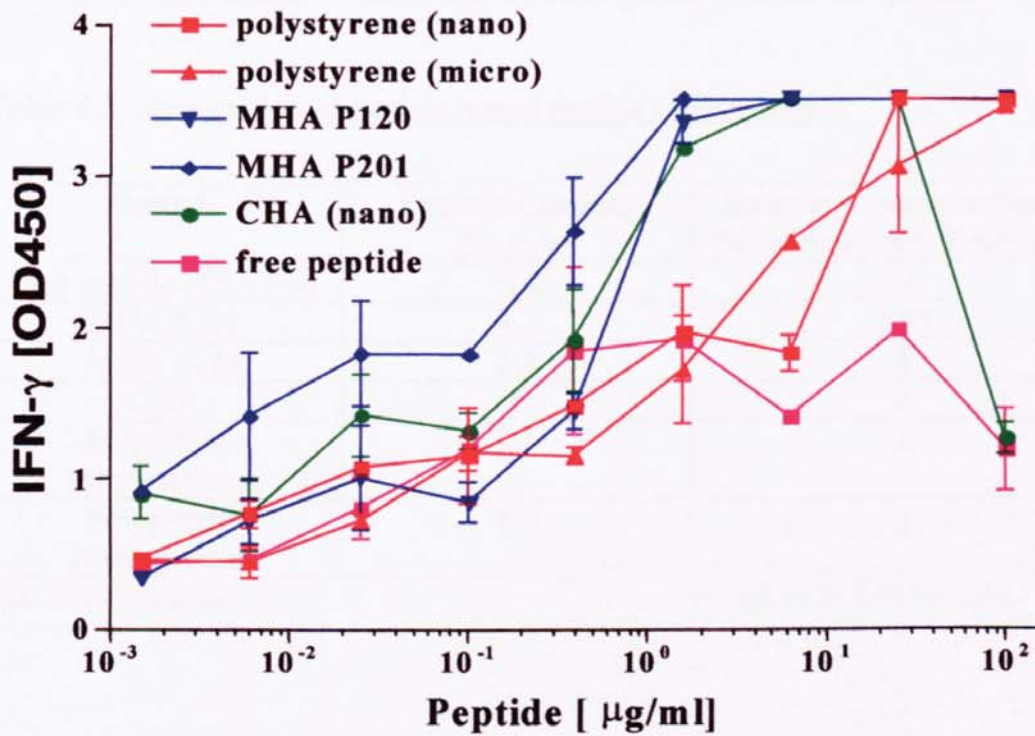


The immune response shown in figure 4.4 suggests that MHA P201 particles could be useful as a carrier for peptide antigens in the Bovigam assay for detecting TB in cattle. The prime objective was to improve the sensitivity of the Bovigam assay by allowing use of small amounts of peptide. Figure 4.4 shows that an adsorbed peptide (ESAT-6,p45) concentration as low as 0.03µg/ml could be used to distinguish between TB infected and non-infected animals using the Bovigam assay. This result also showed to be true in Table 4.3. We assessed whether

particle-bound peptide resulted in more efficient peptide recognition. This was determined by comparing the concentration necessary to achieve 50% of the maximum amount of IFN-$\gamma$ induced with free peptide (ESAT-6,p45). This is expressed as the inverse of the relative concentration needed to achieve 50% of the maximum response with free peptide (e.g. responses of free peptide = 1, more efficient recognition compared to free peptide < 1, less efficient > 1). Only when ESAT-6,p45 was adsorbed to the microparticle MHA P201 did we observe more efficient peptide recognition because we obtained responses equivalent to free peptide with a 25 times lower peptide concentration (Table 4.3). Although ESAT-6,p45 was still recognised when adsorbed to MHA P120, CHA and polystyrene particles, it was about 2 – 5 times less well recognised than the free peptide.

**Table 4.3** <u>Recognition of particle bound peptide (ESAT-6,p45)</u>

| Particle | Peptide Loading (% w/w) | Relative Concentration at 50% Maximum Response* |
|---|---|---|
| Free peptide (ESAT-6) | NA | NA |
| MHA P201 | 4.2 | 0.04 |
| MHA P120 | 3.8 | 2 |
| CHA | 5 | 2 |
| Polystyrene Microparticles | 7.1 | 2 |
| Polystyrene Nanoparticles | 8.3 | 5 |

NA, not applicable                                    *Relative to free peptide

## 4.3  Animal studies to determine the potential of particle-bound peptide (ESAT-6,p45) as a TB vaccine

Mouse studies were performed to determine if the particle systems could be used as a vaccine delivery system. PLA particles were included in the animal experiment since Vordermeier *et al* (1995) have shown them to be a good adjuvant, to deliver the *M.tuberculosis* 38kDa protein. In addition several other studies have used PLA particles to adsorb peptide and proteins as a vaccine delivery system (Vordermeier

146

*et al.*, 1995; O'Hagan *et al.*, 1993). PLA is a synthetic crystalline poly (α hydroxy acid), which had been shown to be non-toxic, non-irritant and biodegradable (Singh *et al.*, 1998). The advantages of synthetic polymers are reproducibility in terms of degradation rates and molecular weights. However PLA degrades more slowly than the copolymers of lactide and glycolide (>1 year) and is more suitable for adsorption of antigens.

Polylactide (PLA) particles were produced as described in the Methods and Materials section 2.3.1. Initially experiments were carried out to determine the loading and release profiles for OVA and peptide (ESAT-6,p45). Similar adsorption levels of OVA and peptide (ESAT-6,p45) onto PLA particles were obtained (Table 4.4).

The increase in pH of the adsorption medium resulted in a decrease in OVA and peptide adsorption. The results for OVA adsorption in this study are slightly higher than those achieved by Coombes *et al* (1998).

**Table 4.4**   Comparison of OVA and peptide (ESAT-6, p45) adsorption on PLA particles (10mg)

| Particle | pH of adsorption medium | % (w/w) Adsorption |
|---|---|---|
| PLA + OVA | 2.7 | 4.9 ± 0.3 |
| PLA + peptide (ESAT-6,p45) | 2.7 | 4.3 ± 0.3 |
| PLA + OVA | 7.4 | 3.5 ± 0.8 |
| PLA + peptide (ESAT-6,p45) | 7.4 | 3.3 ± 0.5 |

Protein adsorption onto particles is influenced by hydrophobic and electrostatic interactions Coombes *et al* (1998). A maximum in protein adsorption is generally reached under pH conditions close to its isoelectric point, where the net surface charge and electrostatic repulsion is at a minimum. It is at this pH that conformational stability of the adsorbed peptide or protein occurs, which leads to increased adsorption. The fact that OVA adsorption increases on lowering the pH to

2.7 suggests that the positively charged OVA molecules interact more effectively with the PLA particles. Similar behaviour is indicated for the (ESAT-6,p45) peptide.

Figure 4.5 represents release profiles of OVA and peptide (ESAT-6,p45) from PLA lamellae in PBS over 24 hours. There is an initial burst of OVA and peptide release, which is due to the decrease in adsorption with increase in ionic strength. It has also been documented that OVA adsorbs in a multi-layer model with the layers closest to the particle surface being more strongly bound than the outer layers of OVA. Hence it is these outer layers that are removed by washing after each time point. It is considered that the protein molecules adsorbed directly onto the PLA lamellae are strongly retained by hydrophobic interactions and the outer layers are bound by electrostatic forces, thus making them susceptible to pH changes. This mechanism may also apply to peptide release particularly the initial burst phase. From the results in Figure 4.5 it is also apparent that the higher the pH of the adsorption media the lower is the release. This behaviour could be due to loss of weakly bound protein or peptide. There is no levelling off of the release curve for peptide or OVA as was noticed when performing release studies using polystyrene, hydroxyapatite, glass and calcium carbonate particles. The retention properties over 24 hours are generally good for the PLA particles, approximately 75 to 90%. Venkataprasad *et al* (1999) and Singh *et al* (1998) have also showed the good retention properties of PLA particles towards adsorbed antigens of M.tuberculosis for vaccine delivery.

PLA particles produced and the peptide added with the adsorption media at pH 2.7 was chosen. This was because it adsorbed slightly larger amounts of peptide and the release profile even though it released more peptide was negligible. However for a vaccine delivery system this is ideal, the release of peptide together with what is bound onto the PLA particles can create an adjuvant effect. PLA has been used extensively as a potential vaccine delivery system and this is represented in publications (Singh *et al.*, 1998; Coombes *et al.*, 1998).

**Figure 4.5**   Desorption of OVA and peptide (ESAT-6,p45) from PLA lamellae in PBS at 37°C
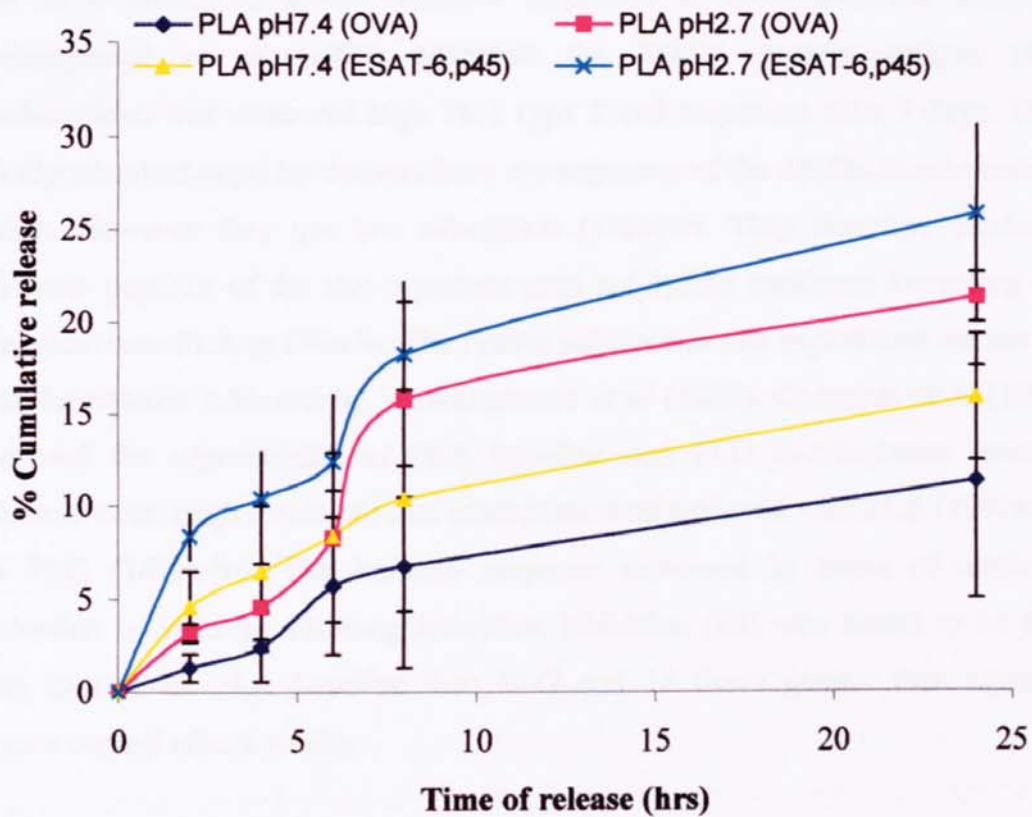


Due to a limited amount of peptide available a single experiment was performed using the following particle adsorbed peptide systems (Polystyrene microparticles 6.5%, polystyrene nanoparticles 8.2%, CHA 2.5%, MHA P201 2.0%, MHA P120 1.9%, PLA 4.1%). The peptide (ESAT-6,p45) emulsified in incomplete Freund's adjuvant (IFA) gave the highest response compared to the other particle carriers (Fig 4.6). IFA is a very strong adjuvant and is used as the benchmark for other potential vaccine delivery candidates (Singh *et al.*, 1998; Venkataprasad *et al.*, 1999). The fact that PBS emulsified with IFA gave very little response indicates that it is the peptide, which interacts with the lymphocytes to produce the response. The response is expressed in terms of stimulation indices (c.p.m with peptide/c.p.m without peptide).

149

The particle adsorbed peptide systems in general gave a very poor response, which was just about detectable. The results do not coincide with those achieved using PLA as a carrier to induce immune responses to other adsorbed antigens. Venkataprasad *et al* (1999) adsorbed the 38kDa protein antigen from *M.tuberculosis* and measured high Th-1 type T cell responses after 7 days. They initially adsorbed peptides derived from the sequence of the 38kDa *M.tuberculosis* protein. However they got low adsorption (1%w/w). They therefore produced polymeric peptides of the above protein onto polylysine backbone increasing the adsorption onto PLA to 6%w/w. The results achieved in this experiment are not far from the 6%w/w achieved by Venkataprasad *et al* (1999). Coombes *et al* (1999) compared the adjuvanticity of PLA lamellae and PLG microspheres towards influenza virus. High levels of virus adsorption were achieved with PLA (19%w/w) and PLG (16%w/w). The immune response measured in terms of antibody production to virus by Haemagglutination inhibition (HI) was found to be five times greater for PLA lamellae than PLG and 14 times greater than aqueous vaccine control after 8 weeks.

**Figure 4.6** Mouse proliferation experiments using peptide (ESAT-6,p45)



150
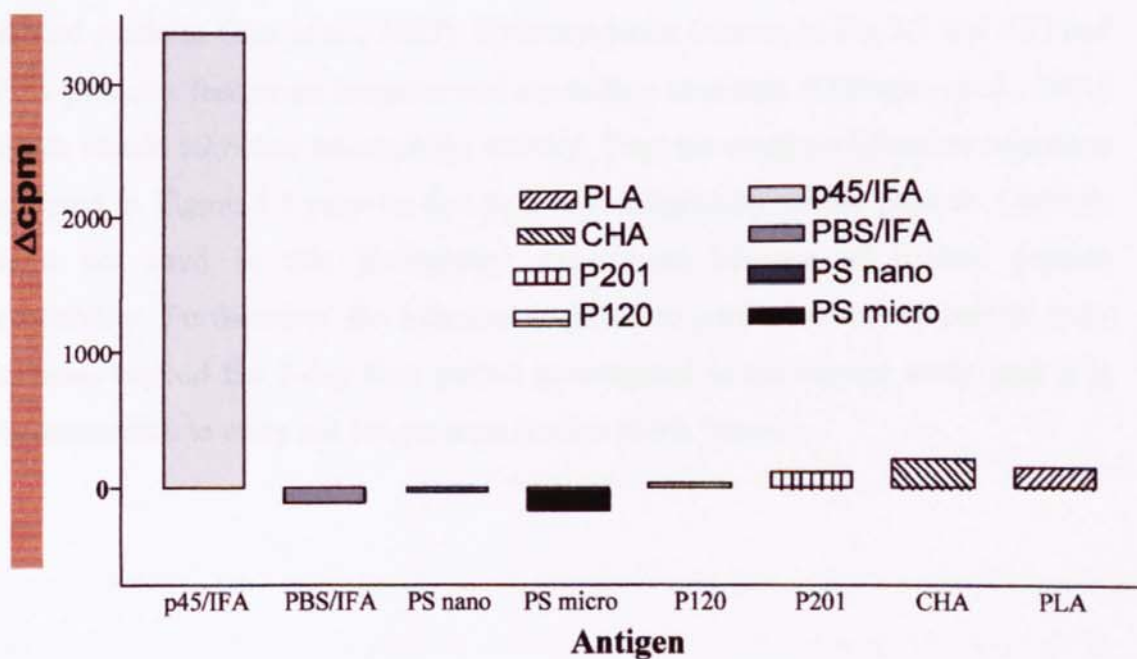
The poor results achieved with the particle-adsorbed peptide vaccines in the present study may be due to the release of large amounts of peptide (ESAT-6,p45) leaving insufficient to interact with the immune cells such as macrophages. The peptide retention properties are in general high for the particles tested (75% adsorbed, Fig 3.12, 3.13, 4.5). However the mice were tested in vitro for T cell responses 7 days after injection while the release studies in comparison were only performed over 24 hours.

Adjuvants tend to work as immunological stimulants due to their capacity to retain antigens, attract and activate macrophages and other immune cells and by modifying the release of cytokines capable of influencing the overall immune response (Coombes *et al.*, 1999). The phagocytic process is probably the most important as it results in antigen transport to the lymph nodes where antigen processing and presentation to T-helper cells occur (Wilkinson *et al.*, 2000). If peptide is lost from the particle surface before particle uptake by phagocytosis there will be less peptide to present to T-helper cells, thus giving a poor immune response. Good peptide retention was demonstrated over short 24h timescales in-vitro by the particle systems used in the vaccine study (Fig 3.12, 3.14 & 4.5). It has also been reported that macrophage activation is readily induced by irregular shaped particles (Yin *et al.*, 2002). Hydroxyapatite (shown in Fig 3.2 and 3.3) and PLA particles feature an irregular and crystalline structure, (O'Hagan *et al.*, 2001) which should stimulate macrophage activity. Thus the small proliferation responses reported in Figure 4.5 may be due to a low antigenicity of the peptide. Controls were not used in this preliminary experiment because of limited peptide availability. Furthermore the immune response to particle-adsorbed peptide may increase beyond the 7-day time period investigated in the current study, and it is recommended to carry out longer-term studies in the future.

## 4.4    Skin test

The skin test is regularly performed in animals to determine whether they are infected with TB. The skin test is performed in conjunction with the Bovigam assay. Hypersensitivity to the organism is detected by injecting a purified fraction of the tuberculin fluid (purified protein derivative obtained from *M.bovis*, PPD-B) into the skin. In infected animals T cells stimulate a local inflammatory response over 1 – 3 days. The T cells responsible for the delayed reaction have been specifically sensitised by a previous encounter and thus they attract macrophages and other lymphocytes to the site of reaction (Section 1.6). A positive result is declared if the difference between skin folds measurements pre-injection and three days after injection is greater than 4mm.

**Figure 4.7**    Median measurements (mm) taken from folds of skin at day 1 and 3 using a) peptide (ESAT-6,p45) bound to MHA P201 particles, b) free polymeric peptide and c) polymeric peptide bound to MHA P201 particles compared to PPD-B in cattle known to have TB
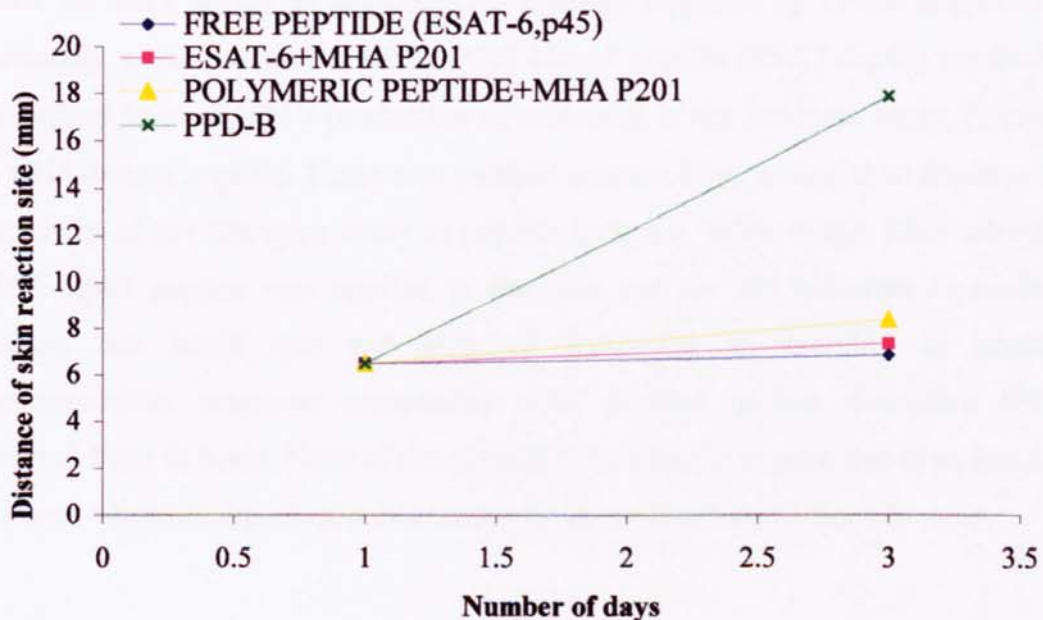


Only PPD-B gave a positive skin-test result in infected animals (3 cows). Hydroxyapatite MHA P201 particles were used as the peptide carrier because of the

high IFN-γ responses to peptide (ESAT-6,p45) achieved using the Bovigam assay. However MHA-bound peptide (ESAT-6,p45) did not induce a positive skin test. A higher response was achieved relative to free peptide (ESAT-6,p45). MHA P201 loaded with polymeric peptide did give a higher response than that achieved with free peptide (ESAT-6,p45) alone or ESAT-6,p45 peptide-bound onto MHA P201. However neither system produced a positive skin test result.

The processing and presentation of particle-bound peptide by APCs appears ineffective for stimulation of skin test responses (Fig 4.7). Peptide destruction during phagocytosis may account for the poor immune response.

## 4.5  Conclusions

Peptide (ESAT-6,p45) was adsorbed onto particle carriers MHA P201, MHA P120, CHA, glass CP03, calcium carbonate and polystyrene micro/nanoparticles and tested for their ability to stimulate an immune response by blood lymphocytes previously sensitised to TB. MHA P201-bound peptide (ESAT-6,p45) resulted in the highest level of IFN-γ production as measured in the Bovigam assay, followed by CHA-bound peptide. These two particle systems have potential to improve the sensitivity of the Bovigam assay to peptide antigens. When MHA P201 adsorbed ESAT-6,p45 peptide was applied in the skin test for TB infection in cattle, a positive test result was not achieved indicating an inability to produce hypersensitivity reactions comparable with purified protein derivative (PPD) obtained from *M.bovis*. None of the peptide-bound particles gave rise to an immune response when administered subcutaneously as vaccine formulations in mice.

# CHAPTER 5

# FORMULATION OF BCG-LOADED
# MATRICES FOR ORAL VACCINE
# DELIVERY: PREFORMULATION STUDIES

# 5 FORMULATION OF BCG-LOADED MATRICES FOR ORAL VACCINE DELIVERY: PREFORMULATION STUDIES

## INTRODUCTION

The most important requirements for a BCG vaccine are, the immunogenicity of the strain, a high proportion of live to dead bacilli and adequate dispersion. The immunogenicity of the BCG strain can be attributed to the viability and stabilisation during storage. New technologies to overcome aggregation have been developed such as cultivation of M.bovis BCG in bioreactors (Dietrich *et al.*, 2002). This method was capable of producing dispersed cultures of BCG in synthetic media in small-scale bioreactors producing single bacilli with a high degree of replication and stability than the conventional steel ball milling method. There are two main procedures of stabilisation that are commonly used due to the production of BCG via bioreactors and not by the conventional ball milling. Complete survival has been obtained when BCG (Pasteur) vaccine was frozen in glycerol solutions and stored for many years at −79°C (Devadoss *et al.*, 1991). More than 50% of the live bacilli are killed after freeze-drying but the remaining live bacilli are preserved at -30°C for twenty years or one year at 4°C. However the problem remains that more than half the BCG bacilli die and thus the immunogenicity of the vaccine will be reduced. A larger dose may therefore have to be administered to induce a reasonable immune response. Ratliff *et al* (1994) also noted a time dependent aggregation of BCG after reconstitution. Generally on reconstitution after storage, the vaccine is used immediately so that clumping does not occur and further loss in viability is avoided. The FDA (Gheorghiu *et al.*, 1988) has approved a time limit of 2 hours for storage of diluted vaccines prior to cancer treatment to preserve viability and hence immunogenicity. Ratliff *et al* (1994) noted that aggregation over time had no effect on c.f.u's but adversely affected antitumour activity.

BCG is a very complicated organism. In order to be formulated into an effective oral vaccine, BCG has to be prevented from aggregating to enhance interaction with mucosal surfaces. Aggregation of BCG is a major problem in general usage, explaining the addition of surfactants to the suspension of the bacteria used in vaccines. The addition of surfactants cetylpyridinium chloride and sodium tauroglycolate prevents aggregation of BCG (Zhang *et al.*, 1988). This is important as aggregation may prevent uptake of BCG via the M-cells in the peyer's patches, where the size is required to be between 2-5µm. If aggregation does occur there is a chance that the BCG bacilli will be too large to be taken up by the M-cells. In addition a false viable count can be recorded because a clump could comprise many BCG bacilli but will only be read as one colony. Thus a measured BCG concentration could be 10 to 100 fold less than the actual value.

## 5.1 Results and Discussion

### 5.1.1 Redispersion characteristics of BCG after centrifugation

The data presented in column iii of table 5.1 indicate that the most suitable excipients for redispersing BCG–Pasteur are SDS and the Tweens 20, 80. Tween 80 resulted in the best redispersion characteristics, as indicated by the data presented in column (v). This particular surfactant was capable of redispersing BCG after centrifugation of a suspension comprising water in most part.

The results in column (iii) of table 5.2 indicate that BCG-Tokyo only redisperses after centrifugation when Tween 20 is used as the dispersant.

Suspending agents are added to BCG cultures whether growing them or formulating them into vaccines to prevent aggregation of BCG and to prevent to some extent death of these cells during culturing (Milstien *et al.*, 1990).

Aggregation influences the immunogenicity of the BCG vaccine and it will also affect the exact quantification and quality (Milstien *et al.*, 1990).

**Table 5.1**  A comparison of the redispersion characteristics of BCG-Pasteur after centrifugation

| Absorbance at 470nm | | | | | | |
|---|---|---|---|---|---|---|
| Suspending Agent (SA) | Molecular weight (MW) | 100µl BCG + 900µl SA (i) | Supernatant (S/N) following centrifugation (ii) | BCG sediment + 1ml SA (iii) | S/N + residual BCG in Eppendorf (iv) | BCG + 1ml SA (v) |
| Distilled Water | | 0.209 ± 0.13 | 0.102 ± 0.12 | 0.052 ± 0.03 | 0.083 ± 0.07 | 0.078 ± 0.08 |
| PBS | | 0.198 ± 0.02 | 0.122 ± 0.02 | 0.068 ± 0.04 | 0.091 ± 0.06 | 0.080 ± 0.03 |
| PVP | 10000 | 0.221 ± 0.03 | 0.060 ± 0.05 | 0.070 ± 0.05 | 0.059 ± 0.08 | 0.064 ± 0.02 |
| Dextran | 60000-90000 | 0.261 ± 0.03 | 0.206 ± 0.05 | 0.095 ± 0.02 | 0.233 ± 0.03 | 0.079 ± 0.08 |
| PEG | 10000 | 0.180 ± 0.04 | 0.150 ± 0.08 | 0.051 ± 0.06 | 0.181 ± 0.06 | 0.045 ± 0.04 |
| Glucose | 180.6 | 0.237 ± 0.01 | 0.134 ± 0.22 | 0.063 ± 0.01 | 0.109 ± 0.03 | 0.086 ± 0.05 |
| SDS | 288.4 | 0.227 ± 0.03 | 0.025 ± 0.04 | **0.143 ± 0.06** | 0.041 ± 0.01 | 0.085 ± 0.03 |
| Tween 20 | | 0.204 ± 0.08 | 0.060 ± 0.05 | **0.137 ± 0.09** | 0.067 ± 0.04 | 0.060 ± 0.07 |
| Tween 80 | | 0.222 ± 0.04 | 0.039 ± 0.07 | **0.142 ± 0.05** | 0.051 ± 0.03 | **0.144 ± 0.02** |
| F-68 | | 0.248 ± 0.09 | 0.122 ± 0.03 | 0.087 ± 0.04 | 0.126 ± 0.06 | 0.081 ± 0.04 |

157

**Table 5.2** A comparison of the redispersion characteristics of BCG-Tokyo after centrifugation

| Absorbance at 470nm | | | | | | |
|---|---|---|---|---|---|---|
| Suspending Agent (SA) | Molecular weight (MW) | 100µl BCG + 900µl SA (i) | Supernatant (S/N) following centrifugation (ii) | BCG sediment + 1ml SA (iii) | S/N + residual BCG in Epindorf (iv) | BCG + 1ml SA (v) |
| Distilled Water | | 0.169 ± 0.04 | 0.122 ± 0.08 | 0.020 ± 0.03 | 0.095 ± 0.02 | 0.049 ± 0.05 |
| PBS | | 0.162 ± 0.12 | 0.033 ± 0.02 | 0.058 ± 0.04 | 0.032 ± 0.02 | 0.053 ± 0.06 |
| PVP | 10000 | 0.165 ± 0.05 | 0.071 ± 0.06 | 0.018 ± 0.08 | 0.056 ± 0.04 | 0.065 ± 0.02 |
| Dextran | 60000-90000 | 0.168 ± 0.06 | 0.044 ± 0.03 | 0.062 ± 0.01 | 0.049 ± 0.07 | 0.067 ± 0.06 |
| PEG | 10000 | 0.152 ± 0.08 | 0.050 ± 0.04 | 0.046 ± 0.02 | 0.045 ± 0.04 | 0.084 ± 0.04 |
| Glucose | 180.6 | 0.162 ± 0.05 | 0.085 ± 0.06 | 0.037 ± 0.05 | 0.085 ± 0.06 | 0.049 ± 0.08 |
| SDS | 288.4 | 0.162 ± 0.03 | 0.046 ± 0.03 | 0.037 ± 0.0 | 0.050 | 0.082 |
| Tween 20 | | 0.154 ± 0.03 | 0.024 ± 0.04 | **0.125 ± 0.05** | 0.034 ± 0.04 | 0.071 ± 0.03 |
| Tween 80 | | 0.160 ± 0.09 | 0.083 ± 0.03 | 0.068 ± 0.02 | 0.086 ± 0.08 | 0.071 ± 0.03 |
| F-68 | | 0.161 ± 0.04 | 0.067 ± 0.03 | 0.063 ± 0.07 | 0.071 ± 0.04 | 0.054 ± 0.07 |

Tween 80 is one of the most common suspending agents used for formulating BCG vaccines (Gheorghiu *et al.*, 1988; Ghoerghiu *et al.*, 1996). Other suspending agents

used are Triton WR 1339, Tween 20 and n-octyl-Glucopyranoside. On the basis of past work and the results presented in Table 5.1 Tween 80 was incorporated in the matrix systems investigated for oral delivery of live BCG Pasteur.

### 5.1.2  Redispersion of BCG after freeze-drying using various surfactants

Freeze-drying has been shown to cause cell death due to clumping of BCG bacteria (Ratliff *et al.*, 1994) and thus this stage has been a stumbling block for many to achieve high levels of dispersed and viable BCG for vaccination purposes.

The results in Table 5.3 and 5.4 suggest that BCG–Pasteur and Tokyo redisperse well after freeze-drying in the presence of the suspending agents listed. Thus freeze-drying was considered to present a useful processing stage for obtaining dispersions of BCG for production of oral delivery systems. The results also indicate that the suspending agents may be generally useful for preventing BCG clumping during 4 hours retention at -20°C.

**Table 5.3**    Sample absorbance after redispersion of freeze dried BCG-Pasteur using various suspending agents

| Suspending agents (SA) | Absorbance of BCG suspensions at 470nm | |
|:---:|:---:|:---:|
| | Before freeze drying | After freeze drying |
| PVP | 0.221 ± 0.05 | 0.236 ± 0.03 |
| Dextran | 0.261 ± 0.04 | 0.212 ± 0.04 |
| F-68 | 0.248 ± 0.01 | 0.180 ± 0.08 |
| SDS | 0.227 ± 0.08 | 0.213 ± 0.04 |
| PEG | 0.150 ± 0.12 | 0.254 ± 0.04 |
| Glucose | 0.237 ± 0.09 | 0.232 ± 0.04 |
| Distilled Water | 0.198 ± 0.06 | 0.187 ± 0.08 |
| Tween 20 | 0.208 ± 0.05 | 0.217 ± 0.02 |
| Tween 80 | 0.231 ± 0.05 | 0.243 ± 0.06 |

**Table 5.4**  Sample absorbance after redispersion of freeze-dried BCG-Tokyo using various suspending agents

| Suspending agents (SA) | Absorbance of BCG suspensions at 470nm | |
|---|---|---|
| | **Before freeze drying** | **After freeze drying** |
| PVP | $0.165 \pm 0.04$ | $0.154 \pm 0.08$ |
| Dextran | $0.168 \pm 0.06$ | $0.170 \pm 0.02$ |
| F-68 | $0.161 \pm 0.01$ | $0.151 \pm 0.08$ |
| SDS | $0.162 \pm 0.04$ | $0.151 \pm 0.02$ |
| PEG | $0.152 \pm 0.08$ | $0.159 \pm 0.02$ |
| Glucose | $0.162 \pm 0.03$ | $0.116 \pm 0.04$ |
| Distilled Water | $0.169 \pm 0.07$ | $0.125 \pm 0.07$ |
| Tween 20 | $0.154 \pm 0.02$ | $0.149 \pm 0.06$ |
| Tween 80 | $0.160 \pm 0.02$ | $0.170 \pm 0.05$ |

For further studies regarding incorporating BCG into Eudragit matrices only BCG Pasteur was carried forward. BCG Pasteur was carried forward because it was the reference strain used in the current DEFRA funded cattle vaccine study and it is the reference strain used in the WHO vaccine development programme for human tuberculosis.

## 5.2 BCG-loaded matrix formulation involving organic solutions of enteric polymers

Enteric coatings are widely used in the pharmaceutical industry (Carino *et al.*, 1999) and have been extensively used in attempts to deliver proteins, peptides and bacteria. Eudragit has been used as an enteric coating delivery system for delivering insulin (Dietrich *et al.*, 2002). CAP and Eudragit have both been used for delivering trypsin inhibitor, growth factors and trefoil peptides (Gheorghiu *et al.*, 1996). These enteric coating systems have been shown to be very good at protecting proteins and peptides against the harsh acidic conditions of the stomach. Yet to achieve therapeutic levels they must disintegrate completely in the small intestine, which has an alkaline environment, and release the protein or peptide effectively so that adsorption can occur. Enteric coating systems such as those mentioned above have

also been used in conjunction with biodegradable microparticles in order to enhance local and systemic immune responses when delivering antigens (Gheorghiu *et al.*, 1996).

### 5.2.1 Production and disintegration behaviour of CAP matrices

Neither CAP nor Eudragit are soluble in water. Formulations of freeze-dried powders for compaction were investigated by mixing acetone solutions of CAP or Eudragit with various surfactants (0.1%w/v) (See section 2.4.3.1). After the acetone evaporated the resulting mixture of enteric coating polymer and surfactant tended to clump together. Tablets in the forms of discs were produced via compression of freeze-dried material.

**Table 5.5** Comparison of macroscopic properties of CAP tablets

| Surfactant/polymer | Weight of CAP | | |
|---|---|---|---|
| | 50mg | 100mg | 200mg |
| PEG | A | D | D |
| Water | A | D | D |
| Dextran | A | D | F |
| Glucose | B | D | F |
| SDS | C | D | F |
| PBS | C | E | C |
| Tween 80 | C | E | D |
| F-68 | C | E | C |
| PVP | C | E | C |

KEY: -
A = Fairly well compacted, a little broken round the sides. Top surface of tablet not uniform, layered structure. B = Very well compacted and complete tablet obtained. C = Fairly well compacted, edges and sides tended to flake. D = Crystalline in appearance, formed layers, but compact. E = Particles stuck to the bottom and sides of tube. Crystalline in appearance, formed layers, broken round the sides. F = Stuck to KBr discs of compression mould, therefore no tablets produced

The freeze-drying step was increased to 16 hours from 4 hours. The preliminary results did however show glucose to form the best tablet at low powder weight.

Tablet compression involving 50mg of CAP powder tended to produce tablets only 1mm in thickness, which was very fragile with a lot of the tablets crumbling around the sides.

The preliminary results using CAP showed the tablets formed to be fragile, particularly around the edges, and to be crystalline in appearance. The surface of the tablet formed a layered structure (Table 5.5). The size of the tablets produced ranged from 13mm x 1mm for 50mg tablet weight to 13mm x 2mm for 200mg tablet weight.

Increasing the weight of the CAP powder to 100 and 200mg respectively resulted in tablets being formed with reduced fragility. The best tablets formed were those containing the surfactants Tween 80, PEG, PVP.

A potential problem with formulation of BCG-loaded CAP matrices using organic solvents is that the acetone may not be completely evaporated and the BCG incorporated into the formulation will be killed or the viability reduced. However Amorim *et al* (2001) showed that encapsulating Casein did not alter the biological activity. Marvola *et al* (1999) also showed that encapsulating Ibuprofen and Furosemide using CAP did not affect the therapeutic values obtained using CAP compared to Eudragit and Hydroxypropyl methylcellulose acetate succinates. CAP is insoluble in water and hence has to be dissolved in acetone.

CAP matrices produced to weights of 200mg were chosen for preliminary disintegration studies using 0.1M HCl pH 1.2 and PBS solution pH 7.2 at 37°C to mimic conditions of the stomach and small intestine. Tablets were added separately to each medium.

The results presented in Table 5.6 reveal that PVP, PEG and Tween 80, which resulted in good tablet compression, also gave rise to good dissolution properties when added to HCl and PBS. Taking the suspending properties of surfactants into

account when dispersing BCG, Tween 80 seems to perform the best. The CAP/Tween 80 matrices hold together for 2 hours in SGF and disintegrate in PBS, which mimics the required action in the small intestine.

**Table 5.6**  Comparison of weights of CAP matrices before and after immersion in SGF or SIF

| Surfactant/ polymer | Weight before addition to HCl (mg) | Weight after addition to HCl for 2hrs (mg) | Weight before addition to PBS (mg) | Weight after immersion in PBS for 4hrs (mg) |
|---|---|---|---|---|
| PEG | $182.4 \pm 12.3$ | $143.3 \pm 18.5$ | $193.3 \pm 14.2$ | $57.2 \pm 12.4$ |
| Water | $193.2 \pm 16.3$ | All dissolved | $188.2 \pm 23.4$ | All dissolved |
| Dextran | $187.1 \pm 23.6$ | $123.1 \pm 8.3$ | $193.2 \pm 7.1$ | $62.4 \pm 11.4$ |
| Glucose | $176.4 \pm 23.2$ | All dissolved | $203.1 \pm 11.3$ | $72.3 \pm 9.4$ |
| SDS | $186.2 \pm 8.5$ | $90.2 \pm 15.5$ | $188.5 \pm 12.6$ | All dissolved |
| PBS | $178.5 \pm 10.2$ | All dissolved | $182.7 \pm 7.4$ | $69.1 \pm 11.6$ |
| Tween 80 | $195.8 \pm 15.3$ | $169.3 \pm 6.5$ | $186.2 \pm 13.5$ | All dissolved |
| F-68 | $190.9 \pm 8.4$ | All dissolved | $193.4 \pm 11.5$ | All dissolved |
| PVP | $187.2 \pm 16.4$ | $149.3 \pm 5.5$ | $189.1 \pm 9.4$ | $72.4 \pm 4.5$ |

### 5.2.2  Production and disintegration properties of Eudragit S100 matrices

The use of solutions of CAP and Eudragit in acetone presented a problem because the addition of the aqueous surfactant to the polymer solution formed a precipitate. This restricts uniform mixing of the enteric coating polymers and the surfactants. This precipitation behaviour may lead to the formation of layered tablet surfaces and inadequate binding, which may lead to fragility.

Eudragit was a better enteric polymer to work with than CAP. It was easier to dissolve in acetone and overall the tablets produced were of better quality than those obtained using CAP. The compressed Eudragit matrices produced using the

surfactants and polymers listed in Table 5.5 were all very well compacted but displayed a layered surface structure. Some of the tablets tended to flake or were broken around the sides.

### 5.2.3 Formulation of BCG-loaded Eudragit matrices using aqueous dispersions

Acetone is likely to be toxic to BCG and may not be removed completely from matrices. Therefore dispersion of Eudragit powder, in PBS and water was investigated for production of powders suitable for compression into matrices.

Eudragit S100 powder (200mg) did not disperse well in water (1ml and 2ml). The majority of the powder tended to sediment and clump together. However Eudragit S100 powder did disperse satisfactorily in 2ml PBS and without too much sedimentation. Gentle shaking easily dispersed the sediment. PBS solution, pH of 7.4, was employed to disperse Eudragit powder prior to BCG addition. The pH of Eudragit S 100 (200mg) in 2ml PBS is approximately 5.0 and the addition of the surfactants and polymer was found to have no influence on pH. The matrices produced by compression of freeze-dried powders obtained by dispersing 200mg Eudragit powder in 2ml PBS containing 0.9ml surfactant and polymer, were similar to those produced using acetone (Table 5.6). However there was no visible surface cracks or surface layers as noted with tablets produced from powders formed using CAP and Eudragit solutions in acetone.

The powder produced after freeze-drying aqueous Eudragit dispersions was much finer than CAP and Eudragit prepared from acetone solution. The tablets produced were as a result more uniform and more compact. Disintegration and dissolution studies in SGF and SIF were performed using Eudragit S100 matrices produced from aqueous dispersions (Table 5.7). A single tablet was placed in 10ml of either SGF or SIF solution in three separate 100ml polypropylene tubes.

164

**Table 5.7** Comparison of Eudragit S100 tablets produced from aqueous dispersions before and after immersion in SGF or SIF

| Surfactant/ polymer | Weight before addition to HCl (mg) | Weight after immersion in HCl for 2hrs (mg) | Weight before addition to PBS (mg) | Weight after immersion in PBS for 4nrs (mg) |
|---|---|---|---|---|
| PEG | 172.2 ± 8.4 | 83.3 ± 12.5 | 183.5 ± 12.7 | 73.2 ± 14.6 |
| Water | 193.4 ± 16.7 | All dissolved | 198.1 ± 7.5 | All dissolved |
| Dextran | 197.8 ± 23.3 | 143.2 ± 18.6 | 188.3 ± 7.4 | All dissolved |
| Glucose | 178.4 ± 13.8 | 156.8 ± 3.7 | 178.4 ± 17.5 | 83.5 ± 9.6 |
| SDS | 182.5 ± 4.7 | 110.5 ± 15.3 | 179.4 ± 10.6 | All dissolved |
| PBS | 188.4 ± 14.5 | All dissolved | 190.5 ± 10.4 | 53.4 ± 5.2 |
| Tween 80 | 195.4 ± 10.5 | 179.4 ± 6.6 | 188.3 ± 15.2 | All dissolved |
| F-68 | 170.4 ± 11.5 | All dissolved | 193.5 ± 8.7 | All dissolved |
| PVP | 189.4 ± 16.6 | 139.5 ± 8.7 | 180.3 ± 8.2 | All dissolved |

The results presented in Table 5.7 suggest that Eudragit S100 matrices containing, Dextran, SDS, Tween 80 or PVP show potential for protecting BCG in SGF and releasing BCG in SIF. PEG is used as a plasticizer in coatings applied to protect drugs, proteins, and peptides from the gastric juices and the acidic pH of the stomach as well as to improve the release characteristics of the enteric coating polymer.

The addition of PEG to Eudragit coatings has been found to increase the permeability of the coating and hence influences the drug release in proportion to the percentage of PEG present (Jenquin *et al.*, 1992). Breitkreutz *et al* (2000) found that 6 out of 10 tablets coated with Eudragit/PEG disintegrated in HCl within half an hour. The present results also show that matrices containing PEG lose around 50% of the initial weight within 2 hours in HCl. The PEG penetration into the Eudragit coating interferes with the hydrogen bonds and hydrophobic interactions, which subsequently allows HCl to enter and cause disintegration (Breitkreutz *et al.*, 2000). Interestingly, PEG restricts dissolution of the Eudragit matrices in PBS suggesting a binding effect.

## 5.3 Conclusion

Pre-formulation was carried out on the stabilisation of aqueous suspensions of BCG using various surfactants (see section 5.2). The aim of these studies was to prevent BCG from aggregating readily into 'cord formations' (Devadoss et al., 1991) or strings of bacteria via the 'cord factor' Trehalose dimycolate (Gheorghiu et al., 1996) during formulation. If this occurs then active BCG is unlikely to be taken up by the Peyer's patches (sites of induction of the immune response) in the small intestine after oral vaccine delivery. Tween 80 was found to be the best surfactant for maintaining dispersion of BCG after preformulation stages involving centrifugation or freeze-drying (see section 5.2.2). However BCG is likely to be inactivated by acidic conditions. Development of an oral BCG formulation was therefore investigated, which would protect the vaccine during transit through the stomach and deliver active bacteria to sites of immune response in the G.I tract.

As a result of favourable powder compaction and matrix disintegration properties in SGF and SIF dispersion of Eudragit in PBS formed the basis of matrix formulations for oral delivery of BCG.

# CHAPTER 6


# EUDRAGIT FORMULATION OF BCG-LOADED EUDRAGIT MATRICES FOR ORAL VACCINE DELIVERY

# 6 FORMULATION OF BCG-LOADED EUDRAGIT MATRICES FOR ORAL VACCINE DELIVERY

## INTRODUCTION

Eudragit polymers are based on methacrylic acid and methyl methacrylate. The Eudragit grades presented in Table 6.1 were selected for investigation of matrix formulations suitable for delivery of dispersed BCG to the small intestine.

**Table 6.1** Characteristics of different grades of Eudragit

| Grade | Characteristics |
|---|---|
| Eudragit S 100 | Insoluble in water, in buffer solutions below pH 6.0 and in natural and artificial gastric fluids. It is soluble in the region of the digestive tract where the fluids are neutral to weakly alkaline, and in buffer solutions above pH 7.0. |
| Eudragit L30 D-55 | Based on methacrylic acid and ethyl acrylate. Aqueous dispersion contains 30 % w/w dry polymer substances. Soluble in intestinal fluids from pH 5.5 upwards. |
| Eudragit L 100 | Freely soluble above pH 6 |
| Eudragit L100-55 | Similar to Eudragit L100 except that it is soluble in aqueous solutions above pH 5.5. |

All the Eudragit grades selected were supplied in powder form except Eudragit L30 D-5.5, which is in suspension. This preparation was selected to try and improve the dispersion qualities in PBS. Eudragit L100 has been used extensively as an enteric coating system for oral delivery preparations (Amorim *et al.*, 2001; Breitkreutz *et al.*, 2000), as have Eudragit L30 D-55 and Eudragit L100-55 (Amorim *et al.*, 2001; Khan *et al.*, 1999; De Jaeghere *et al.*, 2000). Eudragit L30 D-55 and Eudragit L100-55 are soluble at low pH and which could result in release of BCG in the lower regions of the stomach (Lugosi *et al.*, 1992; Delgado *et al.*, 1999). Consequently the BCG may not reach the designated peyer's patches in the small intestine. The pH of Eudragit aqueous dispersions is acidic and thus has to be adjusted to maintain BCG viability. This adjustment could alter the disintegration and dissolution properties of the Eudragit matrix.

## 6.1 Disintegration and dissolution studies performed using Eudragit matrices

When Eudragit powder is added to PBS it does not dissolve but forms a fine, milky suspension. The pH of the final solution (5.0) is probably too low for the BCG to survive and thus was adjusted to pH 7 with 1M NaOH. Disintegration studies were subsequently performed to test whether the matrices produced after pH adjustment, freeze-drying and compression were resistant to low pH, yet dissolved in high pH solutions.

In order to gauge the effect of PBS concentration in Eudragit dispersions on the stability of the tablet, the concentration of PBS was altered in the formulation with and without pH adjustment to 7.

## 6.1.1 Disintegration Eudragit S100 matrices prepared from aqueous dispersions having different PBS concentrations and with pH adjusted to 7

The addition of Tween 80 to Eudragit S 100 aqueous dispersions without pH adjustment (Table 6.1) enhances matrix disintegration in both HCl and PBS at 37°C. The incorporation of 50% PBS in the aqueous dispersion counteracts the effect of Tween and matrix breakdown is reduced in HCl and PBS at 37°C.

Matrices produced from aqueous dispersions with pH adjustment to 7.0 all dissolved in PBS with the exception of the formulations containing 25% PBS.

**Table 6.2** Disintegration of Eudragit S 100 tablets (different concentration of PBS) in HCl or PBS after 2 hours incubation at 37°C (without pH adjustment)

| Composition of Eudragit S 100 dispersion | Disintegration medium | Initial tablet weight (mg) | Tablet weight after 2 hrs incubation (mg) |
|---|---|---|---|
| Eud S100 | HCl | 200.5 ± 10.2 | 175.3 ± 8.4 |
| Eud S100+PBS (100%) | | 200.4 ± 5.6 | 150.2 ± 10.7 |
| Eud S100+PBS (50%) | | 203.4 ± 6.8 | 165.3 ± 12.6 |
| Eud S100+PBS (25%) | | 203.4 ± 10.4 | 178.4 ± 8.6 |
| Eud S100+Tween 80 | | 194.4 ± 14.6 | Disintegrates almost immediately. Dissolved in 45 minutes. |
| Eud S100+PBS (100%)+Tween 80 | | 206.4 ± 6.1 | 113.3 ± 22.5 |
| Eud S100+PBS (50%)+Tween 80 | | 209.3 ± 15.1 | 156.3 ± 11.5 |
| Eud S100+PBS (25%)+Tween 80 | | Dissolved | |
| | | | |
| Eud S100 | PBS | 200.4 ± 8.7 | Dissolved |
| Eud S100+PBS (100%) | | 210.3 ± 12.7 | 160.2 ± 18.6 |
| Eud S100+PBS (50%) | | 210.3 ± 3.6 | 117.1 ± 12.8 |
| Eud S100+PBS (25%) | | 208.5 ± 6.4 | Dissolved |
| Eud S100+Tween 80 | | 204.2 ± 14.9 | Dissolved |
| Eud S100+PBS (100%)+Tween 80 | | 204.4 ± 12.4 | 175.3 ± 7.8 |
| Eud S100+PBS (50%)+Tween 80 | | 213.3 ± 11.4 | 148.3 ± 15.2 |
| Eud S100+PBS (25%)+Tween 80 | | 210.2 ± 9.5 | Dissolved |

The tablets produced after pH adjustments of dispersions were on the whole more stable in HCl than their counterparts without pH adjustment (Table 6.2). Considering the effect of PBS concentration in the tablets with and without pH adjustment, generally for PBS concentration of 50% and 100% there is a similar resistance to tablet degradation in HCl. The addition of Tween 80 has comparatively little effect on degradation resistance in HCl, which is advantageous

for BCG incorporation. If the PBS concentration is decreased to 25% however there is a major decrease in resistance to HCl. Thus formulations produced from Eudragit S 100 and Tween 80 in 50 – 100% PBS dispersions appear promising for oral delivery of BCG.

**Table 6.3**  Disintegration of Eudragit S 100 tablets (different concentration of PBS) in HCl or PBS after 2 hours incubation at 37°C (with pH adjustment to 7.0 using NaOH)

| Composition of Eudragit S 100 dispersions | Disintegration medium | Initial weight (mg) | Weight after 2 hrs incubation (mg) |
|---|---|---|---|
| Eud S100+PBS (100%) | HCl | 190.4 ± 6.8 | 176.3 ± 7.5 |
| Eud S100+PBS (50%) | | 204.3 ± 5.4 | 188.2 ± 6.4 |
| Eud S100+PBS (25%) | | 198.3 ± 4.7 | 121.2 ± 11.5 |
| Eud S100+Tween 80 | | 186.8 ± 7.2 | Dissolved |
| Eud S100+PBS (100%)+Tween 80 | | 193.5 ± 12.4 | 180.2 ± 8.7 |
| Eud S100+PBS (50%)+Tween 80 | | 204.3 ± 10.8 | 188.3 ± 6.7 |
| Eud S100+PBS (25%)+Tween 80 | | 204.5 ± 10.4 | 136.5 ± 5.7 |
| | | | |
| Eud S100+PBS (100%) | PBS | 167.3 ± 11.2 | Dissolved |
| Eud S100+PBS (50%) | | 203.6 ± 8.8 | Dissolved |
| Eud S100+PBS (25%) | | 239.8 ± 16.8 | 203.5 ± 8.6 |
| Eud S100+Tween 80 | | 205.3 ± 7.7 | Dissolved |
| Eud S100+PBS (100%)+Tween 80 | | 200.7 ± 11.7 | Dissolved |
| Eud S100+PBS (50%)+Tween 80 | | 203.5 ± 9.4 | Dissolved |
| Eud S100+PBS (25%)+Tween 80 | | 204.8 ± 12.8 | 165.4 ± 14.2 |

Reports by Arasaratnam *et al* (2000) and Kumar *et al* (1996) have noted that changes in pH of Eudragit can alter its properties, whether it is soluble or insoluble. They also noted that changes in temperature could alter the properties of Eudragit. When the pH of the aqueous dispersion was adjusted the matrix formulation containing Eudragit S 100, Tween 80 and 50-100% PBS displayed good weight

retention after 2 hours in HCl and dissolved after 2 hours in PBS. Therefore release of BCG may be achieved in the small intestine.

### 6.1.2 Disintegration and dissolution studies of Eudragit S100 matrices in SGF and SIF

When Eudragit S100 matrices formulated using 200mg Eudragit S100 + 2ml PBS + 0.9ml Tween 80 (0.1% w/w) were placed in HCl for 2 hours followed by incubation in PBS, the tablets did not break up even when left overnight.

**Table 6.4** The effect of PBS powder incorporation on disintegration properties of Eudragit S100 tablets when incubated for 2 hours in HCl (0.1M, pH 1.2, 37°C) followed by 2 hours in PBS (pH 7.4, 37°C)

| Composition of Eudragit S100 matrices | Weight of Eudragit S100 (mg) (% w/w) | PBS Powder (mg) (% w/w) | Initial weight (mg) | Dry weight (mg) |
|---|---|---|---|---|
| 2ml PBS + 0.9ml Tween 80 (0.1%w/v) | 200 (100%) | 0 (0%) | 202.3 ± 2.9 | 169.3 ± 1.1 |
|  | 150 (75%) | 50 (25%) | 203.7 ± 4.5 | 124.3 ± 2.5 |
|  | 100 (50%) | 100 (50%) | 205.33 ± 0.58 | 88.3 ± 1.2 |
|  | 50 (25%) | 150 (75%) | 203.3 ± 2.1 | 44.3 ± 2.7 |
| 1ml PBS + 1ml water + 0.9ml Tween 80 (0.1%w/v) | 200 (100%) | 0 (0%) | 202.3 ± 2.5 | 174.7 ± 4.6 |
|  | 150 (75%) | 50 (25%) | 204.2 ± 1.3 | 127.3 ± 3.5 |
|  | 100 (50%) | 100 (50%) | 203.6 ± 2.7 | 90.7 ± 1.5 |
|  | 50 (25%) | 150 (75%) | 198.7 ± 1.2 | 48.7 ± 4.5 |

To overcome this problem PBS powder was added to the formulations and the weight of Eudragit S100 powder adjusted according to the figures in the Table 6.3.

The addition of PBS powder increased matrix disintegration relative to the unmodified matrices when incubated in HCl followed by PBS (Table 6.4).

In both formulations an increased content of PBS increased tablet break up in PBS. However excessive PBS powder leads to extra break up in HCl and too little leads to minimal disintegration in PBS. The formulation based on 1ml PBS was selected for further study since disintegration in HCl was reduced compared to the tablet formulated with 2ml PBS and as a 50:50 ratio of Eudragit to PBS powder was chosen.

When the samples are withdrawn from the polypropylene tubes after incubation in HCl for 2 hours and the media replaced with PBS, the HCl associated with the matrix is released into the PBS media and lowers the pH from 7.4 to 2. Eudragit S100 starts to disintegrate at pH 7 and Eudragit L100 at pH 6. Therefore, matrix dissolution is reduced due to the fall in pH (Marvola *et al.*, 1999).

To overcome this problem the PBS medium was replaced with a stronger buffer, 1M HEPES buffer and the tablets were washed five times with water after removal from the SGF before transfer to the HEPES medium. The disintegration results, which were subsequently achieved, are presented in Table 6.5.

All three formulations shown in table 6.5 proved to be relatively stable in HCl with minimal disintegration apparent.

The Eudragit matrices took longer to dissolve when placed in HEPES solution immediately after 2 hours incubation in HCl (Table 6.5) than when placed directly into PBS solution (Table 6.2 and Table 6.4). This was due to residual HCl reducing the pH of the HEPES solution from 7 to 6.2. However it was quicker than placing the tablets in PBS following 2 hours incubation in HCl. This is because even though the pH falls from 7 to 6.2, this pH is still above or at the pH that the Eudragit enteric coating starts to disintegrate.

The tablet formulation based on 100mg Eudragit S100 and 100mg PBS powder displayed better disintegration behaviour than Eudragit S100 tablets. After 2 hours incubation in HEPES solution the tablet had broken up and almost dissolved and the test was continued until complete dissolution had been achieved. The formulation containing 100 mg Eudragit S100 and 100mg PBS was confirmed as a lead formulation.

**Table 6.5** <u>Disintegration of Eudragit matrices when incubated for 2 hours in HCl (0.1M, pH 1.2, 37°C) followed by HEPES solution (1M, pH 7)</u>

| Composition of Eudragit S100 matrices | Weight of Eudragit (mg) | PBS Powder (mg) | Initial weight (mg) | Dry weight after incubation in HCl and HEPES (mg) |
|---|---|---|---|---|
| 2ml PBS + 0.9ml Tween 80 (0.1%w/v) | 200mg Eudragit L100 | 0 | 214.3 ± 3.5 | Dissolved in 4 hours |
| 2ml PBS + 0.9ml Tween 80 (0.1%w/v) | 200mg Eudragit S100 | 0 | 204.2 ± 1.3 | 107.0 ± 18.5 |
| 1ml PBS + 1ml water + 0.9ml Tween 80 (0.1%w/v) | 100mg Eudragit S100 | 100mg | 213.3 ± 5.1 | All tablets dissolved within 4 hours |

### 6.1.3 Disintegration testing of Eudragit L30 D-55 matrices prepared using aqueous dispersions

Eudragit L 30 D-55 was difficult to formulate reproducibly. The weight of solids varied between different aliquots of aqueous dispersion and the pH of the solution was only 3.4, which required a large volume of 1M NaOH solution to adjust to 7.0. This altered the character of the mixture from a milky solution to a colourless, thick, jelly-like consistency, which meant that the BCG suspension, when added, was not mixed effectively. Similar behaviour has been noted by (Arasaratnam *et* al., 2000; Kumar *et al.*, 1994).

**Table 6.6**   Disintegration of Eudragit L 30 D-55 matrices in HCl or PBS after 2 hours incubation at 37°C (without pH adjustment)

| Composition of Eudragit L 30 D-55 | Disintegration medium | Initial weight (mg) | Weight after 2 hours (mg) | Disintegration time |
|---|---|---|---|---|
| Eud L30 D-55 | HCl | 212.3 ± 8.2 | 184.3 ± 6.2 | |
| Eud L30 D-55+Tween 80 | HCl | 189.6 ± 14.4 | 163.6 ± 10.4 | |
| | | | | |
| Eud L30 D-55 | PBS | 211.6 ± 7.7 | 198.2 ± 11.5 | 4hrs 40mins |
| Eud L30 D-55+Tween 80 | PBS | 223.3 ± 12.5 | 213.4 ± 6.8 | 5hrs |

**Table 6.7**   Disintegration of Eudragit L 30 D-55 matrices in HCl or PBS after 2 hours incubation at 37°C (with pH adjustment to 7.0 using NaOH)

| Composition of Eudragit L 30 D-55 | Disintegration medium | Initial weight (mg) | Weight after 2 hours (mg) | Disintegration time |
|---|---|---|---|---|
| Eud L30 D-55 | HCl | 200.4 ± 6.7 | 185.5 ± 8.8 | |
| Eud L30 D-55+Tween 80 | HCl | 206.2 ± 4.8 | 189.4 ± 10.7 | |
| | | | | |
| Eud L30 D-55 | PBS | 203.4 ± 12.4 | 125.8 ± 24.8 | 5hrs 15mins |
| Eud L30 D-55+Tween 80 | PBS | 206.8 ± 6.6 | 187.2 ± 5.6 | 5hrs 45mins |

When disintegration studies were performed on Eudragit L30 D-55 matrices (with and without pH adjustment) in HCl and PBS the tablets were found to be resistant to acid conditions (Table 6.5 & Table 6.6). After 2 hours incubation in PBS, less than 50% of the matrix (with and without Tween 80) had dissolved. Samples no longer exhibited the disk shape but instead became very elongated and split into different layers.

The extended disintegration time in PBS means that BCG will not be released effectively in the small intestine for uptake in the Peyer's patches. Similar behaviour has been noted by (Arasaratnam *et al.*, 2000; Kumar *et al.*, 1994).

### 6.1.4 Disintegration and dissolution testing of Eudragit L100 D-55 matrices prepared using aqueous dispersion

Eudragit L100-D55 is similar to Eudragit L30 D-55 in that it dissolves at pH 5.5 and above. Similar results for disintegration in HCl were obtained for Eudragit L100-55 matrices with and without pH adjustment (Table 6.7 and 6.8). Both sets of tablets were very stable in HCl. The tablets prepared from aqueous dispersions without pH adjustment tended to be more stable in PBS at 37°C after 2 hours with only small amounts of the tablet disintegrating (Table 6.7). Most of the samples prepared with pH adjustment broke into fragments within 30 minutes and actually dissolved in the times noted in Table 6.8. The tablets formulated using Tween 80 in 2ml PBS solution dissolved completely after 2 hours, which may be useful for oral delivery of BCG.

Delgado *et al* (1999) used Eudragit L100-55 solutions at pH 6 to stabilise PLG microparticles and protect surface adsorbed OVA against degradation from pepsin in-vitro. They compared the polymer against carboxymethylcellulose and PVA stabilisers as a control. PLG stabilised with Eudragit improved protection of the protein against pepsin and release of OVA was reduced compared to PVA stabilised particles. Takka *et al* (2001) incorporated Eudragit L100-55 into hydroxypropylmethycellulose (HPMC) to help modify propranolol release. They noted strong interactions between the -COOH group on the Eudragit and propranolol, which influenced the kinetics of drug release.

176

**Table 6.8** Disintegration of Eudragit L 100-55 tablets in HCl or PBS after 2 hours incubation at 37°C (without pH adjustment)

| Composition of Eudragit L100-55 | Disintegration medium | Initial weight (mg) | Weight after 2 hrs incubation (mg) |
|---|---|---|---|
| Eud L100-55 | HCl | 172.5±10.4 | 175.5±8.8 |
| Eud L100-55+2ml PBS | | 204.3±4.7 | 201.2±3.4 |
| Eud L100-55+2ml water | | 170.4±18.6 | 167.6±12.7 |
| Eud L100-55+2ml PBS+Tween 80 | | 208.3±11.4 | 213.6±6.1 |
| Eud L100-55+2ml water+Tween 80 | | 176.2±12.2 | 176.2±8.5 |
| | | | |
| Eud L100-55 | PBS | 206.4±4.5 | Dissolved in 2 ½ hrs |
| Eud L100-55+2ml PBS | | 187.4±6.5 | 124.2±12.4 |
| Eud L100-55+2ml water | | 176.2±8.4 | 146.4±8.3 |
| Eud L100-55+2ml PBS+Tween 80 | | 201.2±6.3 | 135.2±12.2 |
| Eud L100-55+2ml water+Tween 80 | | 182.2±8.7 | 138.6±18.6 |

**Table 6.9** Disintegration of Eudragit L 100-55 tablets in HCl or PBS after 2 hours incubation at 37°C (with pH adjustment to 7.0 using NaOH)

| Composition of Eudragit L100-55 | Disintegration medium | Initial weight (mg) | Weight after 2 hrs incubation (mg) |
|---|---|---|---|
| Eud L100-55 | HCl | 174.2±12.2 | 165.1±6.4 |
| Eud L100-55+2ml PBS | | 208.4±6.6 | 172.6±10.4 |
| Eud L100-55+2ml water | | 182.4±16.3 | 149.2±8.6 |
| Eud L100-55+2ml PBS+Tween 80 | | 203.5±4.4 | 182.4±4.6 |
| Eud L100-55+2ml water+Tween 80 | | 182.6±14.5 | 153.3±6.4 |
| | | | |
| Eud L100-55 | PBS | | Dissolved |
| Eud L100-55+2ml PBS | | 201.4±10.2 | Dissolved in 5.3 hrs |
| Eud L100-55+2ml water | | 201.2±12.7 | 104.7±28.8 |
| Eud L100-55+2ml PBS+Tween 80 | | 201.2±14.8 | Dissolved |
| Eud L100-55+2ml water+Tween 80 | | 201.8±7.8 | 142.8±13.4 |

### 6.1.5 Disintegration and dissolution testing of Eudragit L100 matrices prepared from aqueous dispersions

Eudragit L 100 matrices prepared from aqueous dispersions without pH adjustments (Table 6.10) were unstable in HCl, most of the tablets disintegrating within 2 hours. The tablets containing Tween 80 disintegrated more rapidly in HCl than those without surfactant. This is again most likely to be due to Tween 80 altering the configuration of cohesive bonding with the Eudragit copolymer (Breikreutz *et al.*, 2000).

In contrast Eudragit L100 matrices produced using pH adjustment to 7.0 and containing PBS, Tween 80 appears to act as a binder (Table 6.11). It can also be seen that those tablets produced from PBS dispersions have a greater stability in HCl than those produced from water-based dispersions. The addition of Tween 80 to Eudragit L 100/PBS formulations is not detrimental to tablet stability in HCl. Tween 80 seems however to be producing greater instability of Eudragit L 100 matrices prepared by initial dispersion in water.

**Table 6.10**  Disintegration of Eudragit L 100 tablets in PBS or HCl after 2 hours incubation at 37°C (without pH adjustment)

| Composition of Eudragit L100 | Disintegration medium | Initial weight (mg) | Weight after 2 hrs incubation (mg) |
|---|---|---|---|
| Eud L100 | HCl | 194.2 ± 5.2 | 179.5 ± 10.6 |
| Eud L100+2ml PBS | | 208.3 ± 3.5 | 169.6 ± 11.6 |
| Eud L100+2ml water | | 174.6 ± 15.7 | Dissolved in 2.3 hrs |
| Eud L100+2ml PBS+Tween 80 | | 207.4 ± 5.5 | Dissolved in 1.15 hrs |
| Eud L100+2ml water + Tween 80 | | 195.3 ± 7.6 | Dissolved in 1.5 hrs |
| | | | |
| Eud L100 | PBS | 203.6 ± 4.7 | Dissolved in 1 hr |
| Eud L100+2ml PBS | | 205.8 ± 8.7 | 124.3 ± 9.3 |
| Eud L100+2ml PBS+Tween 80 | | 201.2 ± 9.2 | 138.2 ± 11.1 |

178

Eudragit L100 matrices prepared without pH adjustment from PBS dispersions are relatively intact after 2 hours in PBS at 37°C. However the tablets produced with pH adjustment all dissolved and disintegrated within 2.5 hours and started to break up in the first 30 minutes. On the basis of the above findings Eudragit L 100/PBS/Tween 80 formulations present opportunities for controlled release of BCG in the small intestine following oral administration

**Table 6.11** Disintegration studies involving Eudragit L 100 tablets in PBS or HCl after 2 hours incubation at 37°C (with pH adjustment to 7.0 using NaOH)

| Composition of Eudragit L100 | Disintegration medium | Initial weight (mg) | Weight after 2 hrs incubation (mg) |
|---|---|---|---|
| Eud L100 | HCl | $213.2 \pm 11.2$ | $186.3 \pm 9.4$ |
| Eud L100+2ml PBS | HCl | $221.5 \pm 12.3$ | $184.3 \pm 14.2$ |
| Eud L100+2ml water | HCl | $190.1 \pm 8.1$ | $136.2 \pm 11.6$ |
| Eud L100+2ml PBS+Tween 80 | HCl | $216.3 \pm 8.2$ | $190.6 \pm 4.4$ |
| Eud L100+2ml water+Tween 80 | HCl | $200.1 \pm 7.2$ | $113.5 \pm 12.3$ |
| | | | |
| Eud L100 | PBS | $202.1 \pm 5.4$ | Dissolved and disintegrated in 1 hr |
| Eud L100+2ml PBS | PBS | $201.2 \pm 5.4$ | Dissolved and disintegrated in 2.3 hrs |
| Eud L100+2ml PBS+Tween 80 | PBS | $202.2 \pm 8.4$ | |

The use of PBS in the initial formulation stage seems to stabilise Eudragit L100 matrices in HCl compared with the use of water. This may be due to the stronger electrostatic forces, which occur between the $Na^+$, $PO_3^{4-}$ and $Cl^-$ ions and the ionised carboxyl and ester groups in Eudragit.

Eudragit L100 has been used previously for oral drug delivery. Carelli *et al* (1999) coated hydrogels with Eudragit L100 to deliver drugs to the small intestine. They noted that the stability in HCl was improved and that release of the model drug, prednisolone could be achieved in specific regions of the small intestine. The Eudragit coating disintegrated once the pH above 6 with a peak at 6.8. Similarly

Carino *et al* (1999) noted added protection and good release properties of Insulin coated with Eudragit L100. Since Eudragit L100 dissolves at pH 6 and above, insulin was released early in the duodenum and jejunum and not in the ileum.

## 6.2 S.E.M analysis of Eudragit matrices formulated using aqueous dispersions

Figure 6.1 and 6.2 shows the dense compact structure of matrices formed by direct compression of Eudragit L100 and Eudragit S100 powder respectively. In contrast the Eudragit L100 matrix formulated using aqueous dispersions contains pores and cracks which would allow HCl to penetrate and kill the BCG as well as cause disintegration of the matrix

**Figure 6.1**  S.E.M of internal structure of Eudragit L100 matrices produced by direct powder compaction
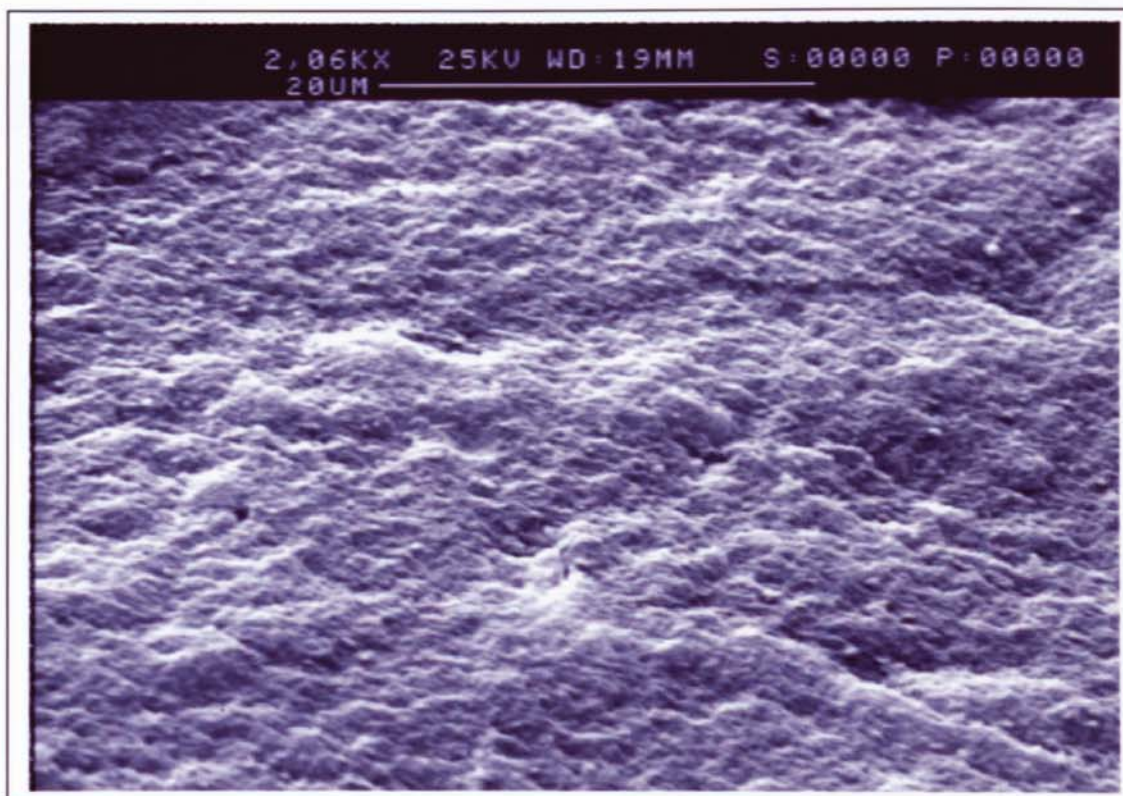
**Figure 6.2** S.E.M of internal structure of Eudragit S100 matrices prepared by direct powder compaction
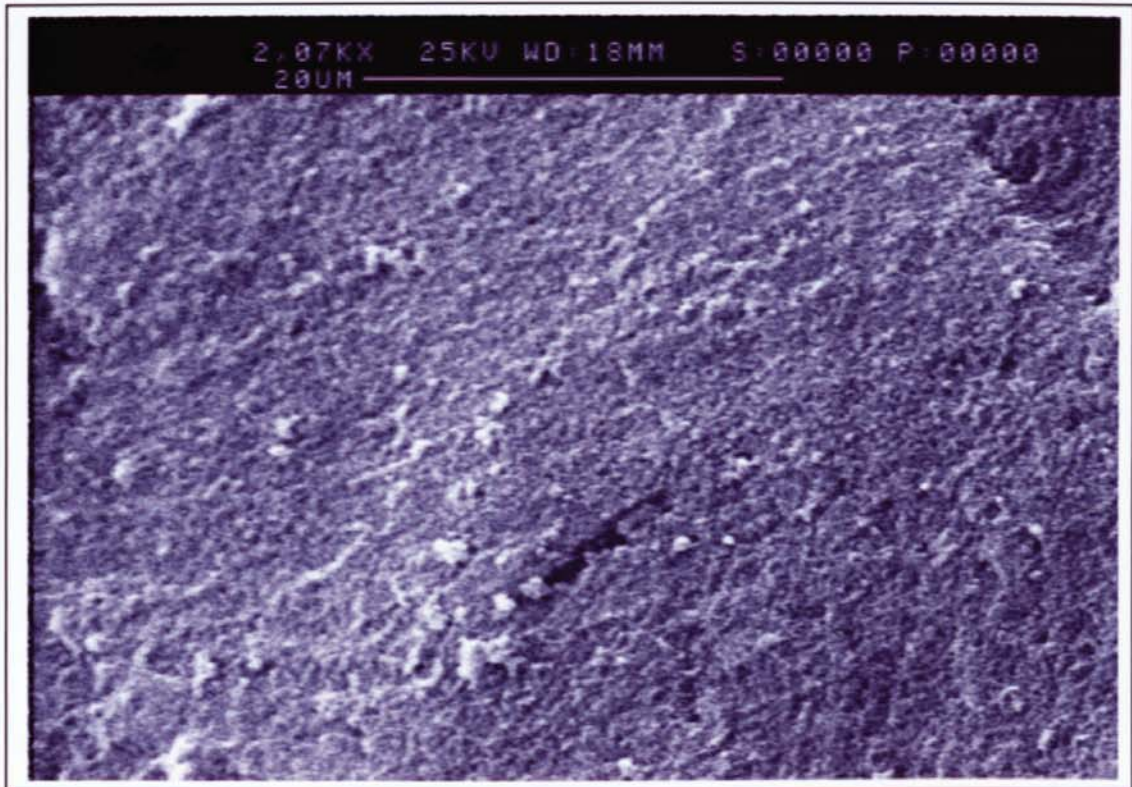


**Figure 6.3** S.E.M of internal structure of Eudragit L100 matrices prepared from dispersions in PBS containing Tween 80 (pH adjusted to 7)



181
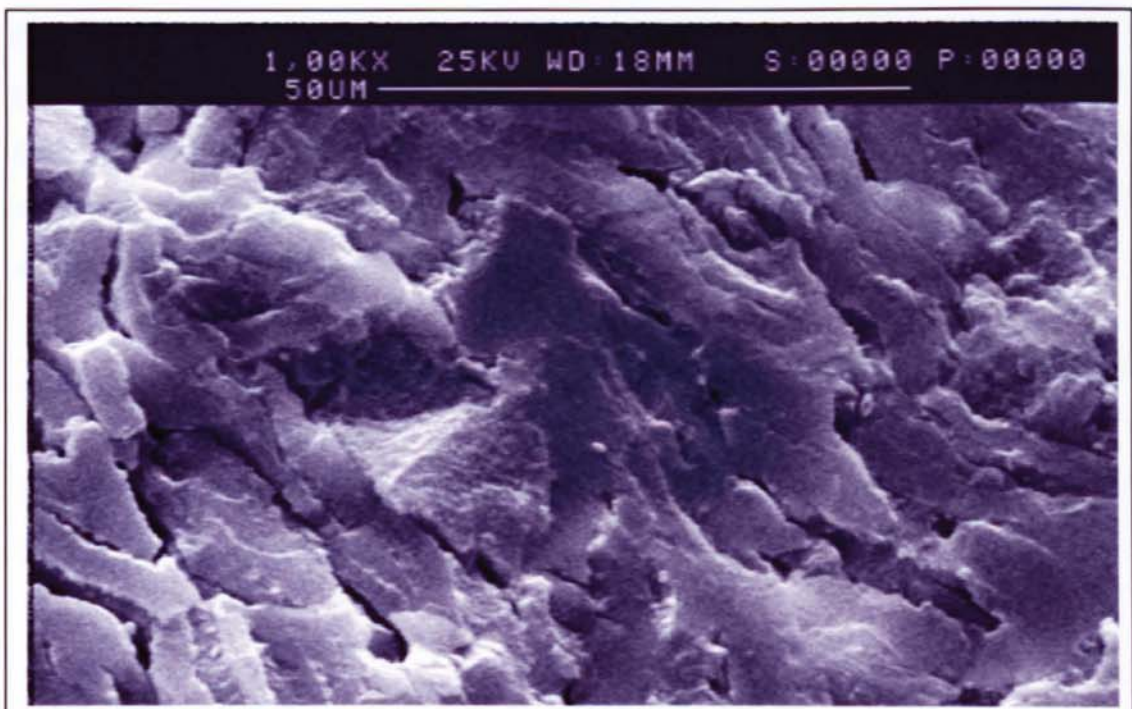
**Figure 6.4** S.E.M of internal structure of Eudragit L100 matrices prepared from dispersions in PBS containing Tween 80 (pH adjusted to 7)
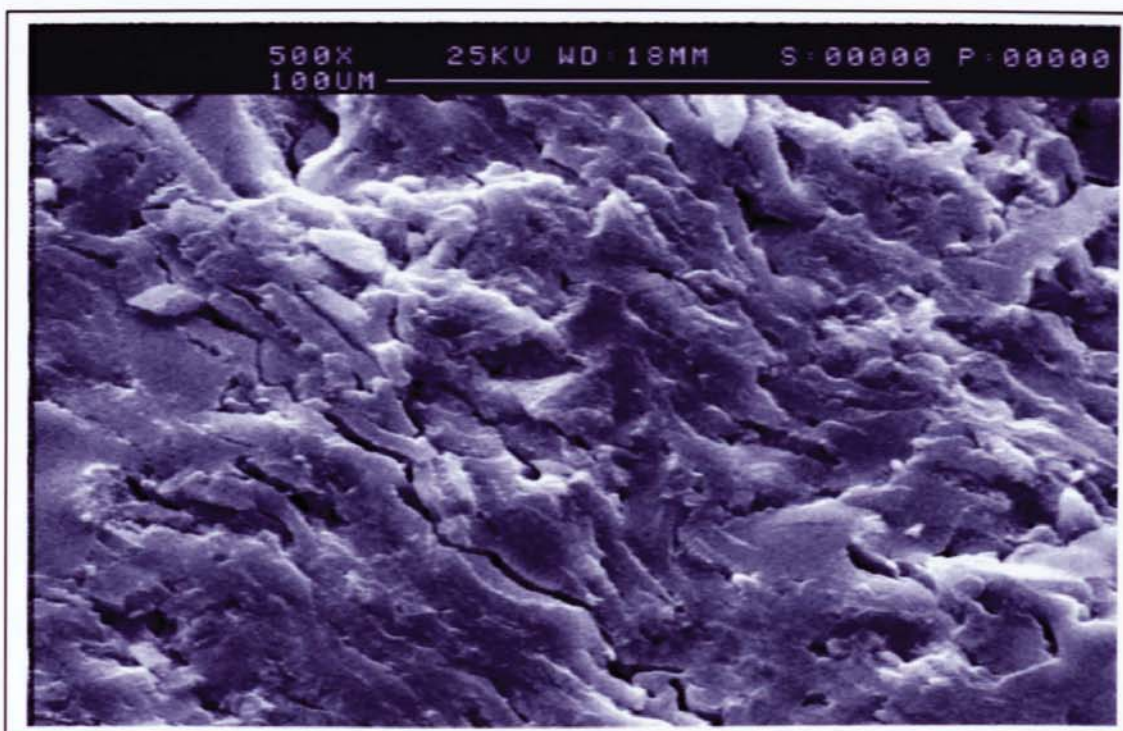


Eudragit coating of PBS produces the morphology shown above. When the Eudragit L100 matrix is placed in PBS, the matrix disintegration occurs due to dissolution of the excipients that have been incorporated and this facilitates overall dissolution of the Eudragit copolymer. According to Kottiyan *et al* (2001) additives, changes in pH and temperature alter the bonds within and between Eudragit molecules. The ester C=O bond becomes stretched, the C-H bond is subjected to bending and C-C multiple bond stretching occurs. These changes contribute to the dissolution of Eudragit in PBS at 37°C.

Figure 6.5 shows an S.E.M of the morphology of Eudragit S100 matrix prepared from dispersions incorporating 100mg PBS powder. The matrix displays a crystalline texture and there seems to be integration of salt crystals with the Eudragit copolymer phase.

**Figure 6.5** S.E.M of internal structure of Eudragit S100 matrices prepared from dispersions in PBS/water (1:1) containing 100mg PBS powder
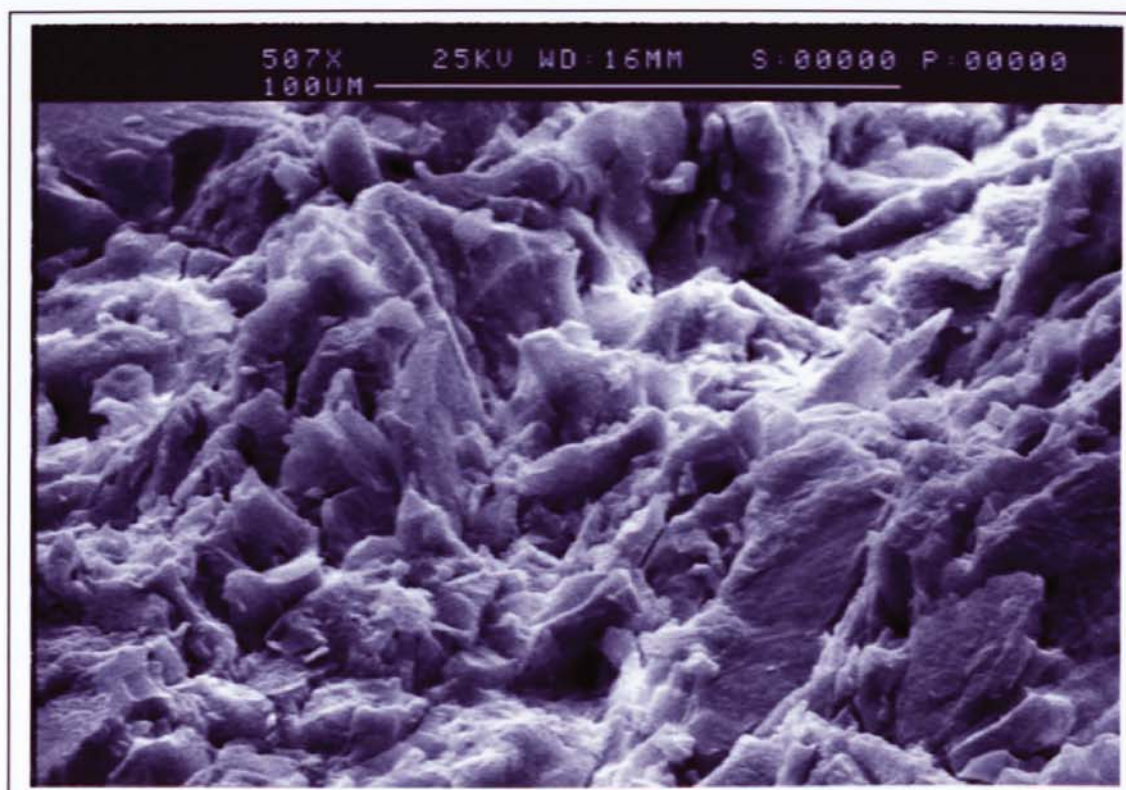


The more crystalline structure of the Eudragit S100 matrices probably means that the cohesive forces in the Eudragit phase are reduced (Takka *et al.*, 2001), hence giving rise to the greater disintegration and dissolution properties in HCl and PBS compared to Eudragit L100. Breitkrutz *et al* (2000) noted that an increase in salt concentration alters the ionic strength and hence the structure of Eudragit S100.

Figure 6.6 shows the Eudragit S100 formulation prepared using 2ml PBS which reveals a similar crystalline morphology.

**Figure 6.6** S.E.M of internal structure of Eudragit S100 matrices prepared from dispersions in PBS containing 100mg PBS powder
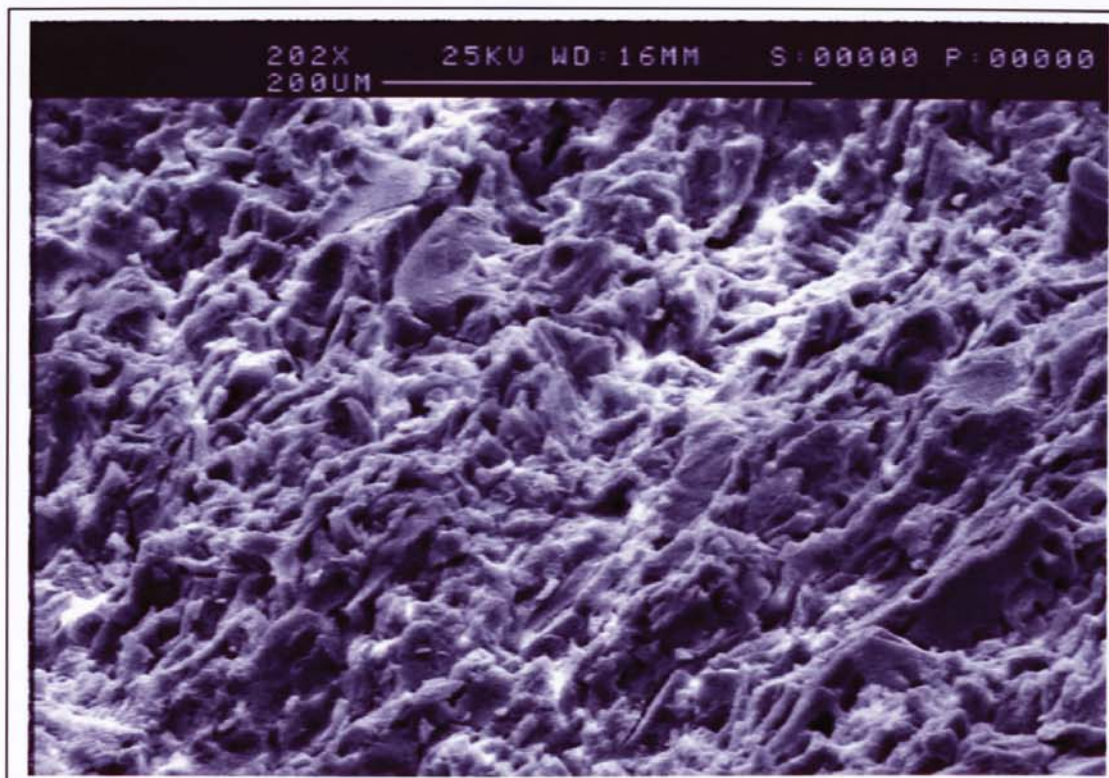


The porous Eudragit S100 and L100 matrices may be useful for encapsulating BCG and providing a controlled release mechanism as well as protection against the acidic conditions of the stomach. The BCG could be protected by the local salt formations, which could offer pH control in HCl media and assist undergo dissolution at pH 7 and above. BCG release may be controlled depending on the salt content. However it is also possible that the crystalline structure of the Eudragit matrices may rupture the cell wall of BCG and thus reduce viability.

## 6.3 Conclusion

On the basis of formulation and disintegration studies in SGF and SIF presented above, two matrix formulations were selected for further investigations of the oral

delivery of BCG. Namely Eudragit S100 and L100 prepared from dispersions in PBS containing Tween 80 (Table 6.5).

**Table 6.12** Represents the composition of the two lead formulations

| Eudragit Grade | Weight of Eudragit | Other excipients |
| :---: | :---: | :---: |
| S100 | 100mg | 100mg PBS powder, 1ml PBS solution, 1ml distilled water, 0.9ml Tween 80 (0.1%w/w), 100μl BCG (pH 7) |
| L100 | 200mg | 2ml PBS solution, 0.9ml Tween 80 (0.1%w/w), 100μl BCG (pH 7) |

# CHAPTER 7

# VIABILITY OF BCG INCORPORATED IN EUDRAGIT MATRICES

# 7 VIABILITY OF BCG INCORPORATED IN EUDRAGIT MATRICES

VLA provided BCG Pasteur at a concentration of $10^8$ c.f.u's per ml. BCG 100μl suspension was incorporated in Eudragit S100 and Eudragit L100 (Table 7.1) described in section 2.10. BCG viability was assessed as described in section 2.4.5.

The formulations were produced under sterile conditions to eliminate or reduce the risk of contamination. The viability of BCG following matrix incubation either in SIF or SGF followed by SIF was measured at around $10^3$ c.f.u's per tablet (Tables 7.1 and 7.2), which equates to a reading of $10^4$ c.f.u's/ml of BCG suspension. This corresponds to a major decrease in viability of approximately x10000.

**Table 7.1** BCG viability after freeze-drying, matrix compression and dissolution in PBS pH 7.4,37°C for 2 hours

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit S100 | 3, 4, 4 | $4x10^3$ | $4x10^4$ |
| Eudragit L100 | 4, 2, 5 | $4x10^3$ | $4x10^4$ |

**Table 7.2** BCG viability (after freeze-drying, tablet compression, placed in HCl (0.1M, pH 1.2) for 2 hours then added to HEPES solution (1M, pH 7, 37°C) for 2 hours)

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit S100 | 3, 4, 4 | $4x10^3$ | $4x10^4$ |
| Eudragit L100 | 4, 2, 5 | $4x10^3$ | $4x10^4$ |

There have been reports by Lima *et al* (2001) that concentrations as low as x10$^5$ c.f.u's are sufficient to induce immune responses in mice. The viability of BCG released from Eudragit matrices is very low and may need to be increased to induce an immune response after oral administration.

Assuming a viability of $10^8$ cfu/ml in the stock BCG sample, experiments were performed to determine at which stage the BCG viability is being lost. It is possible for example that the surfactant Tween is not maintaining BCG dispersion, even though it is widely used for BCG stabilisation (Dietrich et al., 1991; Ratliff et al., 1994). Initial studies (see section 5) did not take into account the effect of pH and salt concentration (at the formulation stage) on the dispersion properties of BCG. It is also possible that the c.f.u counts are not accurate due to clumping of BCG. This has been shown to be a common occurrence when working with BCG suspensions (Kotiyan et al., 2001; Felton et al., 1999).

## 7.1 Viability of BCG at each matrix formulation and dissolution stage

Ratliff et al (1994) have reported that freeze-drying results in loss of viability of BCG. Consequently this stage of the process was investigated to determine any loss in viability. Other stages where loss could potentially occur are at freezing due to crystal formation, which could rupture the bacteria cell walls and also at the disintegration stage when placed in HCl for 2 hours. If the Eudragit copolymer does not encapsulate all the BCG then this could result in exposure to HCl and loss of viability. It has already been shown (section 6.2) that the internal structures of Eudragit matrices are porous.

Experiments were performed to determine if loss in BCG viability occurs at freezing, freeze-drying and disintegration stages of formulation.

Comparing Tables 7.3 and 7.4 it can be seen that adjusting the pH of the Eudragit aqueous dispersions from 4.2 to 7 increases the viability of BCG. The results presented in Table 7.4 also suggest that the formulation containing Eudragit S100 has a decreased BCG viability compared to Eudragit L100. The Eudragit S100 formulation contains 100mg PBS powder. The increased quantity of salt crystals

formed during freezing have potential for rupturing the cell walls and thus killing the BCG (Felton *et al.*, 1999).

**Table 7.3**  BCG viability in Eudragit aqueous dispersions after freezing at -20°C for 6 hours then thawing and plating 100μl of Eudragit suspension resuspended in 10ml PBS (no pH adjustment)

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 | 3, 7, 6 | $5x10^3$ | $5x10^4$ |
| Eudragit S100 | 5, 4, 9 | $6x10^3$ | $6x10^4$ |

**Table 7.4**  BCG viability in Eudragit dispersions after freezing at -20°C for 6 hours then thawing and plating 100μl of Eudragit suspension resuspended in 10ml PBS (with pH adjustment)

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 | 102, 115, 98 | $105x10^3$ | $105x10^4$ |
|  | 56, 61, 58 | $58x10^4$ | $58x10^5$ |
|  | 10, 6, 8 | $8x10^5$ | $8x10^6$ |
| Eudragit S100 | 73, 64, 67 | $68x10^3$ | $68x10^4$ |
|  | 10, 8, 12 | $10x10^4$ | $10x10^5$ |

**Table 7.5**  BCG viability in Eudragit dispersions following freezing at -20°C for 6 hours. Freeze-dried overnight. Powder resuspended in 10ml PBS (with pH adjustment)

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 | 127, 124, 117 | $122x10^3$ | $122x10^4$ |
|  | 16, 24, 30 | $23x10^4$ | $23x10^5$ |
|  | 2, 4, 3 | $3x10^5$ | $3x10^6$ |
|  | 2, 2, 3 | $2x10^6$ | $2x10^7$ |
| Eudragit S100 | 14, 18, 10 | $14x10^3$ | $14x10^4$ |
|  | 4, 6, 2 | $4x10^4$ | $4x10^5$ |
|  | 1, 1, 1 | $1x10^5$ | $1x10^6$ |
|  | 1, 1, 1 | $1x10^6$ | $1x10^7$ |

The BCG viability (cfu/ml) after freezing Eudragit dispersions (Table 7.4) shows a decrease by a factor of approximately 100 and this is carried through to the freeze-

drying stage (Table 7.5). Thus these two steps in matrix formulation result in a loss in viability but not to the level of $10^4$ cfu/ml noted after dissolution of compressed matrices (Table 7.2).

**Table 7.6** BCG viability following freezing of Eudragit dispersions at -20°C for 6 hours. Freeze-dried overnight. Powder compressed at 4 tons for 3mins. Dissolved in 10ml PBS (with pH adjustment)

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 | 1, 3, 2 | $2x10^3$ | $2x10^4$ |
| Eudragit S100 | No viable data | | |

**Table 7.7** BCG viability following freezing of Eudragit dispersions at -20°C for 6 hours. Freeze-dried overnight. Powder compressed at 4 tons for 3mins. Tablet placed in HCl for 2 hours and then in HEPES solution until dissolved (with pH adjustment)

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 | 4, 7, 5 | $5x10^3$ | $5x10^4$ |
| Eudragit S100 | No viable data | | |

The results in Table 7.6 and 7.7 reveal that compressing the powder into a matrix resulted in a major loss of BCG viability. The loss in BCG viability was similar after immersion of matrices in PBS and SGF/SIF. Thus the freeze-drying and compression stage of matrix formulation are resulting in a decrease in BCG activity from $10^8$ (supplied in vial from VLA) to $10^6$ and $10^4$ respectively.

In order to improve BCG viability during matrix formulation the addition of cryo-protectants were investigated. It is possible that during freezing conditions and the freeze-drying process crystals form which during the compression stage rupture the bacteria cell wall similar to the action of the ball grinding method (Gheorghiu *et al.*, 1988) used to kill bacteria.

190

**Table 7.8**  BCG viability in vials supplied by VLA

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| BCG Pasteur | 135, 148, 162 | $148 \times 10^5$ | $\times 10^6$ |
|  | 15, 13, 9 | $12 \times 10^6$ | $\times 10^7$ |

**Figure 7.1**  Colony formation due to sample of BCG (Pasteur) taken from vial grown on Midddlebrook agar at $\times 10^4$ dilution
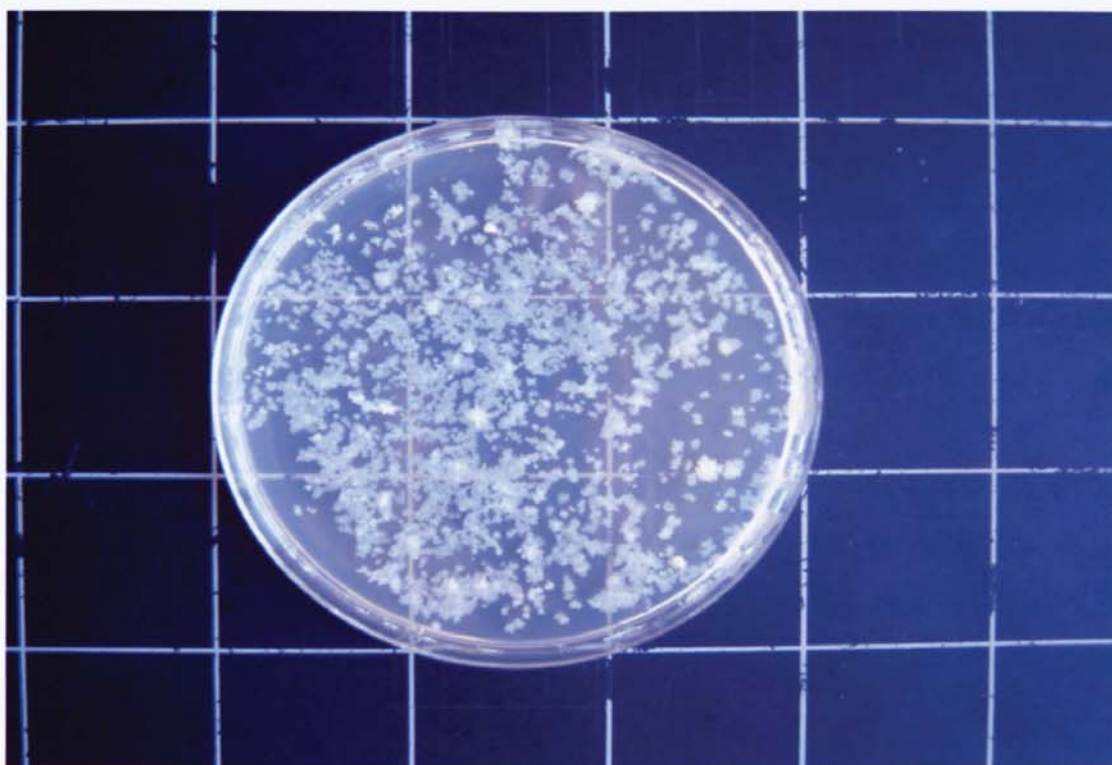


## 7.2  The effect of incorporating cryoprotectants in Eudragit matrices on viability of BCG

Since water is the major component of all living cells and must be available for chemical processes to occur, cellular metabolism stops when all water in the system is converted to ice. Ice forms at different rates during freezing. During slow cooling freezing occurs external to the cell before intracellular ice begins to form. As ice forms: water is removed from the extracellular environment and an osmotic

191

imbalance occurs through the cell membrane leading to water migration out of the cell. The increase in solute concentration outside the cell, as well as intracellularly can be detrimental to cell survival. If too much water remains inside the cell, damage due to ice crystal formation and recrystallisation during warming can occur.

The rate of cooling has a dramatic effect. Rapid cooling minimises the solute concentration effects as ice forms uniformly, but leads to more intracellular ice. Slow cooling, however, results in a greater loss of water from the cell and less internal ice but increases the solute effects. The rate of water loss is dependent on the permeability of the cell. More permeable cells are able to tolerate rapid cooling better than less permeable cells. It is the ice crystal formation and solute concentration, which cause damage to the bacterial cell walls and may lead to cell death. Cryoprotectants are frequently employed to try and control crystal formation at freezing and freeze-drying stages of formulation (Fukui *et al.*, 2001).

Mannitol and Trehalose were added to the lead formulation (Eudragit L100 + 2ml PBS solution + 0.9ml Tween 80 + BCG pH 7) (Section 2.8) to determine if the addition of cryoprotectants had any effect on BCG viability after freezing and freeze-drying and to determine if there is any difference in BCG viability between the two cryoprotectants.

The addition of 20mg mannitol to Eudragit L100 matrix (180mg Eudragit L100 + 2ml PBS solution + 0.9ml Tween 80 pH 7) resulted in a more graining morphology (Fig 7.2 and 7.3) compared with matrices formulated without mannitol (Fig 6.3)

The addition of trehalose (Fig 7.3) also formed a fairly well compact matrix, however the powder compaction of all the constituents is very loose and pores are visible. The constituents are not as compact as the matrix formulation containing mannitol (Fig 7.2).

**Figure 7.2** S.E.M of internal structure cross-section of Eudragit L100 matrices prepared from dispersions in PBS containing Tween 80 and Mannitol (pH adjusted to 7)
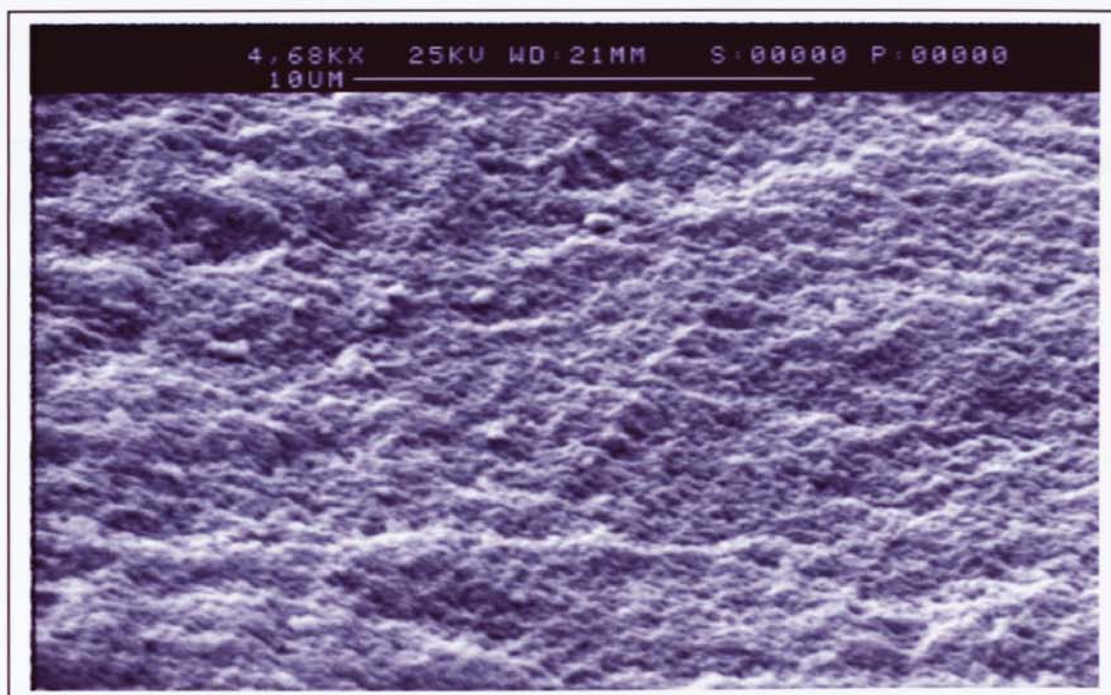


**Figure 7.3** S.E.M of internal structure of Eudragit L100 matrices prepared from dispersions in PBS containing Tween 80 and Trehalose (pH adjusted to 7)
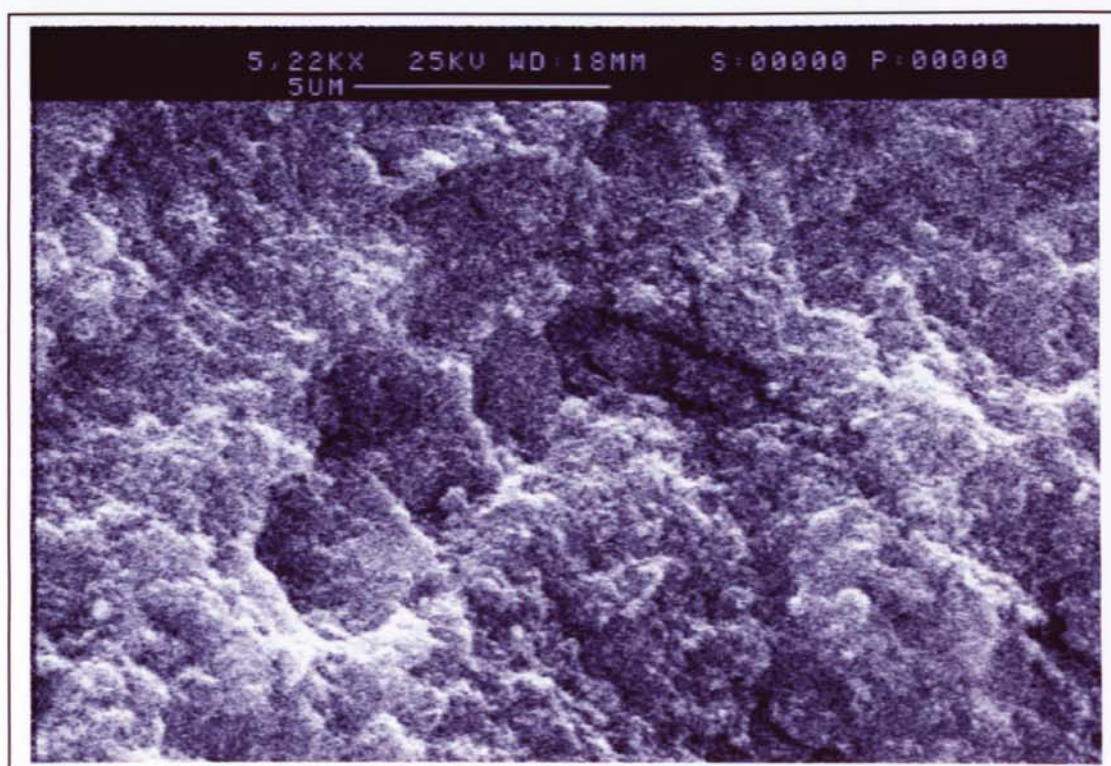
Distinct angular crystalline structures were not visible, which could cause rupturing of the cell wall of BCG under compression. Table 7.9 compares the BCG viability data obtained when the cryoprotectants were added to aqueous Eudragit dispersions without subsequent freezing.

**Table 7.9** BCG viability at initial formulation stage made up to 10ml PBS (with pH adjustment). Plate 100μl of Eudragit matrices

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 + mannitol | Too many | $x10^3$ | $x10^4$ |
|  | 63, 64, 56 | $61x10^4$ | $61x10^5$ |
|  | 10, 11, 18 | $13x10^5$ | $13x10^6$ |
| Eudragit L100 + trehalose | Too many | $x10^3$ | $x10^4$ |
|  | 60, 65, 70 | $65x10^4$ | $65x10^5$ |
|  | 14, 10,10 | $11x10^5$ | $11x10^6$ |
| Eudragit L100 | Too many | $x10^3$ | $x10^4$ |
|  | 58, 63, 55 | $59x10^4$ | $59x10^5$ |
|  | 8, 12, 10 | $10x10^5$ | $10x10^6$ |

**Figure 7.4** Clumping of BCG taken at initial formulation stage shown using methylene blue stain under light microscope x1000 (Eudragit/mannitol)



BCG
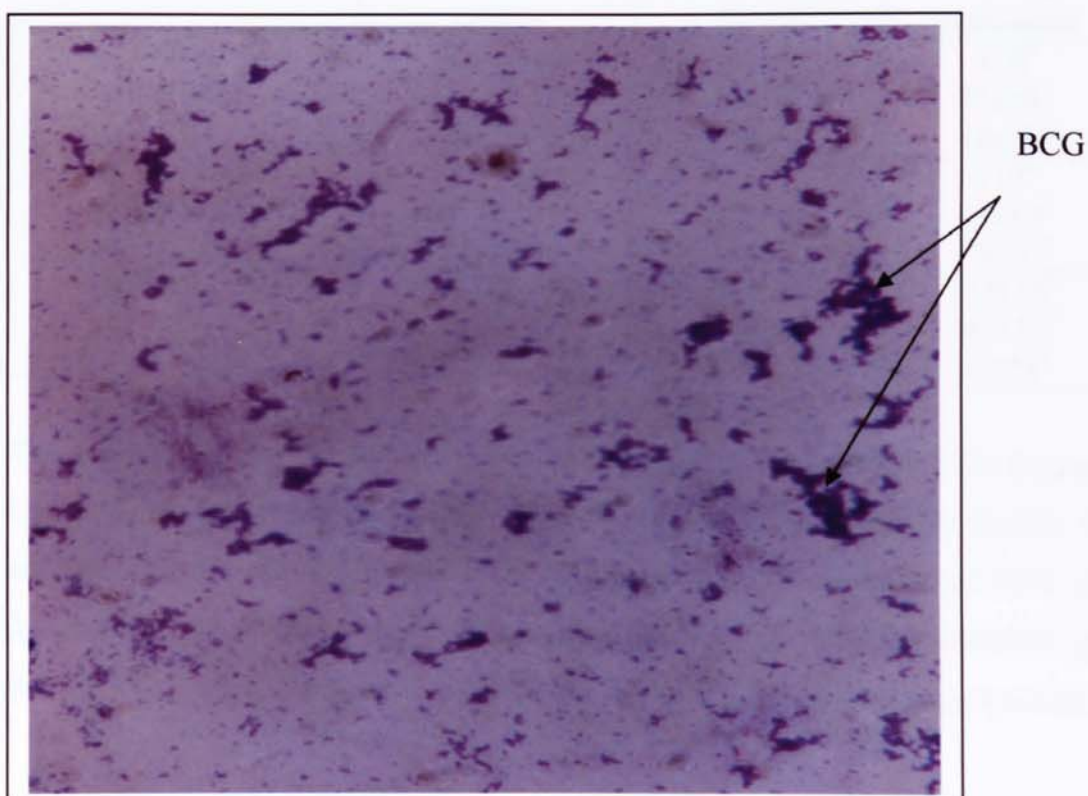
The results suggest that the viability of BCG at this initial stage of the formulation is similar with or without cryoprotectants. However it can also be seen that even at this initial stage of formulation there is a loss of viability from $10^8$ cfu/ml (stock) to $10^6$ cfu/ml in each system.

As mentioned in section (1.9.2 and 5.2) clumping is a major problem when using BCG and this can reduce the colony counts observed on the Middlebrook media (Behr *et al.*, 1999; Groves., 1997; Zhang *et al.*, 1988). Figure 7.4 shows clumping of BCG as seen through a light-microscope at x1000 magnification stained using methylene blue as described in section 2.4.5.5.

Clumping was observed in all three Eudragit matrices shown in Table 7.9 with and without cryoprotectants and no visual difference were apparent at this initial formulation stage.

Table 7.10  BCG viability after freezing at -20°C for 6 hours and thawing (with pH adjustment). Plate 100µl of Eudragit matrix suspension.

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 + mannitol | Too many | $x10^3$ | $x10^4$ |
| | 65, 44, 56 | $55x10^4$ | $55x10^5$ |
| | 14,10,18 | $14x10^5$ | $14x10^6$ |
| Eudragit L100 + trehalose | Too many | $x10^3$ | $x10^4$ |
| | 65, 62, 56 | $61x10^4$ | $61x10^5$ |
| | 20,10,8 | $13x10^5$ | $13x10^6$ |
| Eudragit L100 | 112, 125, 118 | $118x10^3$ | $118x10^4$ |
| | 56, 51, 58 | $165x10^4$ | $165x10^5$ |
| | 10, 16, 8 | $11x10^5$ | $11x10^6$ |

There is no clear loss in BCG viability on freezing and freeze-drying the Eudragit L100 dispersions containing BCG (Table 7.10 and 7.11). However despite the addition of mannitol and trehalose as cryoprotectants, the viability of BCG still decreased at the matrix compression stage (Table 7.12). Therefore mannitol and trehalose are seemingly ineffective at protecting BCG during powder compaction to

produce the Eudragit matrix but are effective in maintaining BCG viability after the freeze-drying stage prior to powder compaction.

**Table 7.11** BCG viability after freezing dispersions at -20°C for 6 hours then freeze-dried overnight. Resuspended in 10ml PBS (with pH adjustment). Plate 100µl of Eudragit matrix suspension.

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 + mannitol | Too many | $x10^3$ | $x10^4$ |
| | 70,72,68 | $70x10^4$ | $70x10^5$ |
| | 8, 5,10 | $3x10^5$ | $3x10^6$ |
| Eudragit L100 + trehalose | Too many | $x10^3$ | $x10^4$ |
| | 62,65,67 | $65x10^4$ | $65x10^5$ |
| | 10,6,7 | $8x10^5$ | $8x10^6$ |
| Eudragit L100 | 117, 124, 127 | $123x10^3$ | $123x10^4$ |
| | 16, 24, 30 | $23x10^4$ | $23x10^5$ |
| | 2, 4, 3 | $3x10^5$ | $3x10^6$ |

**Figure 7.5** Clumping of BCG after the freezing (-80°C) stage shown using methylene blue stain under light microscope x1000 (Eudragit/mannitol)



BCG clumping

196
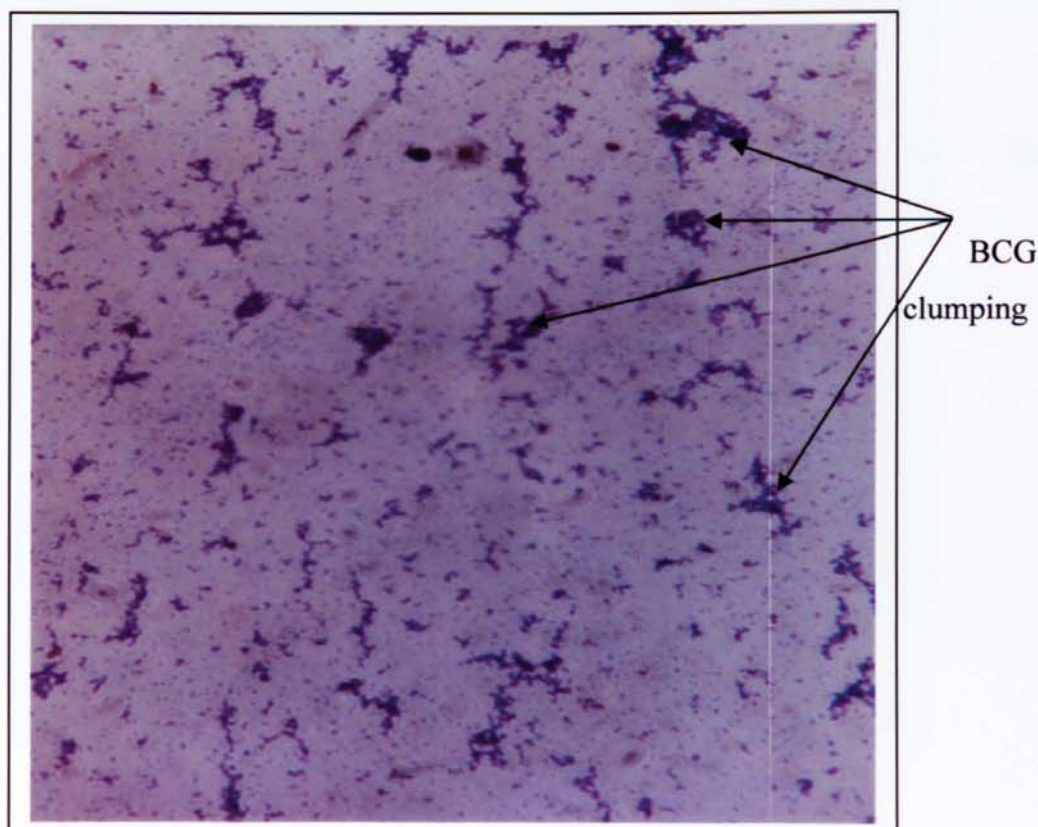
Figure 7.5 shows clumping of BCG after freezing (-80°C) for 6 hours, similar to that obtained prior to freezing.

**Table 7.12**   BCG viability after freezing at -20°C for 6 hours. Freeze-dried overnight. Powder compressed at 4 tons for 3mins. Tablet placed in HCl for 2 hours and then in HEPES solution until dissolved (with pH adjustment). Plate 100µl.

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| Eudragit L100 + mannitol | 4, 6, 1 | $4 \times 10^3$ | $4 \times 10^4$ |
| Eudragit L100 + trehalose | 3, 2, 6 | $4 \times 10^3$ | $4 \times 10^4$ |
| Eudragit L100 | 2, 4, 2 | $3 \times 10^3$ | $3 \times 10^4$ |

**Figure 7.6**   Clumping of BCG after freezing dispersions at -80°C. Freeze-drying, compression and dissolution stage shown using methylene blue stain under light microscope x10000 (Eudragit/mannitol)



BCG
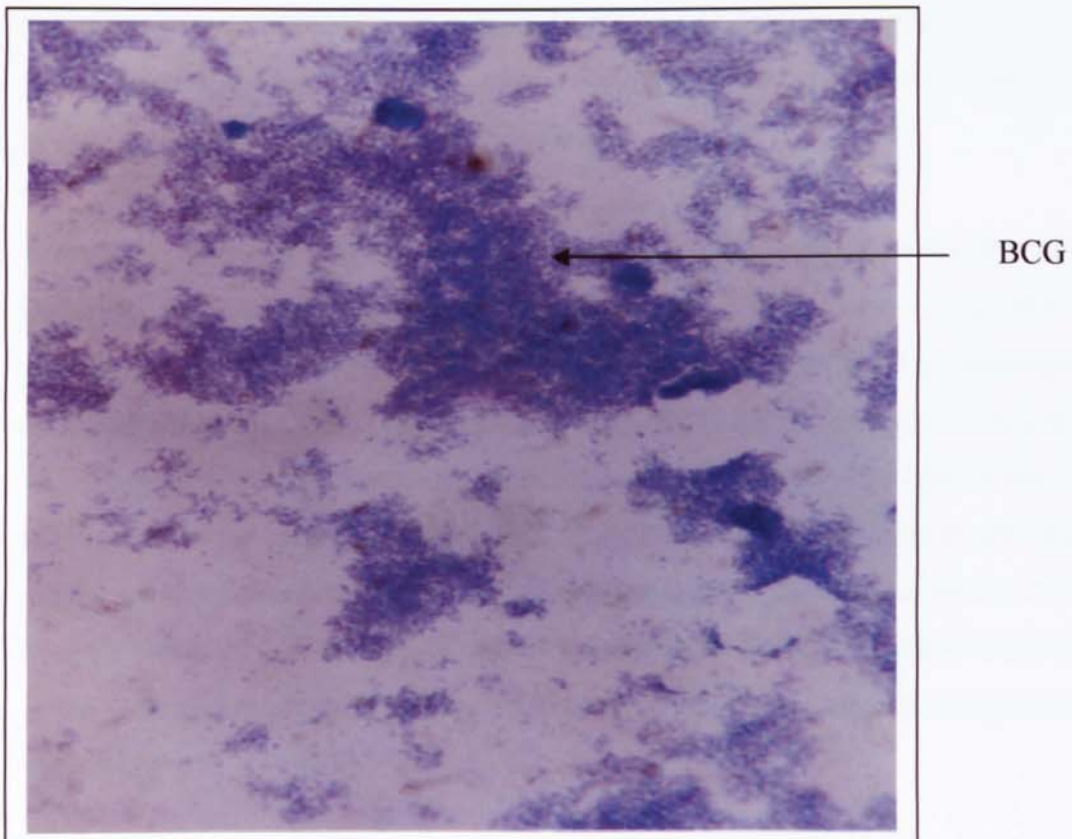
Figure 7.6 shows large aggregates of BCG. These comprise many single BCG colonies, but when grown on the Middlebrook media will only show up as one cfu. Hence there is discrepancy in the results in that the BCG in compressed matrices is alive and in greater concentration that noted in the results (Table 7.12). Having BCG clumping in that item result in reduced immune responses on release in the G.I tract.

## 7.3 Viability of BCG during formulation of Eudragit matrices using concentrated BCG suspensions

The loss in BCG viability and BCG clumping due to powder compaction to form BCG loaded matrices was not overcome by the addition of cryoprotectants. An alternative approach was taken to increase the concentration of the BCG in the initial dispersion. Consequently the loss that occurs after compression, (which according to the results so far has been by a factor of x10000), was expected to lead to a higher residual activity after matrix dissolution. The increase in viability may be sufficient to induce an immune response.

There is a 10000-fold decrease in viability from adding 100µl at the formulation stage ($10^7$ cfu) to disintegration and dissolution studies ($10^3$ cfu) using BCG at a concentration of $10^8$ cfu's/ml. The final concentration of BCG after disintegration and dissolution ($10^3$ cfu) is too low to induce an immune response. Consequently BCG was grown at VLA at a concentration of $10^{10}$ cfu's/ml. The idea being that if at the formulation stage more BCG is incorporated into the formulation and thus the matrix after compression, then after disintegration and dissolution studies the loss in cfu's will not be as low as $10^3$ cfu. It was expected that as the initial BCG added at the formulation stage is $10^9$ cfu's the final concentration after disintegration and dissolution will be $10^5$ cfu.

198

**Figure 7.7** Dispersion of BCG suspension ($10^{10}$ cfu/ml) from vial shown using methylene blue stain under light microscope x1000
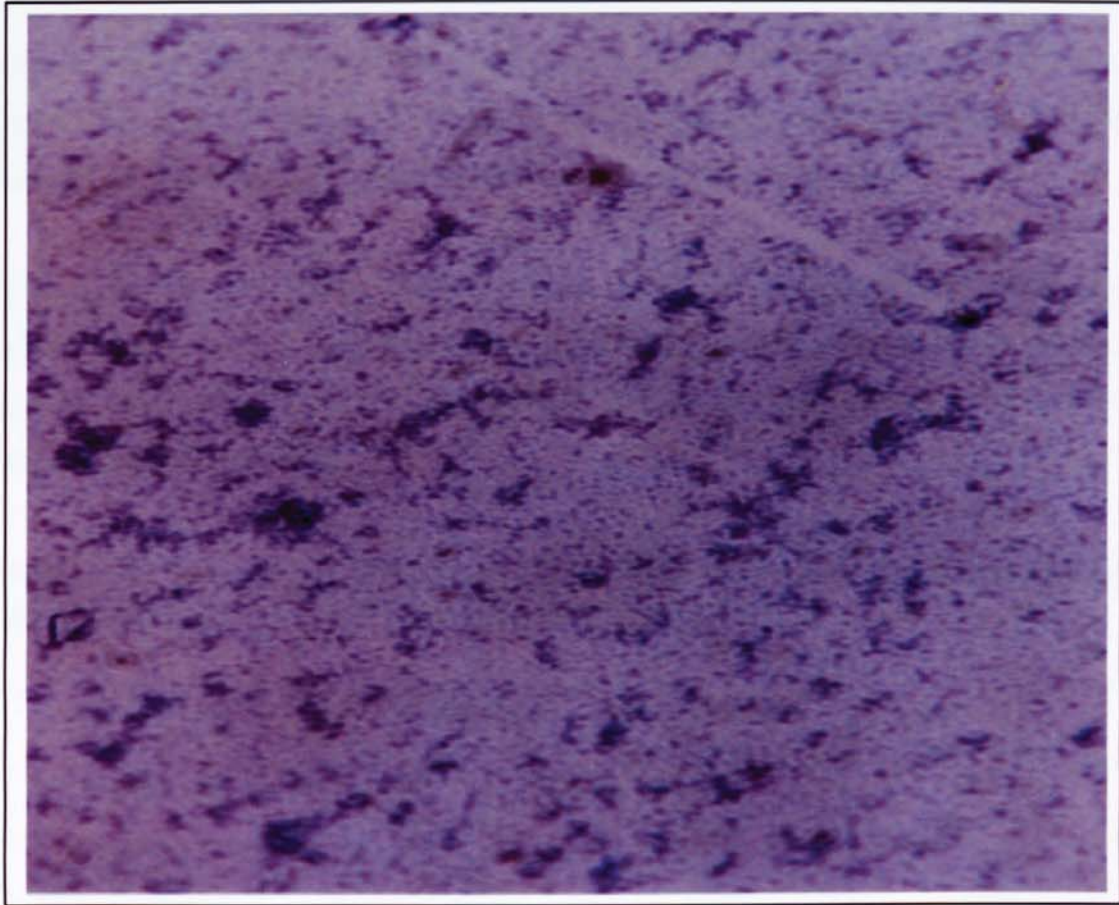


Comparisons were made between BCG-loaded Eudragit L100 matrices containing normal ($x10^8$ cfu/ml) and concentrated ($x10^{10}$ cfu/ml) BCG suspensions at each stage of formulation. The freezing temperature was decreased from -20°C to -80°C and snap freezing using liquid nitrogen was also employed. Once frozen, the samples were freeze-dried. The temperature was decreased from -20°C to -80°C because it was thought that freezing at -20°C was too slow and this allowed large crystals to form. A temperature of -80°C has frequently been used for freezing and storing of BCG (Gheorghiu *et al.*, 1988, 1996; Dietrich *et al.*, 2002; Lugosi *et al.*, 1992).

Figure 7.7 shows an optical micrograph of BCG suspension ($10^{10}$ cfu/ml). Complete dispersion does not occur and some aggregation of BCG is clearly

visible. An explanation for this is most likely due to the fact that BCG ($10^8$ cfu/ml) suspension contains Tween, which acts as a surfactant and keeps the BCG dispersed. The concentrated BCG ($10^{10}$ cfu/ml) suspension does not contain tween and also when it is thawed it does not form a suspension but forms a thick paste, which has to be dispersed in PBS and Tween. The sample was sonicated for 30 seconds to one minute using a sonicator probe to improve dispersion.

The results shown in Table 7.13 and 7.14 reveal that the concentrated BCG sample gives higher c.f.u's at each dilution and especially at $x10^6$ dilution compared to the normal BCG suspension. The concentrated BCG suspension is very thick and its appearance is like a paste.

**Table 7.13** Comparison of viability between BCG normal ($10^8$ cfu's/ml) and BCG concentrated ($10^{10}$ cfu's/ml) taken from vial (plate 100µl)

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| BCG (normal) | 200+ <br> 100, 90, 93 | $x10^5$ <br> $94x10^6$ | $x10^6$ <br> $94x10^7$ |
| BCG (concentrated) | Too many <br> Approx 500+ | $x10^5$ <br> $x10^6$ | $x10^6$ <br> $x10^7$ |

**Table 7.14** Viability of BCG taken from sample vial. Frozen at -80°C or liquid nitrogen and thawed. Resuspend in 10ml PBS (plate 100µl)

| Formulation | Temperature | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|---|
| BCG (normal) | -80°C | 200+ <br> 70, 80, 84 | $x10^5$ <br> $78x10^6$ | $x10^6$ <br> $78x10^7$ |
| BCG (concentrated) | -80°C | Too many <br> 200+ | $x10^5$ <br> $x10^6$ | $x10^6$ <br> $x10^7$ |
| BCG (normal) | Liquid nitrogen | 200+ <br> 70, 80, 88 | $x10^5$ <br> $79x10^6$ | $x10^6$ <br> $79x10^7$ |
| BCG (concentrated) | Liquid nitrogen | Too many <br> 200+ | $x10^5$ <br> $x10^6$ | $x10^6$ <br> $x10^7$ |

The results in Table 7.15 show that the concentrated BCG suspension after freeze drying give higher counts in terms of c.f.u's compared with normal BCG at each dilution. Interestingly the freezing temperature appeared to make a difference to the viability of normal BCG suspensions. There was no noticeable difference in c.f.u's produced with concentrated BCG in terms of number of colonies when frozen at -80°C or liquid nitrogen.

**Table 7.15** <u>Viability of BCG taken from vial. Frozen at -80°C or liquid nitrogen. Freeze-dried. Resuspend in 10ml PBS (plate 100µl)</u>

| Formulation | Temperature | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|---|
| BCG (normal) | -80°C | 104, 82, 89<br>13, 5, 10<br>5, 2, 4 | $97 \times 10^4$<br>$9 \times 10^5$<br>$4 \times 10^6$ | $97 \times 10^5$<br>$9 \times 10^6$<br>$4 \times 10^7$ |
| BCG (concentrated) | -80°C | Too many<br>200+ | $\times 10^5$<br>$\times 10^6$ | $\times 10^6$<br>$\times 10^7$ |
| BCG (normal) | Liquid nitrogen | 220, 180, 190<br>65, 80, 88<br>8, 14, 10 | $197 \times 10^4$<br>$\times 10^5$<br>$\times 10^6$ | $197 \times 10^5$<br>$\times 10^6$<br>$\times 10^7$ |
| BCG (concentrated) | Liquid nitrogen | Too many<br>200+ | $\times 10^5$<br>$\times 10^6$ | $\times 10^6$<br>$\times 10^7$ |

**Table 7.16** <u>BCG viability in aqueous dispersion with Eudragit L100 (plate 100µl)</u>

| Formulation | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | 100, 164, 200<br>38, 27, 31<br>6, 10, 7 | $155 \times 10^4$<br>$32 \times 10^5$<br>$8 \times 10^6$ | $155 \times 10^5$<br>$32 \times 10^6$<br>$8 \times 10^7$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | Too many<br>200+ | $\times 10^5$<br>$\times 10^6$ | $\times 10^6$<br>$\times 10^7$ |

When the concentrated BCG suspension was formulated as an aqueous dispersion with Eudragit L100 (pH adjusted to 7), the c.f.u count at each dilution was higher compared to normal BCG (Table 7.16). Table 7.17 further reveals that when the lower BCG concentration is used, freezing dispersions in liquid nitrogen resulted in higher c.f.u's at each dilution compared to freezing at -80°C.

**Table 7.17** <u>BCG viability in aqueous dispersions with Eudragit L100. Freeze at -80°C or in liquid nitrogen. Thawed. Resuspend in 10ml PBS (plate 100μl)</u>

| Formulation | Temperature | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|---|
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | -80°C | Too many<br>125, 140, 117<br>27, 40, 48<br>7, 3, 5 | $x10^3$<br>$127x10^4$<br>$38x10^5$<br>$5x10^6$ | $x10^4$<br>$127x10^5$<br>$38x10^6$<br>$5x10^7$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | -80°C | Too many<br>200+ | $x10^5$<br>$x10^6$ | $x10^6$<br>$x10^7$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | Liquid nitrogen | Too many<br>180, 175, 200<br>20, 30, 25 | $x10^4$<br>$187x10^5$<br>$25x10^6$ | $x10^5$<br>$187x10^6$<br>$25x10^7$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | Liquid nitrogen | Too many<br>200+ | $x10^5$<br>$x10^6$ | $x10^6$<br>$x10^7$ |

Although freeze-drying of aqueous dispersions containing BCG and Eudragit L100 results in a loss of BCG viability (Table 7.18). The cfu counts in the powder are fairly high and probably adequate to induce an immune response if the BCG is suitably protected during oral administration. However extensive clumping is

apparent in high concentration BCG samples following freezing at -80°C and freeze-drying.

Comparing Tables 7.18 and 7.19 it is evident that powder compaction is resulting in major loss of viability by a factor of 100. Decreasing the freezing temperature was ineffective in maintaining the viability of BCG. However c.f.u's are achieved at $x10^3$ and $x10^4$ dilution, which indicates that viable BCG is present in the Eudragit matrix for possible production of an immune response.

**Table 7.18** BCG viability following freeze-drying in aqueous dispersions with Eudragit L100. Frozen at -80°C or liquid nitrogen. Freeze-dried. Resuspend in 10ml PBS (plate 100μl)

| Formulation | Temperature | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|---|
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | -80°C | 150, 127, 100<br>25, 40, 38<br>7, 4, 8<br>0 | $126x10^3$<br>$34x10^4$<br>$6x10^5$<br>$x10^6$ | $126x10^4$<br>$34x10^5$<br>$6x10^6$<br>$x10^7$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | -80°C | Too many<br>300+<br>94, 64, 78 | $x10^4$<br>$x10^5$<br>$79x10^6$ | $x10^5$<br>$x10^6$<br>$79x10^7$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | Liquid nitrogen | Too many<br>100, 88, 94<br>20, 15, 20<br>3, 7, 8 | $x10^3$<br>$94x10^4$<br>$18x10^5$<br>$6x10^6$ | $x10^4$<br>$94x10^5$<br>$18x10^6$<br>$6x10^7$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | Liquid nitrogen | Too many<br>200, 225, 210<br>74, 64, 78 | $x10^4$<br>$212x10^5$<br>$72x10^6$ | $x10^5$<br>$212x10^6$<br>$72x10^7$ |

**Figure 7.8** Comparison of normal BCG ($10^8$ cfu/ml) and concentrated BCG suspension ($10^{10}$ cfu/ml) following freezing at -80°C and freeze-drying
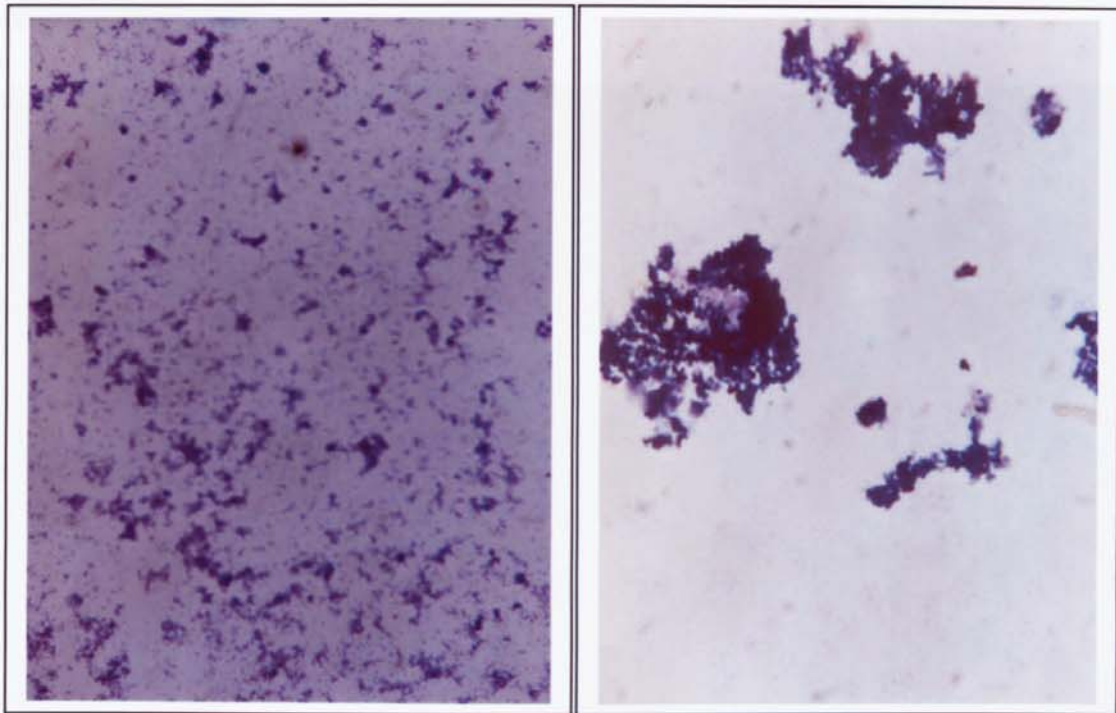


BCG Normal x1000                             BCG Concentrated x 10000

**Figure 7.9** A sample of concentrated BCG (Pasteur), frozen at -80°C and freeze-dried. Grown at $x10^6$ dilution on Midddlebrook agar
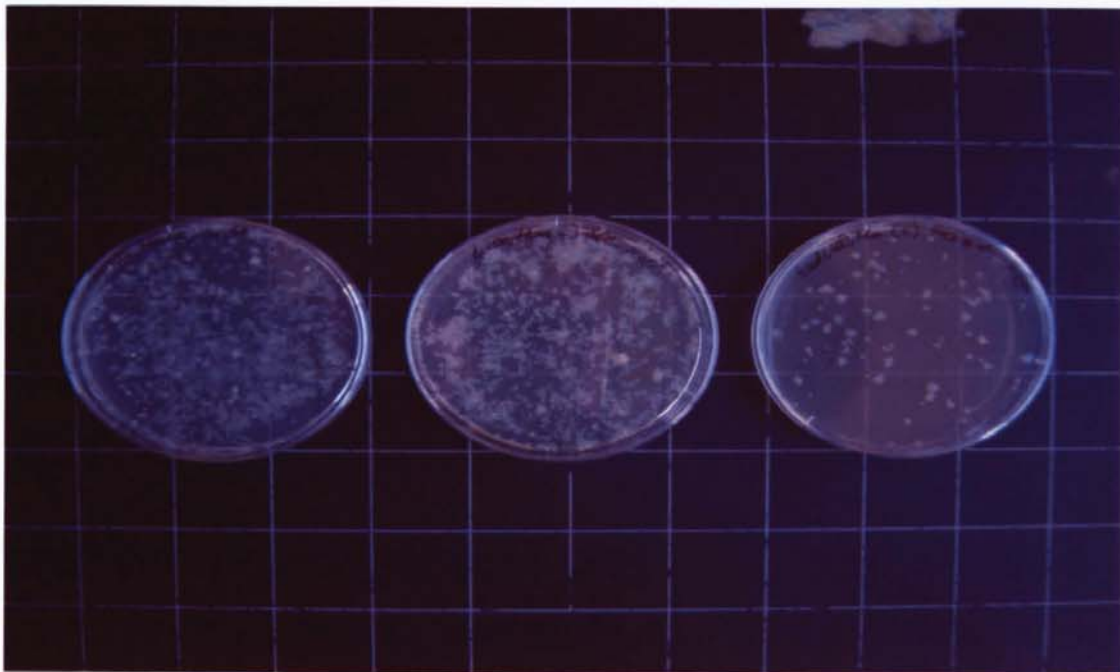
**Figure 7.10**  A sample of concentrated BCG (Pasteur) powder after freeze-drying placed directly into PBS solution at x $10^3$ (A) and $10^4$ (B) dilution and when compressed into tablet and placed in PBS (C) Grown at x $10^3$ dilution on Midddlebrook agar



A                              B                              C
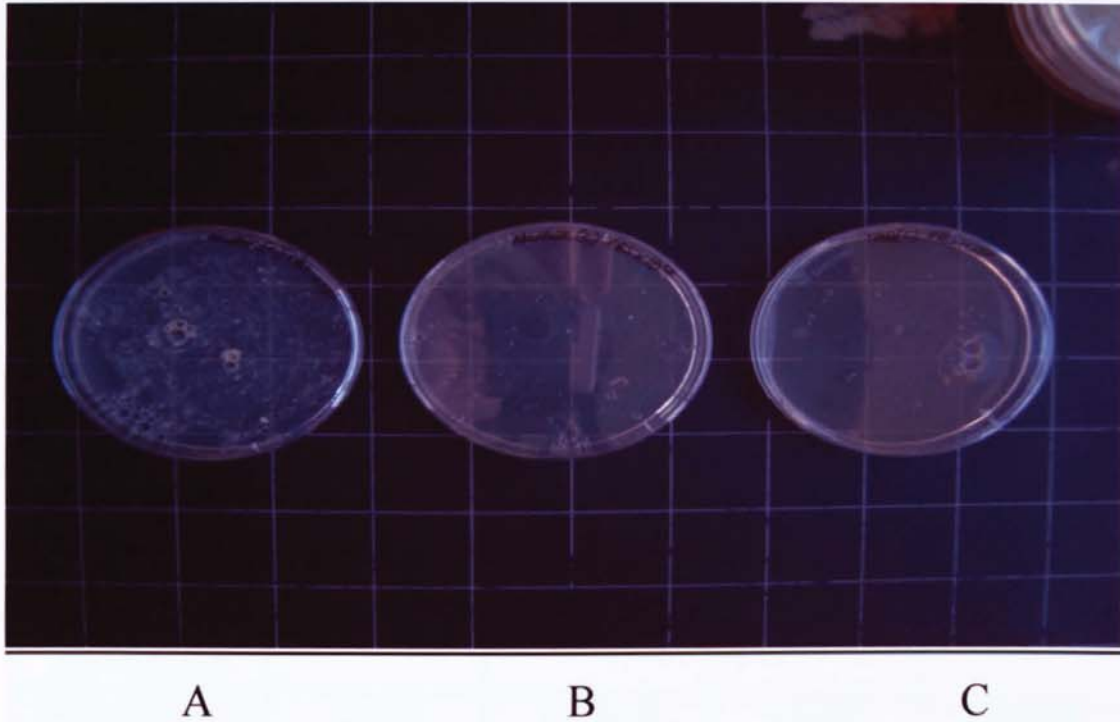
Table 7.20 shows a further loss in BCG viability by a factor of 10 when matrices were incubated in SGF for 2 hours followed by PBS. The viability achieved at $x10^3$ dilution using concentrated BCG suspension is higher than that achieved with normal BCG. Thus there is possibly a greater likelihood using matrices formulated using higher concentration of BCG suspensions of stimulating an immune response. After incubating matrices in HCl at 37°C for 2 hours, they were washed with sterilised water five times and placed in PBS solution at 37°C until dissolved. PBS solution was preferred to HEPES solution because the higher salt concentration in HEPES buffer could affect the cfu count. High salt concentration has been shown to contribute towards clumping of BCG (Felton *et al.*, 1999; Okutgen *et al.*, 1995). Thus the counts of $10^4$ cfu/ml achieved in the previous experiment (see Section 7.2, Table 7.11) may be misrepresented. The results obtained for BCG viability using

PBS as the SIF ($10^4$ cfu/ml equivalent) were similar to those achieved in the previous experiments using HEPES buffer as SIF (Table 7.11).

**Table 7.19** <u>BCG viability after compaction of freeze-dried powders. L100-BCG suspension frozen at −80°C or in liquid nitrogen. Freeze-dry. Compress 200mg powder at 4 tons for 3mins. Matrix dissolved in PBS (plate 100ul)</u>

| Formulation | Temperature | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|---|
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | −80°C | 8, 12, 10 | $10 \times 10^3$ | $10 \times 10^4$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | −80°C | 50, 40, 48 <br> 8, 10, 15 | $46 \times 10^3$ <br> $11 \times 10^4$ | $46 \times 10^4$ <br> $11 \times 10^5$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | Liquid nitrogen | 15, 10, 8 | $11 \times 10^3$ | $11 \times 10^4$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | Liquid nitrogen | 55, 40, 45 <br> 7, 5, 8 | $47 \times 10^3$ <br> $7 \times 10^4$ | $47 \times 10^4$ <br> $7 \times 10^5$ |

**Table 7.20** Viability of BCG after compression of freeze-dried powders. L100-BCG dispersion frozen at -80°C or liquid nitrogen. Freeze-dried. Compress 200mg powder at 4 tons for 3mins. Matrix immersed in HCl for 2hrs then in PBS. (plate 100µl)

| Formulation | Temperature | Number of counts | cfu at dilution factor | cfu's/ml equivalent |
|---|---|---|---|---|
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | -80°C | 3, 5, 7 | $5 \times 10^3$ | $5 \times 10^4$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | -80°C | 25, 28, 32 | $28 \times 10^3$ | $28 \times 10^4$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^8$ cfu/ml) | Liquid nitrogen | 5, 2, 4 | $4 \times 10^3$ | $4 \times 10^4$ |
| 200mg Eudragit L100 + 2ml PBS + 0.9ml Tween 80 + 100ul BCG ($10^{10}$ cfu/ml) | Liquid nitrogen | 23, 25, 18 | $22 \times 10^3$ | $22 \times 10^4$ |

## 7.4 Viability of BCG in 25mg Eudragit matrices formulated for animal studies

The Eudragit L100 matrix formulation was selected as the best overall regarding disintegration-dissolution properties and BCG viability retention at each stage of formulation. Consequently this formulation was carried forward for animal experiments to determine the immune response to 25mg matrices containing high concentrations of BCG. The Eudragit matrices with incorporated BCG were intended for oral delivery to mice. The mice would then be killed and an immune response measured to determine if BCG had been taken up. Mannitol was added at

the initial formulation stage as it has been shown to increase cfu's of BCG at the early stages of formulation (initial formulation, freezing and freeze-drying) using 200mg Eudragit L100 (see section 7.3).

In this experiment different compression forces and the amount of time the compressed force was applied to 25mg powder was studied to see the effect it has if any on the characteristics of the tablet in 0.1M HCl pH 1.2 and PBS solution pH 7 at 37°C. 25mg Eudragit L100 matrices were produced for in-vivo studies to be performed on mice. The initial work involved studying disintegration and dissolution studies followed by incorporating BCG and counting cfu's of live BCG.

### 7.4.1 Effect of compression force and compression time on disintegration and dissolution of 25mg Eudragit L100 matrices

The Eudragit matrices where placed in HCl for 2 hours followed by PBS. Table 7.21 shows that when the compression force is reduced to 2 tons, matrix disintegration in HCl increases. High compression forces increase material compaction, hence there is likely to be an increase in cohesive forces between the enteric coating polymer and other excipients (Fukui *et al.*, 2000). The forces involved include hydrogen bonding and Van Der Waals forces (Felton *et al.*, 1999). Felton *et al* (1999) have stated that there is a certain force below which interactions between molecules do not occur as effectively as at higher forces.

The amount of time for which the compression force was applied also proved to be a significant factor. Generally the longer the force was applied the less matrix disintegration occurred in HCl. If the force is not applied for long enough then adequate material compaction does not occur and weak inter-molecular forces or no forces exist between the compact excipients (Lima *et al.*, 2001). If the powder is not compact and is free flowing within the compact or on the surface then this

contributes to an unstable structure due to stresses between molecules (Lima *et al.*, 2001) which when placed in HCl causes the breakage of bonds.

**Table 7.21**  Effect of compression force and time of compression on weight loss of Eudragit L100 matrices in 0.1M HCl pH 1.2 followed by PBS at 37°C

| Compression force (tons) | Time compression force applied (mins) | % Weight loss in HCl after 2 hours | Time to dissolution in PBS (hrs) |
|---|---|---|---|
| 4 | 3 | 6.4 | >16 |
|   | 2 | 8.6 |   |
|   | 1 | 9.1 |   |
| 3 | 3 | 6.0 | >16 |
|   | 2 | 4.9 |   |
|   | 1 | 5.0 |   |
| 2 | 3 | 8.0 | ~12 |
|   | 2 | 12.8 |   |
|   | 1 | 11.3 |   |
| 1 | 3 | 10.0 | ~6 |
|   | 2 | 18.0 |   |
|   | 1 | 20.2 |   |

High compression forces can result in slow dissolution of matrix and exceed the G.I.T transit time in the small intestine of 2 to 3 hours (Marvola *et al.*, 1999). The results in Table 7.21 show that only the compression force influences matrix dissolution rate in PBS. The higher the applied force the more compact the matrix becomes and thus the stronger the interaction forces between excipient molecules (Carelli *et al.*, 1999; Felton *et al.*, 1999). Hence a very low dissolution rate was obtained. Although the higher force applied may contribute to greater stability in acidic conditions; it seems to inhibit the dissolution properties of the tablet in PBS solution. The results in Table 7.21 indicate that the dissolution of the 25mg matrices investigated would occur well outside the G.I.T transit time and thus effective BCG delivery to the Peyer's patches and immune responses would not be achieved (Clarke *et al.*, 1993). Modification of the compressive force is required to improve the dissolution properties of 25mg Eudragit L100 matrices.

Despite the low dissolution rate of 25mg Eudragit L100 matrices (Table 7.21), BCG viability was investigated to analyse the effects of smaller matrix formulations since future animal studies could utilise this configuration. Concentrated BCG suspensions ($10^{10}$ cfu/ml) were used due to the fact that there was an increase in viability at each stage of formulation (prior to compaction) compared to normal BCG suspension ($10^8$ cfu/ml). Also the freezing temperature of -80°C was selected since freezing using liquid nitrogen did not enhance the BCG viability in previous experiments.

BCG viability was investigated prior to formulation and then also at freezing (-80°C) and freeze-drying stages. The results were found to be similar to those observed in the previous section 7.3 (Table 7.13, 7.14 and 7.15).

Compression of the freeze-dried powder into 25mg matrices (1 ton, 3 minutes) resulted in complete loss of BCG viability. The smaller matrix diameter results in a higher compressive stress, which may effectively lead to damage the majority of BCG present in the formulation by the salt crystals formed during freezing and freeze drying.

Freeze-dried powders containing Eudragit and viable BCG were incubated in HCl followed by PBS solution to determine whether the powder alone can protect BCG. This would enable direct incorporation into the bait used for oral badger vaccines. This approach was unsuccessful (no cfu content at $10^3$ dilution, underlining the need for additional protection of powdered formulations against low pH environments.

## 7.5 Conclusion

Eudragit L100 and S100 based aqueous dispersions proved to be effective in retaining BCG viability in freeze-dried powder format [cfu/ml were reduced by a

factor of 100 relative to stock BCG ($10^8$ cfu/ml)]. Matrix formulation by compression resulted in BCG activity retention of $10^4$ cfu/ml. However a major problem encountered during this process is the formation of crystals during freezing and freeze-drying, which upon powder compression ruptures the bacterial cell wall, resulting in inactivation of a high proportion of the bacteria [cfu/ml count reduced by a factor of 10000 relative to BCG stock ($10^8$ cfu/ml)].

The addition of cryoprotectants (mannitol, trehalose), lowering the freezing temperature from -20°C to -80°C and snap freezing using liquid nitrogen were ineffective in maintaining the BCG viability in matrices prepared by compression of freeze-dried powders. BCG viability was of the order $10^4$ cfu/ml. Crystal formations under freezing and freeze-drying conditions probably damage the cell walls of BCG during powder compaction. Increasing the stock BCG concentration ($10^{10}$ cfu/ml) in the formulation was ineffective in increasing the number of colony forming units in the matrices. When producing 25mg Eudragit L100 matrices the BCG was rendered totally inactive probably due to the higher stress experienced during matrix compaction resulting in efficient rupturing of the cell walls of BCG bacteria. Hence increasing BCG concentration, lowering the freezing temperature or adding cryoprotectants did not increase the viability as expected and thus after compression the viability was lost. The results of Eudragit formulations and matrices containing BCG are summarised in tables 7.22, 7.23 and 7.24.

Further work is necessary to improve the viability of BCG incorporated in Eudragit matrices possibly by means of binder selection to reduce the force experience by the BCG. Alternatively investigations using other enteric coating polymers such as CAP could be pursued to avoid incorporation of a crystalline salt component in the formulation for pH adjustment.

**Table 7.22**  Viability of BCG at each stage of formulation

| Eudragit matrix | Viability of initial aqueous formulations (pH 7) (cfu/m)l | Viability after freezing (-20°C) (cfu/ml) | Viability after freeze-drying (cfu/ml) | Viability after 2 hours incubation in HCl and dissolved in PBS (cfu/ml) |
|---|---|---|---|---|
| Eudragit L100 + 2ml PBS solution + 0.9ml Tween 80 (0.1%w/v) | $10 \times 10^6$ | $11 \times 10^6$ | $3 \times 10^6$ | $3 \times 10^4$ |
| Eudragit L100 + 2ml PBS solution + 0.9ml Tween 80 (0.1%w/v) + mannitol | $14 \times 10^6$ | $14 \times 10^6$ | $8 \times 10^6$ | $4 \times 10^4$ |
| Eudragit L100 + 2ml PBS solution + 0.9ml Tween 80 (0.1%w/v) + trehalose | $13 \times 10^6$ | $13 \times 10^6$ | $7 \times 10^6$ | $3 \times 10^4$ |

**Table 7.23**  Summary of BCG viability of stock

| BCG concentration | Temperature of freezing (°C) | Viability from vial (cfu/ml) | Viability after freezing (cfu/ml) | Viability after freeze-drying (cfu/ml) |
|---|---|---|---|---|
| $\times 10^8$ | -80 | $100 \times 10^7$ | $70 \times 10^7$ | $5 \times 10^7$ |
| $\times 10^8$ | Liquid nitrogen | $100 \times 10^7$ | $70 \times 10^7$ | $8 \times 10^7$ |
| $\times 10^{10}$ | -80 | $500 \times 10^7$ | $200 \times 10^7$ | $200 \times 10^7$ |
| $\times 10^{10}$ | Liquid nitrogen | $500 \times 10^7$ | $200 \times 10^7$ | $200 \times 10^7$ |

**Table 7.24** Summary of BCG viability (normal and concentrated) loaded matrices with mannitol

| Eudragit matrix | Viability after initial formulation (pH 7) (cfu/ml) | Viability after freezing (cfu/ml) | | Viability after freeze-drying (cfu/ml) | | Viability after 2 hours incubation in HCl and dissolved in PBS (cfu/ml) | |
|---|---|---|---|---|---|---|---|
| | | -80°C | Liq. $N_2$ | -80°C | Liq. $N_2$ | -80°C | Liq. $N_2$ |
| Eudragit L100 + 2ml PBS solution + 0.9ml Tween 80 (0.1%w/v) + mannitol ($10^8$ cfu/ml) | $8 \times 10^7$ | $5 \times 10^7$ | $25 \times 10^7$ | $6 \times 10^6$ | $6 \times 10^6$ | $5 \times 10^4$ | $4 \times 10^4$ |
| Eudragit L100 + 2ml PBS solution + 0.9ml Tween 80 (0.1%w/v) + mannitol ($10^{10}$ cfu/ml) | $\sim200 \times 10^7$ | $\sim200 \times 10^7$ | $\sim200 \times 10^7$ | $79 \times 10^7$ | $72 \times 10^7$ | $28 \times 10^4$ | $22 \times 10^4$ |

Liq. $N_2$ = Liquid Nitrogen

# CHAPTER 8

# APPENDICES

# 8 APPENDICES

**Appendix 1** Preparation of Borate and Saline buffers

Saline buffer was prepared by dissolving 35g of sodium chloride in 1000ml to give a 1M solution. Borate buffer was prepared by dissolving 61.84g of Boric acid crystals in 1000ml to give a 1M solution.

**Appendix 2** Properties of Eudragit S100, Eudragit L30 D-55, Eudargit L100 and Eudragit L100-55

| Type | Commercial form | Commercial content | Particle size | Solubility | Solubility in small intestine (pH) |
|---|---|---|---|---|---|
| Eudragit S100 | Solid in the form of white, very fine, free flowing powders with weakly sour odour | At least 95% of dry substance | At least 95% less than 0.25mm | 1g of the substance dissolve in 7g of methanol, ethanol, isopropyl alcohol or acetone. It is also soluble in liquids above pH 7 | pH 7 and above |
| Eudragit L30 D 55 | Aqueous dispersion, milky-white liquid of low viscosity with a weakly sour odour | Aqueous dispersion containing 30% dry substance. The dispersion contains 0.7% of Sodium Lauryl Sulphate and 2.3% of Polysorbate | Not available | The aqueous dispersion is miscible with water and a viscous solution is obtained on mixing one part of Eudragit L 30 D with 5 parts acetone, isopropyl alcohol or ethyl alcohol. | pH 5.5 and above |
| Eudragit L100 | Solid in the form of white, very fine, free flowing powders with weakly sour odour | At least 95% of dry substance | At least 95% less than 0.25mm | 1g of the substance dissolve in 7g of methanol, ethanol, isopropyl alcohol or acetone. It is also soluble in liquids above pH 7 | pH 6.0 upwards |
| Eudragit L100-55 | Solid in the form of white, very fine, free flowing powders with weakly sour odour | The product contains 0.7% of Sodium Lauryl Sulphate and 2.3% of Polysorbate as emulsifiers | At least 95% less than 0.25mm | 1g dissolves in 7g methanol, alcohol, isopropyl alcohol and acetone and in sodium hydroxide 1M to give a clear opalescent solution. | pH 5.5 upwards |

# CHAPTER 9

# REFERENCES

# 9 REFERENCES

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223

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