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FORMULATION AND CHARACTERISATION OF AN EFFECTIVE PARTICULATE DELIVERY VEHICLE FOR THE NOVEL SUB-UNIT VACCINE ANTIGEN, Ag85B-ESAT-6

DANIEL JON KIRBY

Doctor of Philosophy

ASTON UNIVERSITY

July 2007

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This research focused on the formulation of particulate delivery systems for the sub-unit fusion protein, Ag85B-ESAT-6, a promising tuberculosis (TB) vaccine candidate.

Initial work concentrated on formulating and characterising, both physico-chemically and immunologically, cationic liposomes based on the potent adjuvant dimethyl dioctadecyl ammonium (DDA). These studies demonstrated that addition of the immunomodulatory trehalose dibehenate (TDB) enhanced the physical stability of the system whilst also adding further adjuvanticity. Indeed, this formulation was effective in stimulating both a cell mediated and humoural immune response.

In order to investigate an alternative to the DDA-TDB system, microspheres based on poly(DL-lactide-co-glycolide) (PLGA) incorporating the adjuvants DDA and TDB, either alone or in combination, were first optimised in terms of physico-chemical characteristics, followed by immunological analysis. The formulation incorporating PLGA and DDA emerged as the lead candidate, with promising protection data against TB.

Subsequent optimisation of the lead microsphere formulation investigated the effect of several variables involved in the formulation process on physico-chemical and immunological characteristics of the particles produced. Further, freeze-drying studies were carried out with both sugar-based and amino acid-based cryoprotectants, in order to formulate a stable freeze-dried product. Finally, environmental scanning electron microscopy (ESEM) was investigated as a potential alternative to conventional SEM for the morphological investigation of microsphere formulations.

Results revealed that the DDA-TDB liposome system proved to be the most immunologically efficient delivery vehicle studied, with high levels of antibody and cytokine production, particularly gamma-interferon (IFN-γ), considered the key cytokine marker for anti-mycobacterial immunity. Of the microsphere systems investigated, PLGA in combination with DDA showed the most promise, with an ability to initiate a broad spectrum of cytokine production, as well as antigen specific spleen cell proliferation comparable to that of the DDA-TDB formulation.

Key words: liposome; microsphere; PLGA; adjuvant; immunisation.

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Publications resulting from this and related work

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Abbreviations list

Ag85B	Antigen 85B
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
APC	antigen presenting cell
BCG	Bacille Calmette-Guérin
CD4	cluster of differentiation 4
CTAB	cetyl trimethyl ammonium bromide
CTL	cytotoxic T lymphocytes
DCs	dendritic cell
ddH2O	double distilled water
DDA	dimethyldioctadecyl ammonium
DNA	deoxyribose nucleic acid
DOTAP	dioleoyl-trimethyl-ammonium-propane
DOTS	directly observed treatment, short-course
ELISA	enzyme linked immunosorbent assay
EPI	expanded programme on immunisation
ESAT-6	6 kDa early secretory antigenic target
ESEM	environmental scanning electron microscopy
Fab	antigen-binding fraction
Fc	crystallisable fraction
FCA	Freund's complete adjuvant
GIVS	global immunisation vision and strategy
HBV	hepatitis B virus
HIV	human immunodeficiency virus
IFN-γ	gamma-interferon
Ig	immunoglobulin
IL	interleukin
ISCOM	immune-stimulating complex
MDP	muramyl dipeptide
MDR	multidrug-resistant
MHC	major histocompatibility complex
MMR	measles, mumps and rubella combination vaccine
MPL	monophosphoryl lipid A
Mtb	*Mycobacterium tuberculosis*

NaCl	sodium chloride
NICE	National Institute for Clinical Excellence
NISV	non-ionic surfactant vesicles
ODN	oligodeoxynucleotides
o/w	oil-in-water
PAMP	pathogen associated molecular pattern
PAS	para-amino salicylic acid
PBS	phosphate buffered saline
PC	phosphatidyl choline
pI	isoelectric point
PLA	poly(lactide)
PLG	poly(glycolide)
PLGA	poly(DL-lactide-co-glycolide)
PRR	pattern recognition receptor
PVA	poly(vinyl alcohol)
RNA	ribonucleic acid
rpm	revolutions per minute
s.d.	standard deviation
SEM	scanning electron microscopy
TB	tuberculosis
TDB	trehalose 6,6'-dibehenate
TDM	trehalose dimycolate
TEM	transmission electron microscopy
Th1	T-helper type 1
Th2	T-helper type 2
TLR	toll-like receptor
VLP	virus-like particles
WHO	World Health Organisation
w/o/w	water-in-oil-in-water
XDR	extensively drug-resistant
ZP	zeta potential

Chapter 1

General introduction

1.1. Vaccines

1.1.1. Brief history

The term vaccine, derived from the latin *vacca*, meaning cow (Mackett & Williamson, 1995), originates from the first recorded successful inoculation experiment by Edward Jenner in 1794. Jenner realised that pre-exposure to the animal related disease cowpox (*vaccinia*), led to protection against the more aggressive and deadly human disease, smallpox. This event can be seen as the birth of vaccinology, and the term vaccination has subsequently become synonymous with inoculation, although such practices had existed since the ancient Chinese practice of inoculation with pus from smallpox patients, whilst lay persons utilised Jenner's technique long before he presented his findings (Hilleman, 2000; Plotkin, 2005).

However, it was not until 80 years after Jenner's studies that other vaccines began to be developed, following the studies of Robert Koch, who gave credibility to the 'germ theory of disease' in 1876 (Mackett & Williamson, 1995), and the advent of the 'golden age' of immunology (Bramwell & Perrie, 2005b). The first evidence of the use of this new knowledge and the consequent rational design of vaccines was in the 1870's, when Louis Pasteur described the use of attenuated chicken cholera vaccines, following an accidental injection of an old culture of chicken cholera microorganisms (Goldsby et al., 2003; Plotkin, 2005). Subsequent vaccines against rabies and anthrax were then developed to significant economic benefits (Bramwell & Perrie, 2005b). Further seminal studies by such workers as von Behring, Ehrling and Metchnikoff both advanced

knowledge of immunological phenomena and focussed efforts in vaccinology (Hilleman, 2000; Plotkin, 2005).

Nevertheless, it was not until the cell-culture revolution between 1950-1980 that really kick-started the use of attenuated viral vaccines and the isolation of sub-units of infectious agents (Baker & Katz, 2004; Plotkin, 2005; Bramwell & Perrie, 2005b). In addition, further advances in immunology, a greater understanding of host-pathogen interactions and the dawn of genetics and recombinant DNA technology has led to a broadening of horizons in vaccine design (Liu, 1999; Plotkin, 2005; Bramwell & Perrie, 2005b).

1.1.2. Outline of vaccine function in preventing disease

Although antibiotics and improved living conditions have reduced deaths from infectious agents in developed countries, vaccination remains the 'gold standard' in preventing rather than treating diseases (Mackett & Williamson, 1995; Perrie et al., 2007a). The purpose of any vaccine is to exploit the natural defence mechanisms of a body's immune system to induce memory and long-term protection against a pathogen without causing disease (Playfair & Bancroft, 2004; Perrie, 2006).

The first line of defence against pathogens is referred to as innate immunity, which acts in a rapid, 'non-specific' manner and includes physical (skin and mucous membranes), physiological (pH, temperature), phagocytic and inflammatory barriers (Goldsby et al., 2003; Storni et al., 2005).

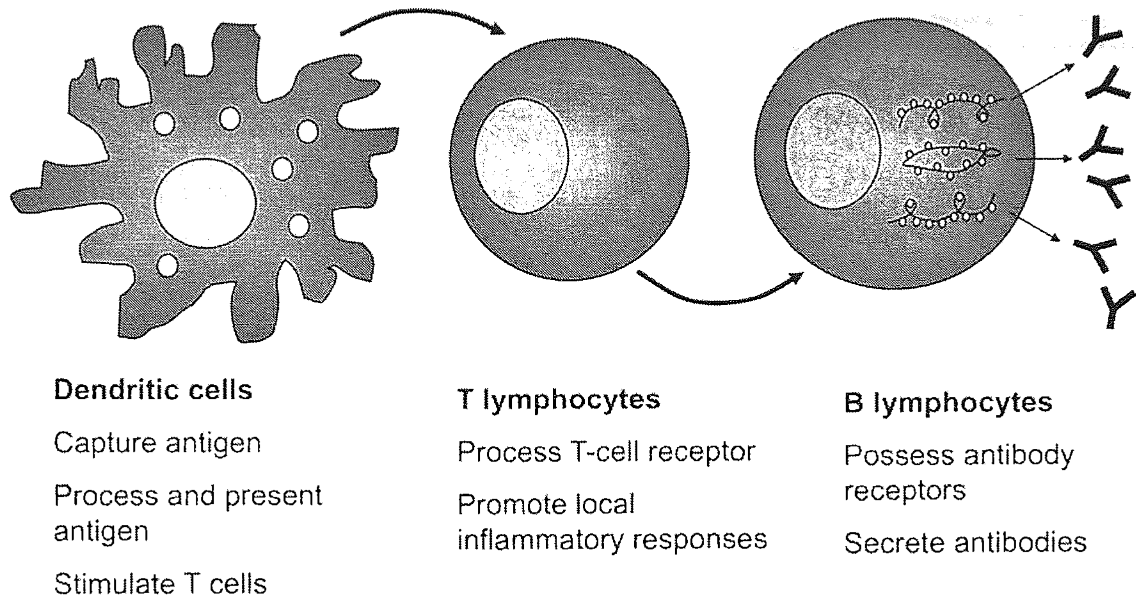

Fig. 1.1. Cell types that collaborate in immune responses. Antigen presenting cells (APCs), and dendritic cells in particular, are responsible for capturing and processing antigen, followed by subsequent stimulation of thymus derived lymphocytes (T cells). T cells, in turn, promote inflammatory responses and secrete cytokines, which can lead to activation of antibody secreting bone marrow derived lymphocytes (B cells). *Figure adapted from (Storni et al., 2005).*

Intruding micro-organisms that evade the physical and physiological barriers display certain pathogen associated molecular patterns (PAMPs) on their surface (Hashimoto et al., 1988), which assist the immune system in distinguishing between self and non-self through interaction with pattern recognition receptors (PRR). Molecules capable of pattern recognition can be either soluble (lysozyme, complement) or cell-associated, particularly on the surface of antigen-presenting cells (APCs) (e.g. macrophages, dendritic cells) (Medzhitov & Janeway, 1998; Goldsby et al., 2003; Playfair & Bancroft, 2004; Storni et al., 2005). These cell-associated receptors have been termed Toll-like receptors (TLRs), and their stimulation activates important mediators of innate and adaptive immunity, leading to a transmission of a 'danger signal' to cells of the immune system (Takeuchi & Akira, 2001; Akira, 2003; Storni et al., 2005; Lendemans et al., 2005). Of the APCs, dendritic cells are considered the most important in recognition,

capture and processing of foreign antigens in innate immunity (Nossal, 1997; Playfair & Bancroft, 2004; Perrie et al., 2007a) (Fig. 1.1.).

Innate immunity is followed by adaptive (or acquired) immunity, which is a more specific response regulated by thymus-derived lymphocytes (T cells) and bone-marrow derived lymphocytes (B cells) (Playfair & Bancroft, 2004; Storni et al., 2005; Perrie et al., 2007a) (Fig. 1.2.). However, adaptive and innate immunity are not independent, since direct interaction with APCs is required in order for an effective adaptive immune response (Goldsby et al., 2003).

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Fig. 1.2. Cellular interactions involved in the induction of adaptive immunity. (a) T-helper cells (T_H) are activated through antigen presentation via MHC II molecules. Subsequent differentiation can lead to effector and memory T cells, or (b) activation of B cells through the secretion of certain cytokines (IL-4, IL-5 and IL-6). (c) Alternatively, T cells can differentiate into cytotoxic T-lymphocytes (CTL) through antigen presentation via MHC I molecules, an important facet in anti-tumour activity. *Figure from (Kuby, 1997).*

Following uptake by APCs, the pathogen is degraded by lysosomal enzymes, and fragments presented on the surface of the APCs via class II major histocompatibility complex (MHC II) molecules (Pierre et al., 1997), which, along with costimulatory molecules (particularly cell-surface protein B7), stimulate CD4 T-helper cells (Th) required to initiate most adaptive immune responses (Storni et al., 2005; Perrie et al., 2007a) (Fig. 1.2. and 1.4.). An alternative pathway, mediated by class I MHC and CD8 T cells, leading to cytotoxic T lymphocytes (CTLs), is important for immunity against viruses and tumours so will not be dealt with in the remit of this thesis (Fig. 1.2.).

The consequent adaptive immune response can further be divided into two sub-categories depending on the subsequent differentiation of the Th cell: humoural and cell-mediated immunity.

APC mediated T cell activation can lead to subsequent B-cell stimulation, which possess membrane-bound antibody molecules that act as antigen receptors. The humoural branch of the immune system relies on interaction of B cells with antigen through these receptors, leading to B-cell proliferation and differentiation into antibody-secreting cells (Goldsby et al., 2003).

Antibodies (also called immunoglobulins (Ig)) act as effector molecules, which specifically bind to antigen and facilitate its clearance from the body by several methods (e.g. opsonisation, neutralisation and activation of the complement system) (Goldsby et al., 2003; Playfair & Bancroft, 2004).

There are five different classes of antibody (IgM, IgG, IgD, IgE and IgA), each associated with different functions, although all related by a common basic structure (Fig. 1.3.). Antibodies are made up of four polypeptide chains, two identical light chains (molecular weight ~25,000) and two identical heavy chains (molecular weight $\geq$ 50,000), which determine antibody type.

Each heavy and light chain contains an amino-terminal variable region (V) and a constant region (C) as its stem. It is the variable region that distinguishes specificity (Fab), whereas the constant region determines biological activity (Fc) (Goldsby et al., 2003; Playfair & Bancroft, 2004) (Fig. 1.3.).

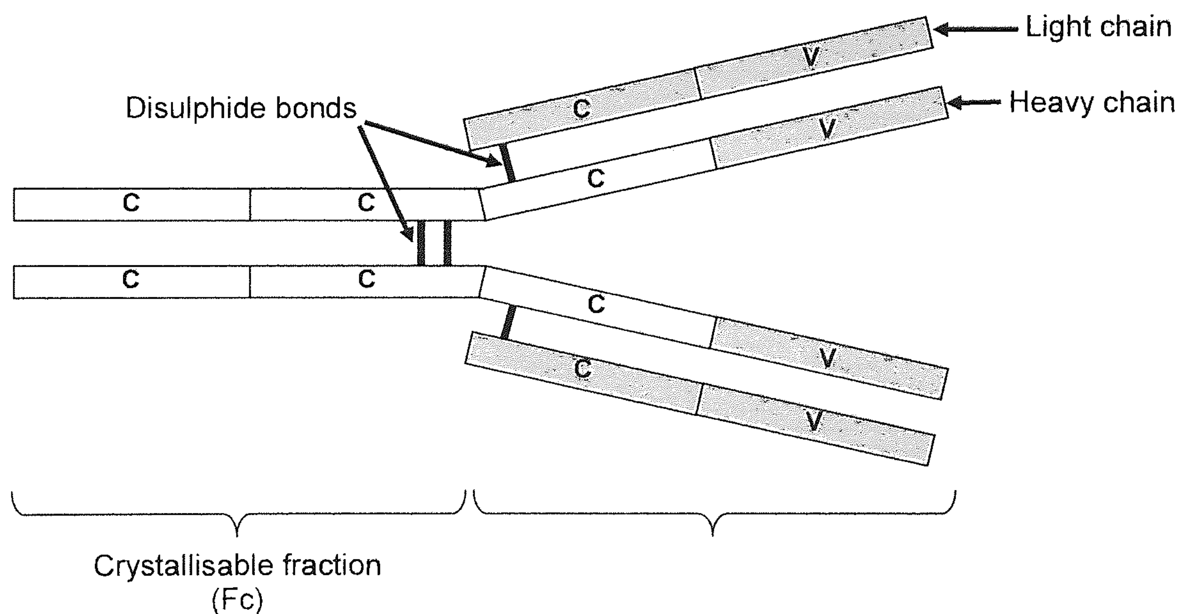

Fig. 1.3. Schematic representation of common antibody structure. The structure is characterised by two identical heavy and light chains. Each chain contains an amino-terminal variable region (V), which is responsible for binding antigen, and a constant region (C), which defines the class of antibody, and thus the biological function.

In contrast to B cells, T cells can only recognise antigen associated with MHC molecules (Fig. 1.4.). Following activation, T cells begin to proliferate and secrete various cytokines, giving rise to a clone of effector cells (Goldsby et al., 2003). Depending on the duration of antigen stimulation and the cytokine environment to which the cells are exposed, the subsequent response can be polarised to yield either a T-helper type 1 (Th1) or type 2 (Th2) response (Storni et al., 2005) (Table 1.1.).

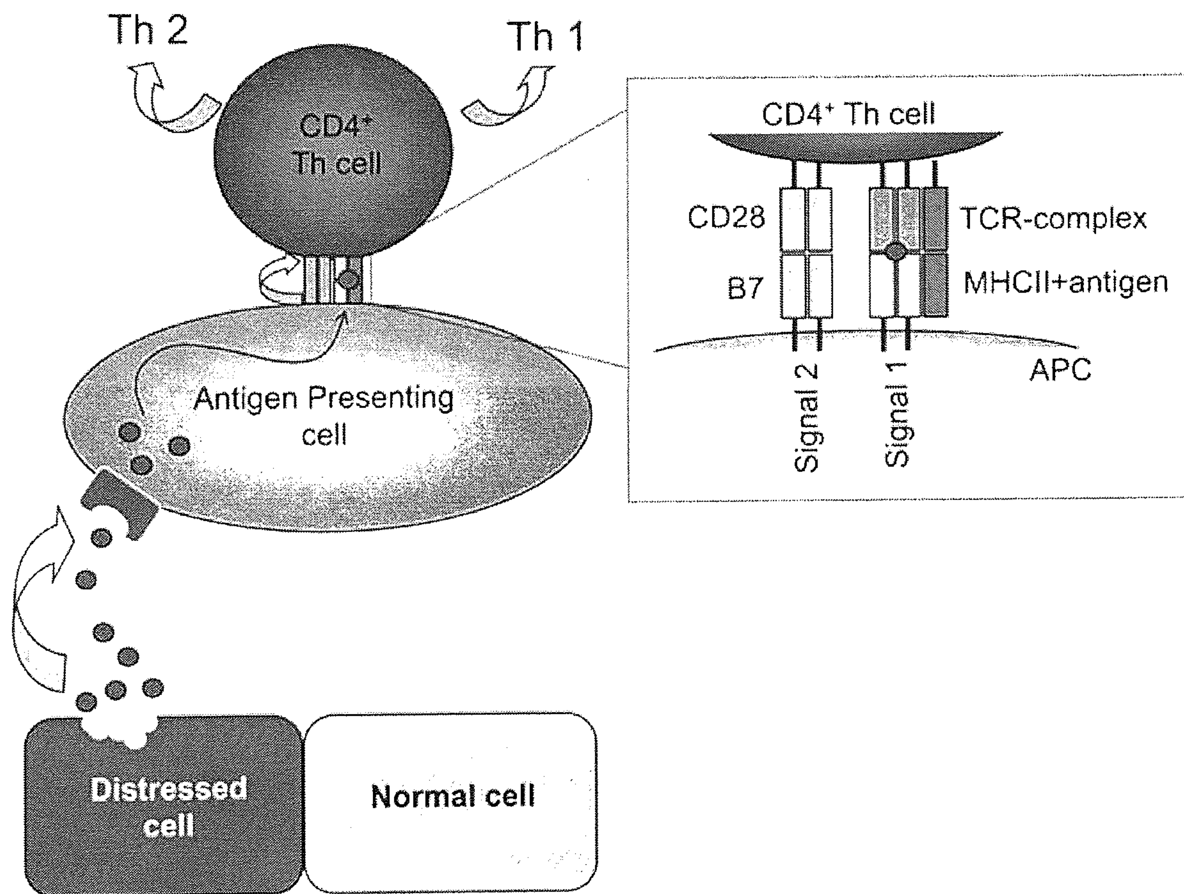

Fig. 1.4. Schematic representation of antigen uptake and presentation. Immunity is primarily activated by danger signals (Signal 0), which are typically mediated by Toll-like receptors and enable innate and adaptive immunity through antigen presentation (Signal 1) and subsequent co-stimulation (Signal 2) of $CD4^+$ T-helper (Th) cells. Activation of Th cells results in the secretion of cytokines, which can polarise the immune response to yield either a Th1 (mainly through T cells and macrophages) or Th2 (mainly through B cells) type response. *Figure adapted from (Storni et al., 2005).*

	Immune response	
	T helper type 1 (Th1)	**T helper type 2 (Th2)**
Cytokines	IL-2, IL-12, IL-18, IFN-γ, IFN-α	IL-2, IL-4, IL-5, IL-6, IL-10, IL-13
Main cell type	T cells Macrophages	B cells

Table 1.1. Summary of the major cytokines and cell types involved in T helper type 1 (Th1) and T helper type 2 (Th2) immune responses. Following T cell activation, the subsequent immune response can be polarised to either a Th1 or Th2, depending on duration of antigen stimulation and the resulting cytokine environment.

The Th1 response is characterised by secretion of cytokines such as gamma-interferon (IFN-γ), which supports inflammation and activates mainly certain T cells and macrophages, and is thus active in cell mediated immunity. This enhanced activation of phagocytic cells is particularly important in anti-bacterial immune responses. On the other hand, Th2 cells activate mainly B cells and immune responses that depend on antibody, through secretion of cytokines such as interleukin-4 (IL-4), IL-5 and IL-6 (Goldsby et al., 2003; Playfair & Bancroft, 2004).

As well as differentiating into effector cells, certain lymphocytes remain as memory cells (Fig. 1.2.), perhaps the most important consequence of adaptive immunity, particularly for successful vaccines (Nossal, 1997; Storni et al., 2005). Upon second entry of the antigen, the immune system will react quickly to provide a faster, stronger and specific immune response, thus preventing symptoms of the disease from occurring (Perrie et al., 2007a) (Fig. 1.5.).

However, despite this complex and well developed defence system, the primary immune response can be slow and limited, leaving the body vulnerable to infection and often inadequate to prevent the development of serious symptoms. Therefore, as mentioned previously, the purpose of a vaccine is the induction of appropriate and effective immunity to an infectious agent without the need to suffer an initial infection (Mackett & Williamson, 1995; Bramwell & Perrie, 2005b; Perrie, 2006) (Fig. 1.5.).

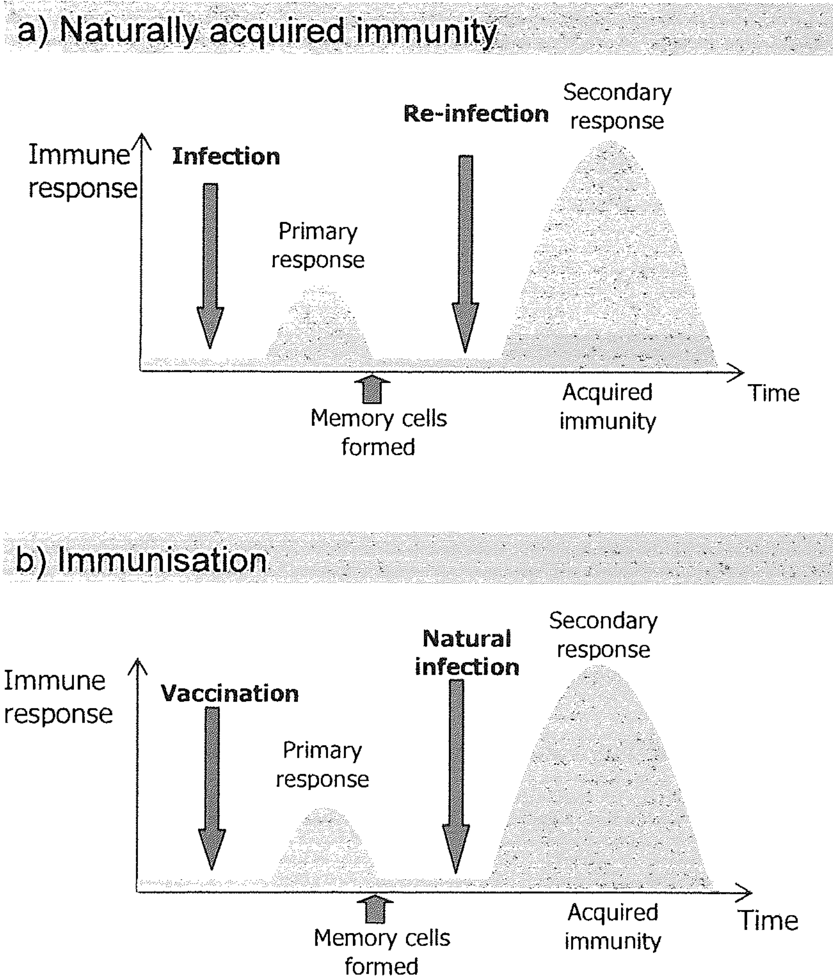

Fig 1.5. The principle of vaccination. (a) During natural infection the immune system will initiate a primary immune response and generate memory cells. On subsequent infections, memory cells are re-stimulated allowing the body to mount a stronger and more effective response on subsequent re-infection. (b) Administration of a vaccine allows the body to generate memory cells without exposure to a primary infection. Thereafter when the body is presented with a natural infection it is able to produce a faster and more intense immune response than the unvaccinated primary response. *Adapted from (Roitt et al., 1996).*

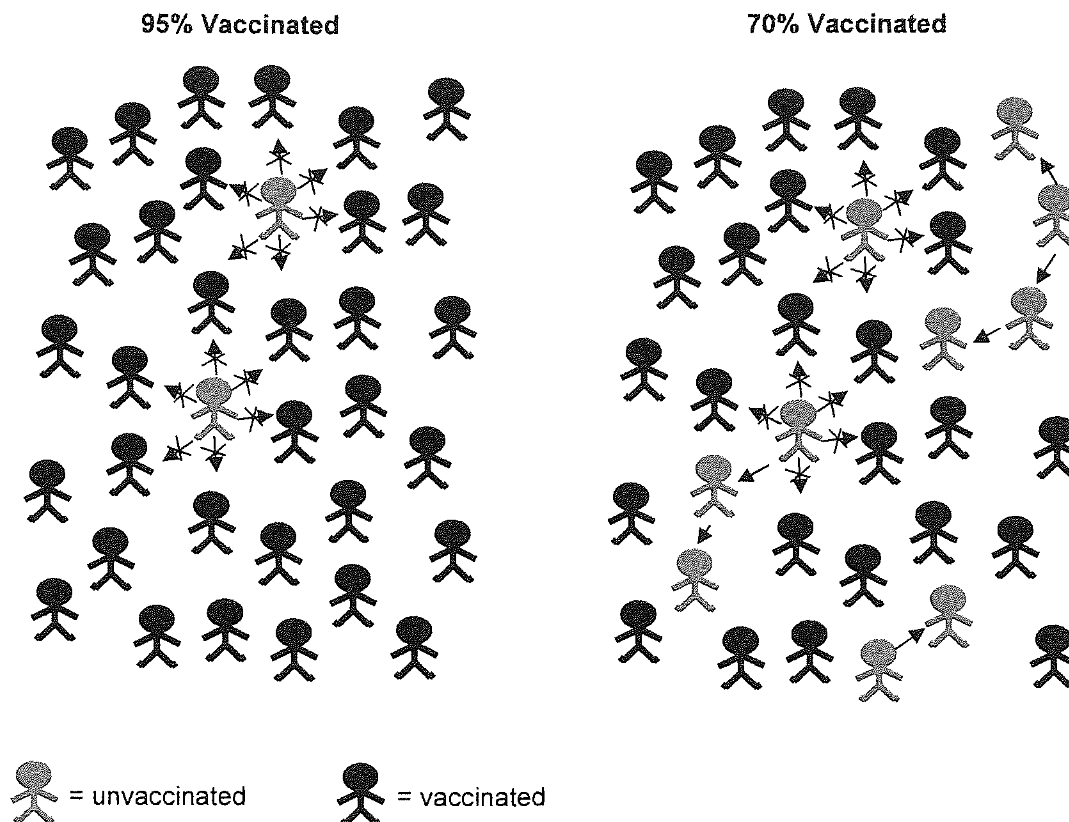

Fig 1.6. Schematic representation of herd immunity. Protection is conferred on unvaccinated people within a population when a certain threshold percentage of the population is vaccinated. For the MMR vaccine, that percentage is 95%. At levels below this, a reservoir persists and outbreaks of infection from non-vaccinated individuals occur. *Adapted from (Lewis, 2004).*

In addition to preventing infection in the individual, vaccination can also reduce the spread of disease by imparting 'herd immunity' on a community due to fewer infected individuals, lower excretion by vaccinees and even vaccination by contact (Mackett & Williamson, 1995; Plotkin, 1999; Perrie, 2006) (Fig. 1.6.).

1.1.3. Different types of vaccines

There are three main types of vaccines currently available: live attenuated vaccines; inactivated vaccines; and vaccines containing cellular extracts or toxoids (summarised in Table 1.2.).

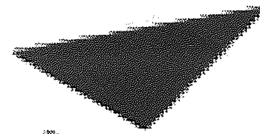

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Table 1.2. Examples of vaccine types currently available. The live (Oral) Poliomyelitis[1] vaccine has now been replaced by the inactivated poliomyelitis vaccine within the UK for routine immunisation. Similarly the Acellular pertussis[2] vaccine has replaced whole-cell pertussis vaccine used in the combination products employed in childhood vaccination programmes. *Table from (Perrie, 2006).*

1.1.3.1. Live attenuated forms

Due to their strong potency, the majority of vaccines currently in use are based on live organisms (Goldsby et al., 2003; Perrie et al., 2007a). The practice of attenuation (weakening) of live infectious agents has been in use since Jenner's time, and can be achieved by several methods including heat, chemical treatment and repeated passage in artificial media or cell culture. The subsequent vaccine retains the antigenicity of the infectious agent whilst reducing the virulence (Hilleman, 2000; Plotkin, 2005; Perrie, 2006). Vaccines produced in this manner include the bacillus Calmette-Guérin (BCG)

and the measles, mumps and rubella combination vaccine (MMR), and such vaccines are generally capable of stimulating both a humoural and cell-mediated immune response. However, there is a risk of reversion to virulence, and this type of vaccine is not considered safe for use in immunocompromised individuals (e.g. HIV sufferers, chemotherapy patients) (World Health Organisation, 2005b; Perrie et al., 2007a).

1.1.3.2. Inactivated forms

The use of inactivated vaccines also started at the end of the 19th century and consists of killing whole bacteria or viruses, usually by heat or chemicals such as formaldehyde, thus destroying infectivity while retaining immunogenicity (Mackett & Williamson, 1995). While offering advantages in terms of safety, such vaccines are generally less effective than live attenuated vaccines, usually only stimulating humoural immunity and often requiring booster doses (Mackett & Williamson, 1995). Nevertheless, inactivated polio and cholera vaccines have recently been introduced into the UK vaccine programme, replacing previously used live preparations, due to the low risk of contracting the disease compared to the live-vaccine associated risks (Perrie, 2006).

1.1.3.3. Cellular extracts and toxoids

An adaptation of this approach is the use of inactivated bacterial exotoxins, such as those produced by tetanus and diphtheria (Mackett & Williamson, 1995). In addition to being successful vaccines in their own right, toxoids may also be used to increase the immunogenicity of some other vaccines, such as the *Haemophilus influenzae* type B

(Hib), which contains a polysaccharide unit from the virus conjugated to diphtheria or tetanus toxins (Perrie, 2006).

Cellular extracts, or sub-unit vaccines, comprise highly purified proteins, surface antigens, synthetic peptides or parts of cells, which offer reduced side-effects compared to live attenuated vaccine systems (Perrie, 2006). With the advent of genetic engineering, greater opportunities have arisen for safer and more efficient production of vaccines of this type at reduced costs (Plotkin, 2005; Perrie et al., 2007a), such as the hepatitis B vaccine which is cloned into yeast and manufactured using biotechnology rather than, as previously, purified from the blood of hepatitis B carriers (Perrie, 2006).

In addition, the ability to sequence the genome of whole organisms has provided a new tool for identifying which regions of the pathogen – known as epitopes – should be included in vaccines as well as the potential of DNA vaccines encoding for specific proteins (Liu, 1999). Such systematic identification of potential antigens without the need for cultivating the pathogen has been termed "reverse vaccinology", and is likely to play a major role in future vaccine development (Mora et al., 2003; Bramwell & Perrie, 2005b).

Despite offering several advantages in terms of vaccine safety, these simple subunit constructs lack the immunostimulatory components of whole-cell vaccines, such as the PAMPs that are essential in activating the innate immune response, and therefore fail to induce an effective immune response when administered alone. As a result, such antigens

rely on effective adjuvants to elicit the appropriate immunity (Demana et al., 2005; Lendemans et al., 2005; Vangala et al., 2006).

1.2. Vaccine adjuvants

Immunological adjuvants were first described in 1924 by Ramon as "substances used in combination with a specific antigen that produced a more robust immune response than antigen alone", which encompasses a large range of materials (O'Hagan et al., 2001). Despite this, very few adjuvants have achieved regulatory approval.

Currently, the most commonly used vaccine adjuvants are aluminium based (Alum) due to their demonstrated safety profile and role in successful vaccine programmes (Bramwell & Perrie, 2005a). However, there exists concerns over severe local reactions and stability issues of such adjuvants, as well as the observation that the immune responses generated with sub-unit antigens are generally weak humoural responses, with a bias towards Th2 immunity, which is not desirable for diseases such as TB where a cell-mediated response is required (O'Hagan et al., 2001; Bramwell & Perrie, 2005a; Myschik et al., 2006; Perrie et al., 2007a). Therefore, further development and application of adjuvants is still required.

In this regard, it seems appropriate to separate the types of adjuvants used into two general categories (Table 1.3.). Those adjuvants that fall into the category of delivery systems are generally particulate in nature, and can promote uptake, transport or presentation of the antigen to Antigen Presenting Cells (APCs), or may even deliver

antigen directly to the lymph node (O'Hagan et al., 2001). Those adjuvants belonging to the other category are often soluble molecules derived from pathogens, and thus possess pathogen associated molecular patterns (PAMPs), which activate innate immunity and interact with pattern recognition receptors (PRR) on phagocytes (Medzhitov & Janeway, 1998; Lendemans et al., 2005), activating pro-inflammatory pathways and directing the induction of acquired immunity (Aderem & Ulevitch, 2000). Furthermore, it has been shown that the immunogenicity of a combination of subunit antigen plus immune potentiator can be even further enhanced if these are delivered by particulate carrier systems (Lendemans et al., 2005).

Therefore, adjuvants can play a crucial role in rendering vaccines immunogenic, and the selection of the appropriate adjuvant can control the type of acquired immune response induced (Yip et al., 1999).

Adjuvant category	Principal mechanism of action	Examples
Delivery systems	Up-regulation of antigen presentation (Signal 1)	Microspheres, nanospheres, liposomes, niosomes, virosomes, ISCOMS, emulsions
Immunostimulatory/ immunomodulatory	Signalling through PRR (Signal 0) Stimulation of cytokine release or the expression of co-stimulatory molecules on APC (Signal 2)	Saponins, MDP derivatives, LPS, DDA, MPL, TDM, TDB, CpG DNA motifs

Table 1.3. A brief summary of the sub-categories of adjuvants available and their mechanisms of action. Innate immunity is activated by danger signals (Signal 0), followed by initiation of adaptive immunity through antigen presentation (Signal 1) and subsequent co-stimulation (Signal 2) of $CD4^+$ T-helper (Th) cells.

1.2.1. Particulate delivery systems

Taking a lesson from nature, in that all pathogens are particulate, it is unsurprising that all the pharmaceutical vaccine adjuvant formulations presently being tested are particulate based. Indeed, particulates are capable of passively targeting APCs, whilst having the ability to provide persistent antigen due to slow degradation (Storni et al., 2005; Perrie et al., 2007a). Further, this can be seen as an explanation as to why antigens in solution generally fail to provide an effective immune response, since if an antigen does not reach lymphoid organs it is ignored by immune cells (Zinkernagel, 2000; Perrie et al., 2007a).

1.2.1.1. Liposomes

Liposomes were first identified as effective immunological adjuvants over 30 years ago for administration of diptheria toxoid (Allison & Gregoriadis, 1974), and have received much attention since. Generally composed of phospholipids, such as phosphatidyl choline (PC) and its derivatives, liposomes are vesicular structures consisting of concentrically oriented bilayers alternating with aqueous compartments (Kersten & Crommelin, 1995; Gregoriadis et al., 1999).

Due to the ability of liposomes to entrap both water-soluble and lipid-soluble molecules, they have previously been employed for the delivery of a range of synthetic drugs (e.g. actinomycin (Gregoriadis, 1973); oestradiol (El Maghraby et al., 1999); ibuprofen, (Mohammed et al., 2004)) and biologicals (e.g. DNA, (Perrie & Gregoriadis, 2000; Perrie et al., 2001; Perrie et al., 2002); proteins (Parmar et al., 1999)). Indeed, entrapment within liposomes can circumvent issues pertaining to direct drug use (e.g. toxicity, drug

targeting), whilst also resolving solubility issues (Gregoriadis et al., 1999; Mohammed et al., 2004).

Further, depending on the nature of the amphiphile chosen, characteristics of the resulting liposome formulation, such as bilayer rigidity and surface charge, can be significantly altered (Gregoriadis et al., 1999; Bramwell & Perrie, 2005a). Indeed, cationic liposomes have been extensively researched for their role in enhancing transfection of DNA plasmids (Perrie et al., 2001; Perrie et al., 2003), and more recently as efficient adjuvants for sub-unit vaccine delivery (Davidsen et al., 2005; Smith Korsholm et al., 2007). Moreover, additional material such as cholesterol (for enhanced rigidity) or supplementary adjuvants can be incorporated within the bilayer in order to further tailor the formulation for the particular application (Kersten & Crommelin, 1995).

In addition, the several methods of formulation of liposomes can give rise to vesicles of varying lamellarity (uni- or multi-lamellar vesicles) and size (from a few nanometres to several microns), further enhancing the flexibility of such systems for numerous applications (Perrie & Gregoriadis, 2000). However, the ability to elicit an immune response can be antigen specific (Bramwell & Perrie, 2005a).

1.2.1.2. Niosomes

Niosomes, or non-ionic surfactant vesicles (NISV), exhibit many similarities to liposomes, and are often mooted as a potentially more stable alternative due to the

relative chemical stability and the relatively low costs of the surfactants employed compared to lipids (Bramwell & Perrie, 2005a; Perrie et al., 2007a).

The most common composition of niosomes is the combination of 1-monopalmitoyl glycerol, cholesterol and dicetyl phosphate, although the incorporation of the latter, charged moiety adds an element of ambiguity to the term "non-ionic" (Baillie et al., 1985; Bramwell & Perrie, 2005a; Perrie et al., 2007a). Despite there being little evidence of the use of niosomes in the vaccine field (Bramwell & Perrie, 2005a), there have been reports of effective delivery of both DNA (Perrie et al., 2004) and sub-unit (Baillie et al., 1985; Vangala et al., 2006) vaccines with niosome-based vesicle formulations.

1.2.1.3. Microspheres

The use of polymeric microparticles for the delivery of vaccines has been the subject of much research in recent years (Sinha & Trehan, 2003; Tamber et al., 2005), thanks in part to the wide range of synthetic (e.g. polymethyl methacrylate, polyesters, polyanhydrides, polyamides) and natural (e.g. albumin, collagen, starch, chitosan, dextran) polymers available for their production. Of these polymers available, the polyesters, and in particular the poly lactides (PLA), polyglycolides (PLG) and their copolymers (PLGA), have been the most widely used, since these polymers have been tested for toxicity and safety in extensive animal studies, and are currently used to prepare controlled release delivery systems, e.g. Zoladex® (I.C.I), Decapeptyl® (Debiopharm), Prostap® (Lederle) Lupron Depot® , Nutropin Depot® and Sandostatin LAR®, which are licensed for use in humans in Europe and the USA (Furr & Hutchinson, 1985; Burns, Jr. et al., 1990).

Moreover, they have also found use in humans for resorbable sutures, bone implants and screws (Hanafusa et al., 1995; Furukawa et al., 2000), graft materials for artificial organs and as supporting scaffolds in tissue engineering research (Langer & Folkman, 1976).

Following introduction into the body, PLGA hydrolysis produces lactic and glycolic acids, which are metabolised in the Krebs cycle to CO_2 and water. This degradation process occurs in two stages – first hydrolytic scission of the ester bonds generates oligomers and monomers and a general decrease in the polymer molecular weight. Secondly, the microspheres lose mass, and the rate of polymer chain scission may increase due to autocatalysis in the presence of acidic degradation products (Gopferich, 1996).

Biodegradable poly(DL-lactide-co-glycolide) (PLGA) microspheres, therefore, appear to be an ideal candidate for the delivery of sub-unit vaccines, due to their relative biocompatibility, adjuvanticity and prolonged drug release profile. Given their particulate nature, such vehicles can promote uptake, transport or presentation of the antigen to Antigen Presenting Cells (APCs). In addition, it has been reported that PLGA microparticles exhibit an adjuvant effect for both humoural (Eldridge et al., 1991; O'Hagan et al., 1991) and cell-mediated immunity (Audran et al., 2003). Indeed, sub-10 μm PLGA microspheres are readily recognised and ingested by macrophages and dendritic cells, an important property for stimulating the immune response (Storni et al., 2005).

Numerous methods have been developed for the production of microparticles, including double emulsion – solvent evaporation (Ogawa et al., 1988), nano-precipitation (Ren et al., 2005), cross-flow filtration (Fessi et al., 1989), salting-out techniques (Allemann et al., 1992), emulsion-diffusion methods (Choi et al., 2002), jet milling (Nykamp et al., 2002) and spray drying (Quaglia et al., 2003).

However, the most common method of preparation is the double emulsion process (w/o/w), whereby an initial primary w_1/o emulsion is formed by dispersion of an aqueous antigen solution (w_1) into an organic polymer solution. This primary emulsion is then mixed by high-speed homogenisation into a secondary water phase (w_2), often containing an emulsion stabiliser or surfactant such as poly(vinyl alcohol) (PVA), in order to form a secondary $w_1/o/w_2$ emulsion. The organic solvent is then allowed to evaporate to facilitate the formation and hardening of the microparticles. This formulation technique, originally developed by Vranken and Claeys (1970) and modified by Ogawa and co-workers (1988), prevents the partition of hydrophilic drugs/antigens into the aqueous phase, thereby achieving efficient and reproducible entrapment. Other advantages include the fact that the protein is encapsulated as an aqueous solution, scaling down is possible, and high yields and encapsulation efficiencies are obtained. Nonetheless, arguments pertaining to shelf life of antigens and stability of microspheres also apply to this process (Sinha & Trehan, 2003).

Using the above technique, antigen may be entrapped within the carrier, thus acting as a shield from the hostile external environment (Jilek et al., 2005), potentially reducing

adverse reactions caused by the vaccine strain in immunocompromised individuals (Bramwell & Perrie, 2005a), whilst also exhibiting controlled release of antigen, thereby possibly eliminating the need for multiple vaccination doses (Langer & Folkman, 1976; Preis & Langer, 1979; Jiang et al., 2005).

Conversely, a variation of w/o/w process is the single oil-in-water process (o/w), whereby the initial formation of the w_1/o emulsion is omitted, with antigen being adsorbed to the surface of the microspheres following harvesting (Jabbal-Gill et al., 1999; Fearon et al., 2003; Mandal et al., 2004). This alternative process eliminates exposure of antigen to organic solvents during the formulation process, enabling delivery of several antigens simultaneously on the same formulation, at a range of different loading levels, and offering the ability to deliver either anionic or cationic moieties through the appropriate choice of polymer and/or surfactant/emulsifier. In addition, the surface characteristics and the internal morphology/porosity may be modified to yield the desired release profiles (Kim et al., 2006). Further details of both of the emulsion-based preparation techniques are given in section 2.7.

1.2.1.4. ISCOMs

Immune-stimulating complexes (ISCOMs) have the advantage of being both particulate in nature and contain saponins derived from *Quillaja saponaria* (Quil-A), which function as built-in adjuvant (Kensil, 1996; Konnings et al., 2002; Myschik et al., 2006). The saponins, along with cholesterol, phospholipid and target antigen, form symmetrical colloidal particles with a generally open cage-like structure in the size range of 30-100nm

(Konnings et al., 2002; Morein et al., 2004). ISCOMs formed in the absence of antigen are termed ISCOM matrices, indicating that the polar lipids determine the structural elements of such systems (Demana et al., 2005).

Hydrophobic interactions between the cholesterol and the saponins lead to the cage-like structure, and consideration of the relevant compositions of each constituent, usually through the construction of pseudo-ternary phase diagrams, will determine the successful formation of ISCOMs (Demana et al., 2004; Lendemans et al., 2007).

Furthermore, although generally characterised by a highly negative surface due to the presence of glucuronic acid in the *Quillaja* glycosides (Kersten & Crommelin, 1995), and thus limited in terms of the type of antigen that can be associated, recent results describe the formation of cationic cage-like complexes through the use of the cationic DC-Cholesterol in place of cholesterol, or the substitution of PC with the cationic lipid dioleoyl-trimethyl-ammonium-propane (DOTAP). The resulting cationic complexes bear resemblance to the classical anionic ISCOMs, whilst enabling the association of a greater diversity of antigens, including DNA (Lendemans et al., 2005; Lendemans et al., 2007).

1.2.1.5. Emulsions

First used in 1916 for an inactivated *Salmonella typhimurium* vaccine (Le Moignic, 1916), emulsions have a long history and are widely used as immunopotentiating delivery systems for veterinary vaccine antigens (McKercher & Graves, 1976), and there has been a recent resurgence in their use as nano-emulsions (Solans et al., 2005).

Emulsions generally consist of three components, the oil phase, the aqueous phase, and an emulsifier and the selection of each of these components and the method of preparation of the emulsion greatly determine the type and stability of the emulsion produced. These can be single (e.g. oil-in-water (o/w) or water-in-oil (w/o)) or multiple (e.g. water-in-oil-in-water (w/o/w)) emulsions, and are often used as depot systems for the delivery of bioactive agents. However, their potential advantages in drug delivery can be counter-balanced by an increased complexity of the dosage form and the attendant problems of optimal formulation and acceptable stability.

A commonly used emulsion adjuvant is the squalene based oil-in-water emulsion (MF59), which is licensed for use in humans. However, as with Alum, this system generally promotes Th2 immunity that limits its potential use as a vaccine adjuvant (Singh et al., 1992; Holten-Andersen et al., 2004).

1.2.1.6. Virus-like particles and Virosomes

Virus-like particles (VLPs) are formed by the self-assembly of envelope or capsid proteins from viruses, and retain many of the structural characteristics of authentic viruses, whilst being non-infectious and non-replicating due to the absence of the genetic material. These "pseudovirions" can be produced by transfection of DNA plasmids encoding for the necessary proteins into mammalian cells, yeast cells or recombinant baculoviruses in insect cells (Noad & Roy, 2003; Bramwell & Perrie, 2005a; Grgacic & Anderson, 2006; Young et al., 2006).

VLPs represent an interesting and promising method for the production of viral vaccines, and, indeed, there exists a licensed hepatitis B virus (HBV) vaccine, which is a particulate vaccine prepared by expression of the S gene of HBV in yeast (McAleer et al., 1992; Noad & Roy, 2003).

Virosomes, in contrast to VLPs which are typically "grown" rather than formulated, are membranous vesicles that carry viral fusion proteins in their membrane (Daemen et al., 2005; Bramwell & Perrie, 2005a). The majority of virosomes are generated from influenza viruses, following the same basic preparation technique based upon: (1) detergent solubilisation of the viral membrane, (2) sedimentation of the internal viral proteins and the viral RNA genome by ultracentrifugation and (3) reconstitution of the phospholipids and membrane proteins into a biological membrane by selective removal of the detergent (Mischler & Metcalfe, 2002; Daemen et al., 2005).

The currently licensed influenza vaccine, Inflexal V® (Berna), is a trivalent virosomal influenza vaccine, composed of 70% naturally occurring phospholipids and 30% envelope phospholipids originating from the influenza virus, providing the antigenic determinants neuraminidase and haemagglutinin glycoproteins (Mischler & Metcalfe, 2002; Bramwell & Perrie, 2005a; Perrie, 2006). However, the use of VLPs or virosomes may be affected by pre-exposure, thus leading to implications for multiple administrations (Bramwell & Perrie, 2005a).

1.2.2. Immunomodulatory adjuvants

Since the structural requirements and immunological mechanisms of action of immunomodulatory adjuvants are poorly understood, they have thus been referred to as "the immunologist's dirty little secret" (Janeway, Jr., 1989; Storni et al., 2005). Nevertheless, a plethora of vaccine adjuvants exists, some of which are outlined below, and includes directly purified or synthetic derivatives of cell wall components, bacterial DNA extracts (CpG motifs) and cytokines.

1.2.2.1. Dimethyldioctadecyl ammonium bromide (DDA)

Discovered as an adjuvant by Gall in the mid 1960s (Gall, 1966), dimethyldioctadecyl ammonium bromide (DDA) is a synthetic amphiphilic lipid, comprising a hydrophilic positively charged dimethylammonium headgroup attached to two hydrophobic 18-carbon alkyl chains (Fig. 1.7.). DDA is known to induce cell-mediated immunity and delayed-type hypersensitiviy (Snippe et al., 1982), and along with its cationic nature and surfactant properties, has been shown to be an effective adjuvant in numerous applications, including mucosal immunisaton (Klinguer et al., 2001), gene delivery (Esposito et al., 1999) and sub-unit vaccine delivery (Lindblad et al., 1997; Brandt et al., 2000; Holten-Andersen et al., 2004; Rosenkrands et al., 2005).

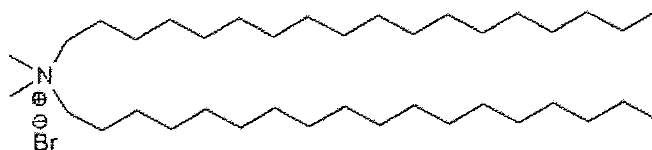

Fig. 1.7. Structure of dimethyldioctadecyl ammonium bromide (DDA).

1.2.2.2. Trehalose 6,6'-dibehenate (TDB)

Being a synthetic derivative of the mycobacterial cell wall component trehalose dimycolate (TDM), trehalose 6,6'-dibehenate (TDB) (Fig. 1.8.) has been shown to retain much of the bioactivity of the native form, whilst showing less toxicity as a result of the shorter fatty acid chains (Pimm et al., 1979; Olds et al., 1980). Indeed, the combination of the DDA with TDB has been previously shown to be an efficient adjuvant for TB subunit vaccines (Holten-Andersen et al., 2004), inducing a strong gamma interferon (IFN-γ) response, considered to be the key cytokine for induction of a Th1 immune response, essential for effective anti-mycobacterial immunity (Flynn et al., 1993; Cooper et al., 1993).

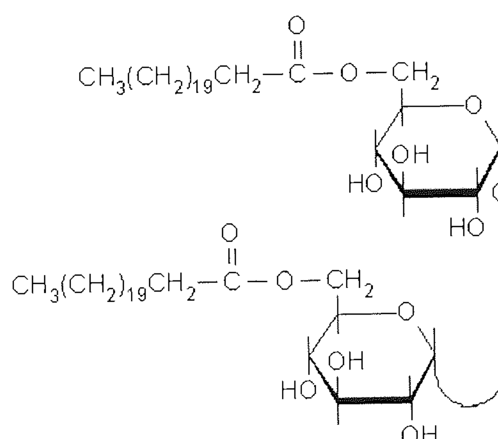

Fig. 1.8. Structure of trehalose 6,6'-dibehenate (TDB).

1.2.2.3. Monophosphoryl lipid A (MPL)

Another adjuvant derived from mycobacterial cell wall components is monophosphoryl lipid A (MPL) (Fig. 1.9.), which is obtained from *Salmonella minnesota*. MPL has also been shown as a potent Th1 stimulator, inducing enhanced secretion of IFN-γ and IL-2, whilst also increasing migration and maturation of dendritic cells (DCs) (Ulrich & Myers, 1995).

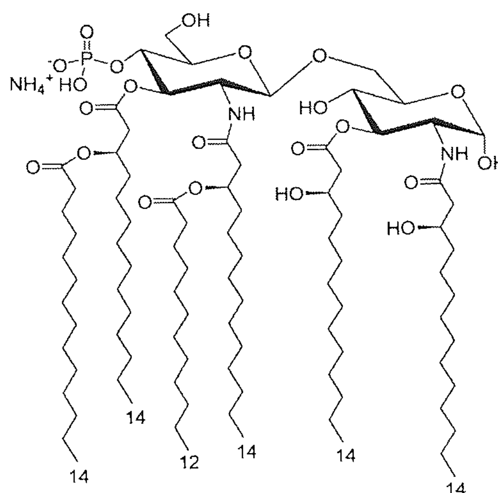

Fig. 1.9. Structure of monophosphoryl lipid A (MPL).

As a consequence, MPL has been extensively investigated for its role in vaccine adjuvanticity (Brandt et al., 2000; O'Hagan et al., 2001; Holten-Andersen et al., 2004).

1.2.2.4. CpG motifs

The immunostimulatory effects of CpG motifs, derived from bacterial DNA or synthetic oligodeoxynucleotides (ODN), have attracted much interest, and include activation and maturation of DCs, induction of Th1 response through stimulation of cytokines and co-stimulatory molecules, and enhancement of mucosal immune responses (Davis et al., 1998; McCluskie & Davis, 1998; O'Hagan et al., 2001).

1.2.2.5. Cytokines

Alternatively to using cytokine inducing adjuvants, cytokines themselves may also be used directly. Although several cytokines have been investigated, such as IFN-γ, IL-1, IL-2 and IL-12, issues pertaining to stability, toxicity, *in vivo* half-life and cost have

limited their use as vaccine adjuvants (O'Hagan et al., 2001). Nevertheless, progress in their use for immunotherapy of cancer has been made (Salgaller & Lodge, 1998).

In brief, several tools exist for improving vaccine efficacy, particularly of sub-unit antigens, and careful consideration of the immunological requirements of the vaccine and disease function is evidently a prerequisite in designing future vaccines. Nevertheless, despite this wealth of knowledge and the relative success of vaccines throughout their history, there remain several problems relating to their use.

1.3. Successes and challenges

It would not be unreasonable to state that vaccines represent one of the greatest successes of modern medicine, revolutionising public health and saving millions of lives each year by what is one of the most cost-effective means (Poland & Jacobson, 2001; World Health Organisation, 2002; Andre, 2003; Perrie, 2006). Indeed, vaccinology is the only science to have eradicated an infectious disease when, on 9th December 1979, the World Health Organisation (WHO) certified that smallpox – one of the worst scourges of humanity and responsible for 8-20% of all deaths in Europe before the introduction of the vaccine – had been wiped out, nearly 200 years after Jenner's prediction of the same (Fenner, 1982; Mackett & Williamson, 1995; Henderson & Moss, 1999; Andre, 2003; Plotkin, 2005).

Furthermore, with the projected eradication of poliomyelitis (Wack & Rappuoli, 2005; Bramwell & Perrie, 2005b), virtual disappearance of diseases such as diphtheria, tetanus, pertussis, measles, mumps and rubella, and twenty-six infectious diseases now considered

vaccine preventable (Andre, 2003), it is estimated that immunisation saves over 3 million lives each year and greatly reduces the risks of disability caused by infectious diseases (World Health Organisation, 2002; Andre, 2003).

Moreover, initiatives by groups such as the WHO have fuelled this success. The Expanded Programme on Immunisation (EPI), launched by the WHO in 1974, led to an increase from 5% to 80% of children being vaccinated worldwide (Andre, 2003; World Health Organisation, 2005a), and with new initiatives such as the Global Immunisation Vision and Strategy (GIVS) aiming to provide more vaccines to more people, particularly in developing countries, even more lives are likely to be saved by effective vaccination (World Health Organisation, 2006b).

However, with the successes of vaccination programmes, public opinion has turned to scepticism. Indeed, such scepticism was evident in Jenner's time following fears that inoculation with bovine material could create cow-like human hybrids (Andre, 2003). More recently, low rates of disease, particularly in developed countries, and scaremongering associated with potential adverse effects has gathered pace, leading to anti-vaccination campaigns, which have inevitably led to increases in disease rates (Andre, 2003; Plotkin, 2005).

The most recent incidence of such a campaign in the UK was the opposition to the MMR vaccine as a consequence of suggestions of a link with autism, and although the evidence was seemingly unfounded, there was still a decrease in uptake of the vaccine by as much

as 20% (Perrie, 2006). Indeed, acceptability of adverse reactions is directly dependent upon the perception of the severity and risk associated with disease (Bramwell & Perrie, 2005a).

On the global scale, the polio eradication programme, set up by the WHO in 1988, was dealt a huge blow in 2003-4 following a boycott of the immunisation program by predominantly Muslim states in Nigeria, after local imams claimed that the vaccine was part of US plot to depopulate Muslim lands by causing sterility and spreading AIDS (Fleck, 2004; Dyer, 2005). Consequent polio outbreaks have led to concerns of the spread of disease to much of Western and Central Africa and beyond, delaying the WHO's target to eradicate polio by the end of 2005 (Dyer, 2005; Bramwell & Perrie, 2005b).

Further, with more stringent regulatory requirements, the cost to research, develop, manufacture and launch a new vaccine is estimated at between $200-800 million (Andre, 2003; Plotkin, 2005), although this must be counterbalanced by the fact that the world market for influenza vaccines alone is worth between $1.1-1.5 billion per year (Perrie et al., 2007a).

Additionally, there remains an urgent need for an effective vaccine against several of the most deadly infectious diseases, including malaria, HIV/AIDS and, as outlined below, tuberculosis (TB).

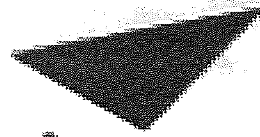
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Fig. 1.10. Global incident rates of tuberculosis. Although TB is a global problem, there is an evident correlation between incident rates and economic factors. *Figure from (World Health Organisation, 2006c)*

1.4. Tuberculosis

1.4.1. Facts and figures

Globally, TB kills two million people a year, with one third of the world's population (around 2 billion) currently infected with the bacillus, *Mycobacterium tuberculosis* (Mtb), of whom nearly 9 million are diagnosed with TB every year (World Health Organisation, 2006a). To further heighten the impact of this epidemic, TB is the leading cause of death in HIV patients in poor countries, with individuals who are co-infected with HIV and the TB bacillus at least 30 times more likely to develop active TB disease (World Health Organisation, 2006a; World Health Organisation, 2006c). In addition, TB kills more adults each year than any other pathogen and creates more orphans than any other infectious disease (Young, 1998; O'Brien, 1998).

Although TB remains a global threat (Fig. 1.10.), economic factors remain a determining factor in disease management, with around 98% of TB related deaths occurring in the developing world (World Health Organisation, 2006c). Nevertheless, TB is on the increase in the UK, with the number of TB cases in London doubling since the 1980s (NHS, 2005).

1.4.2. Brief history of TB

Evidence of tuberculosis can be dated back to the Egyptian mummies over 4000 years ago (Zimmerman, 1979; Dutau, 2005; Daniel, 2006; NIAID, 2006; UMDNJ, 2007) and, indeed, it is estimated that *Mycobacterium tuberculosis* (*Mtb*) may have infected early hominids as early as 3 million years ago (Gutierrez et al., 2005). Tuberculosis is clearly noted in the biblical books Deuteronomy and Leveticus, and was well known to the ancient Greeks, including Hippocrates who wrote of 'phthisis' (consumption) as it was then known (Dutau, 2005; Daniel, 2006; UMDNJ, 2007). Tuberculosis continued to blight mankind throughout history, earning the disease such pseudonyms as the "white plague" and the "captain of all the men of death", further emphasising the devastating impact of the disease through the ages (Young, 1998).

However, it was not until 1865 that French military surgeon Jean-Antoine Villeman first convincingly showed that TB was caused by a specific infectious microorganism (Dutau, 2005; Daniel, 2006; UMDNJ, 2007), and it was not until March 24, 1882, that Robert Koch first identified the causative agent, the tubercle bacilli (Fig. 1.11.) (Kaufmann, 2003; Dutau, 2005; Daniel, 2006; NIAID, 2006).

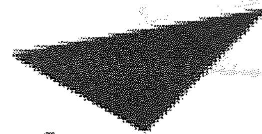

Fig. 1.11. Electron micrograph of *Mycobacterium tuberculosis*. *Mtb* is shown in blue, whereas animal tissue is shown in brown. *Figure from (Young, 1998).*

This discovery had a great impact on both the medical profession and the public, since almost one-third of adult mortality in the capitals of the Western world at the time was caused by tuberculosis (Kaufmann, 2003), and this scientific advancement was heralded as the beginning of the end of TB, although subsequent history tells a different story, with the WHO declaring TB a global emergency in 1993 (World Health Organisation, 2006c).

1.4.3. Disease function

As already stated, TB is caused by the infectious organism *Mycobacterium tuberculosis* (*Mtb*) (Fig. 1.11.). The disease is contagious and spreads through airborne droplet nuclei containing *Mtb* expectorated by infected individuals, although prolonged contact is necessary to initiate infection (Frieden et al., 2003; NHS, 2005; World Health

Organisation, 2007). Following inhalation, the *Mtb* containing droplets lodge in the alveoli of the distal airways, and the pathogen is then taken up by alveolar macrophages, which in turn interact with T lymphocytes (predominantly $CD4^+$) to release cytokines (particularly IFN-γ), leading to a subsequent cell mediated immune response with immunity developing after 2-8 weeks (Frieden et al., 2003).

In individuals whose immune response cannot contain the replication of *Mtb*, active TB occurs. This affects primarily the lungs, although extra-pulmonary tuberculosis can affect the central nervous system, the bones and joints (particularly the spine), and the genitourinary tract (Frieden et al., 2003). Further, if not treated, each person with active TB infects on average 10-15 people each year (World Health Organisation, 2006d).

Due to the characteristic slow replication of *Mtb*, the progression of the disease severity is also slow, and has thus been referred to as "a disease that does not start but one that finishes" (Cole et al., 1998; Dutau, 2005). In addition, *Mtb* can remain in a dormant state within the host for several years, and this latent form of the disease can be converted to the active disease upon weakening of the immune system (Frieden et al., 2003).

1.4.4. Treatments

Prior to Koch's discovery of *Mtb*, early treatments centred on improved housing and nutrition and the use of sanatoria. However, the basis of sanatorium treatment was founded on the beliefs of Hermann Brehmer, who, following a period of convalescence in the Himalayas, became convinced that enforced rest and exposure to fresh air, sun and

cold could effectively cure tuberculosis (Daniel, 2006; NIAID, 2006; UMDNJ, 2007). Nevertheless, the sanatorium treatment did have beneficial effects, in that it isolated the infected individuals, thus preventing the spread of disease (Dutau, 2005; UMDNJ, 2007).

As a consequence of Koch's discovery, there was a great deal of concerted effort in the attempts to finally find a cure for this persistent menace. However, despite some advances, including Koch's discovery of tuberculin which would later play a role in diagnosis rather than a cure, it would be another 40 years before Albert Calmette and Camille Guérin began trials of their vaccine, BCG (Daniel, 2006; NIAID, 2006). In the meantime, as a consequence of the First World War, screening by the use of chest radiographs and subsequent surgical procedures to collapse lungs were widely used in the management of TB (Daniel, 2006; NIAID, 2006).

Indeed it was not until the end of World War II that effective antibiotics, streptomycin and para-amino salicylic acid (PAS), were developed by Selman Waksman and Jorgen Lehmann, respectively (Daniel, 2006; NIAID, 2006). The subsequent development of isoniazid, ethambutol and rifampicin throughout the 40's, 50's and early 60's led to the closing of sanatoria, and revived hope for the eradication of TB (Dutau, 2005; Daniel, 2006; NIAID, 2006).

However, although TB is curable with antibiotics, the slow replicating nature of *Mtb* dictates that the courses are long-term and, hence, compliance is often poor. In attempt to rectify the poor patient compliance, the WHO have initiated the DOTS (Directly

Observed Treatment, Short-course) scheme throughout the world, including the UK, with more than 26 million TB patients treated under the project since 1995 (World Health Organisation, 2006a; World Health Organisation, 2007; Frieden et al., 2003; NICE, 2006).

In spite of this, due to non-compliance of antibiotic regimes, the situation is further complicated by the emergence of multidrug-resistant (MDR) and more recently extensively drug-resistant (XDR) strains of *M. tuberculosis*. Accounting for about 3% of all TB cases, it has been reported that MDR strains are resistant to both isoniazid and rifampicin, the two first-line drugs used to treat TB, and has been observed in virtually all countries, with 450,000 new cases each year (World Health Organisation, 1997; World Health Organisation, 2006d). Further, XDR TB is resistant to second-line treatments, and has also been confirmed worldwide (Centers for Disease Control and Prevention, 2006; World Health Organisation, 2007).

Despite the severity of this problem, the only current vaccine against TB is the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine, whose efficacy is highly variable (Bramwell & Perrie, 2005a; Wu et al., 2007). Indeed, estimates of protection imparted by BCG vary from nil to 80% (Fine, 1995), and the consensus is that BCG vaccination can provide protection against pulmonary TB, but that this protection wanes over time (Colditz et al., 1995; Doherty et al., 2002). As a consequence, research into new and improved TB vaccines remains a global priority recognised by the World Health Organisation.

1.4.5. Sub-unit vaccines for TB

Amongst the most promising candidate vaccine strategies is the use of sub-unit vaccines, which have been further aided by the sequencing of the total genome of several pathogens, including *Mtb*, and subsequent "reverse vaccinology" (Cole et al., 1998; Mora et al., 2003). Derived from purified mycobacterial proteins or DNA molecules coding for them, these 'non-live' preparations offer great advantages over mycobacterial whole-cell vaccines in terms of safety and quality control, as already stated. One such protein subunit vaccine, Ag85B-ESAT-6 – a fusion protein of the immunodominant antigens 6-kDa early secretory antigenic target (ESAT-6) and antigen 85B (Ag85B) – has previously been shown to have much potential as a vaccine against *Mtb*, facilitating good levels of protection in mice when combined with other adjuvants (Olsen et al., 2001; Holten-Andersen et al., 2004).

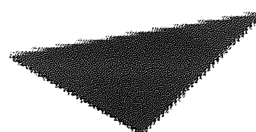

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Fig. 1.12. Circular map of the chromosome of *Mtb*. Figure represents the best characterised strain of *Mtb*, H37Rv. Figure from (Cole et al., 1998)

1.5. Importance of a stable product

As stated above, although the EPI led to 80% of children receiving vital immunisation, there still remains the 20% of infants who missed out on vaccines – particularly against diphtheria, pertussis, polio, measles, tetanus and tuberculosis – which accounts for around two million unnecessary deaths each year, especially in the most remote and impoverished areas (Liu, 1999; Langridge, 2000). Amongst the several causes of this gap in accessibility to vaccination is the formulation of the vaccine itself, which has spurred efforts to make more stable vaccines that do not need refrigeration or devices for delivery (Liu, 1999).

Indeed, inadequate infrastructure and a lack of effective distribution chains results in the loss of millions of vaccine doses each year, and it is therefore essential early in the development of vaccines against third world diseases (e.g. TB, HIV/AIDS, malaria) to incorporate vaccine stability as a key success criterion (Christensen et al., 2007).

As a consequence, several technologies, such as auto-disable syringes and safety boxes, mono-dose pre-filled injection devices, needle-free injections and point-of-use sharps processing all aim to facilitate vaccination whilst eliminating potential safety risk (e.g. accidental needle-stick or needle re-use) (Lloyd, 2000). In addition, the development of thermo-stable vaccines with enhanced shelf-life, through techniques such as freeze-drying, targets the preclusion of the cold-chain, and thus aiding universal coverage of high quality immunisation services (Lloyd, 2000; Mohammed et al., 2006; Christensen et al., 2007).

1.6. Aims and Objectives

The aim of this research was to formulate and rigorously characterise, both physico-chemically and immunologically, particulate delivery systems for the promising sub-unit TB vaccine, Ag85B-ESAT-6. Investigations focussed on both liposome and microsphere based formulations, incorporating additional adjuvants, whilst also attempting to produce a vaccine product with enhanced shelf life.

Therefore, the structure of the thesis is as follows:

- Formulation of cationic liposome formulations based on the immunomodulator DDA, with emphasis on improving stability and immunogenicity through the addition of TDB.
- Formulation and optimisation of an alternative particulate delivery system based on PLGA microspheres, and subsequent comparison of physico-chemical and immunological characteristics of the different delivery systems.
- Further optimisation of formulation parameters of the lead microsphere formulation, including the effect of preparation technique and formulation components.
- Production of a vaccine product with enhanced shelf-life by means of freeze-drying in the presence of various cryoprotectants.

Chapter 2

Materials and methods

2.1. Materials

Poly(DL-lactide-co-glycolide) (PLGA) (75:25) (Mw 90,000-126,000), poly(vinyl alcohol) (PVA) (Average Mw 13,000-23,000, 87-89 % hydrolysed), Chitosan (Low molecular weight), cetyl trimethyl ammonium bromide (CTAB), N-acetylmuramyl-L-alanyl-D-isoglutamine hydrate (muramyl dipeptide (MDP)), carbohydrates (dextrose, sucrose and trehalose), amino acids (lysine, histidine, arginine, glycine, valine and leucine), Phosphate Buffered Saline (PBS) tablets, sodium chloride (NaCl) and Sephadex® G-75 were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). Methanol (extra pure), chloroform (extra pure) and 1 M hydrochloric acid, used to adjust pH in the Tris-buffer, were purchased from Fisher, UK. Tris (ultra pure) was from ICN Biomedicals (Aurora, OH). Dimethyl dioctadecylammonium bromide (DDA) and α,α'-trehalose 6,6'-dibehenate (TDB) were obtained from Avanti Polar Lipids (Alabaster, AL). The purity of the compounds was > 99% by HPLC. Non his-tagged protein Ag85B-ESAT-6, supplied by Statens Serum Institut, Copenhagen, was produced in *Escherichia coli* as described previously for the His-tagged version (Olsen et al., 2001), purified by column chromatography and dissolved in 10 mM Tris-buffer, pH 7.4, at a concentration of 0.5 mg/ml. ^{125}I (NaI in NaOH solution) was purchased from Amersham Biosciences (Bucks., UK). All other reagents used were of analytical grade.

2.2. Preparation of adjuvant liposomes

2.2.1. Lipid film hydration method

DDA liposomes in the presence or absence of TDB were prepared by the film method. Weighed amounts of DDA (1.25 mg/ml) and TDB (0.25 mg/ml) were dissolved in

chloroform/methanol (9:1, v/v) and the organic solvent was removed using a rotavapor under reduced pressure. The obtained lipid film was then flushed with N_2 to remove trace amounts of the organic solvent. Vesicles were formed by hydrating the lipid film (Blandamer et al., 1998; Feitosa et al., 2000; Barreleiro et al., 2002) in either 10 mM Tris-buffer at pH 7.4 or 5% (w/v) dextrose where appropriate. The lipid film was hydrated for 20 min at a temperature 10 °C above the main phase transition of DDA (Tm ≈ 47 °C) (Benatti et al., 1999; Feitosa et al., 2000) to ensure complete hydration. Ag85B-ESAT-6 was added to the preformed liposomes at a fixed concentration of 0.01 mg/ml.

2.2.2. Aqueous heat method

For comparison, DDA adjuvant liposomes were also prepared as described previously (Holten-Andersen et al., 2004). Briefly, DDA was mixed into either ddH_2O, 0.9% (w/v) NaCl, 5% (w/v) dextrose or 10 mM Tris-buffer at pH 7.4 to a concentration of 1.25 mg/ml, heated to 80°C for 20 minutes with intermittent shaking, and then cooled to room temperature. Where appropriate, 0.25 mg/ml of TDB (250 μl of a 1 mg/ml solution in 0.2% triethylamine) was then added, and the resulting solution was vortexed briefly. The Ag85B-ESAT-6 was finally added at a concentration of 0.01 mg/ml.

2.3. Dynamic light scattering: Determination of liposome size and zeta potential

The z-average diameter of DDA liposomes, in the presence or absence of TDB, was determined using a *ZetaPlus* (Brookhaven Instrument Corporation, NY) by dynamic light scattering using the photon correlation spectroscopy (PCS) technique. PCS is based on the theory that the observed time dependency of the fluctuations in intensity of scattered light from a colloidal dispersion is a function of the rate of diffusion, or Brownian

motion, of the scattering particles, and hence their size in suspension (Tscharnuter, 2000; Gun'ko et al., 2003). For analysis, the liposome suspension (as prepared in Section 2.2.) was diluted as appropriate using ddH_2O and the measurements recorded at 25°C. The size reported for each sample was the average of three readings and each reading was a mean of measurements recorded for 3mins.

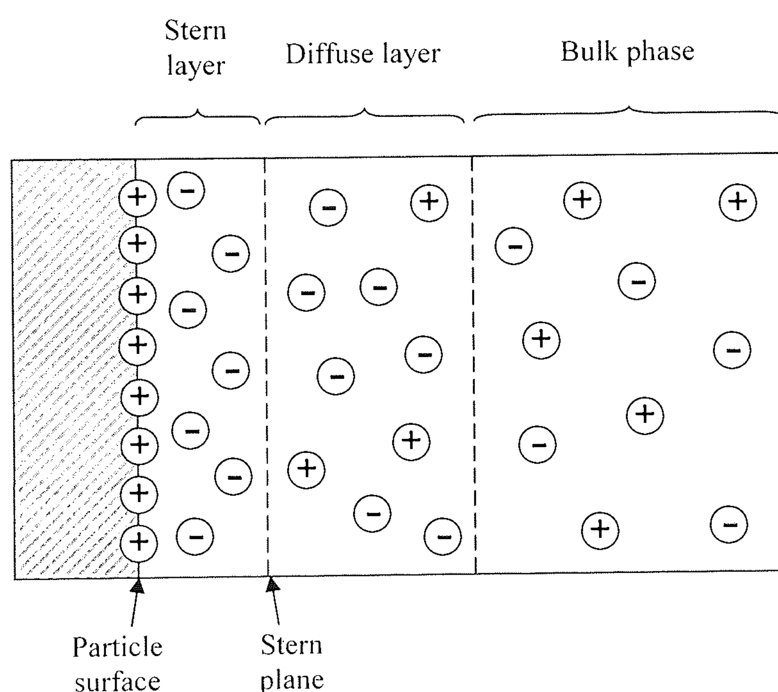


Fig. 2.1. Schematic representation of the electric double layer arising from charged particles in solution. Zeta potential is the difference in potential between the stern plane and the bulk phase.

Surface charge on the vesicles was measured indirectly as zeta potential. Charged particles in solution attract solvent counter-ions, which form a fairly immobile layer around the particle (Stern layer), which in turn attract further mobile counter-ions (diffuse layer), resulting in an electrical double layer (Fig. 2.1.). The zeta potential is defined as the difference in potential between the surface of the Stern layer (Stern (or shear) plane) and the distant electroneutral region of the bulk solution, and is measured by means of

electrophoretic mobility in response to an electrical field (Shaw, 1992; Martin, 1993; Atkins, 1998).

The measurements were performed at 25 °C using a *ZetaPlus* instrument (Brookhaven Instrument Corporation, NY) using 50 μL of the liposome dispersion diluted to 2 mL in a 1/10 solution of either 0.9% NaCl, 10mM Tris-buffer or 5% (w/v) dextrose where appropriate. The reported measurements are the mean of three samples, each of which are the average values of 10 readings.

2.4. Transmission electron microscopy (TEM) of liposome formulations

Morphological analysis was carried out by TEM using a JEOL 1200EX TEM fitted with a LaB6 filament, with an operating voltage from 40 to 120 kV. A small drop of sample (10 μL) was placed on a polymer filmed copper grid and allowed to stand for 2 minutes. The excess sample was removed using a filter paper, followed by addition of 10 μL of uranyl acetate. The grid was then allowed to stand for another 2 minutes, washed in distilled water and air dried, forming a thin film which was viewed at 70 kV.

2.5. ^{125}I radio labelling of Ag85B-ESAT-6

Radiolabelling of Ag85B-ESAT-6 was performed using the Iodo-gen® pre-coated iodination tubes (Pierce Biotechnology, Rockford, IL). Briefly, Ag85B-ESAT-6 was diluted with 50 μL Tris-buffer (25 mM, pH 8) and added to the pre-coated iodination tube. A pre-determined activity of ^{125}I (3.7 MBq) was then diluted up to 30 μL with 25 mM Tris-buffer and added to the iodination tube. This mixture was then left for 15 minutes, with intermittent shaking, to facilitate radio labelling of Ag85B-ESAT-6.

Removal of the unlabelled Ag85B-ESAT-6 was performed by Sephadex G-75 gel column separation. In order to make the column, Sephadex G-75 (1%, w/v) was first soaked in double distilled water at 90°C for 1 hour, with stirring. The swollen gel was then packed into a 5 mL column and equilibrated with the 25 mM Tris-buffer.

Prior to separation, the reaction mixture from the iodination tube was further diluted with the Tris-buffer, and then passed through the column with 25 mM Tris-buffer as mobile phase. Aliquots of the eluted solution (0.5 mL) were collected and measured for gamma radiation using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA) and also for UV absorbance at 280 nm, so as to confirm the presence of radiolabelled Ag85B-ESAT-6 (see Appendix 1). The appropriate aliquots were then pooled and stored at –20°C until required for further use.

2.6. Adsorption of antigen to adjuvant liposomes

The degree of adsorption of Ag85B-ESAT-6 to the liposomes was determined by ^{125}I radiation. Radiolabelled Ag85B-ESAT-6 (prepared as in Section 2.5.) was added to liposomes prepared as described above (Section 2.2.), mixed, and then allowed to stand for 10 minutes at ambient conditions. The formulation was then pelleted by ultracentrifugation (100,000 x *g* for 1 hour), resuspended in the original volume of 10 mM Tris-buffer, and then measured for gamma radiation. Adsorption of Ag85B-ESAT-6 was determined on the basis of ^{125}I radioactivity recovered in the suspended pellets. At regular time intervals, a similar procedure was adopted to determine Ag85B-ESAT-6 retention under different storage conditions (4°C and 25°C).

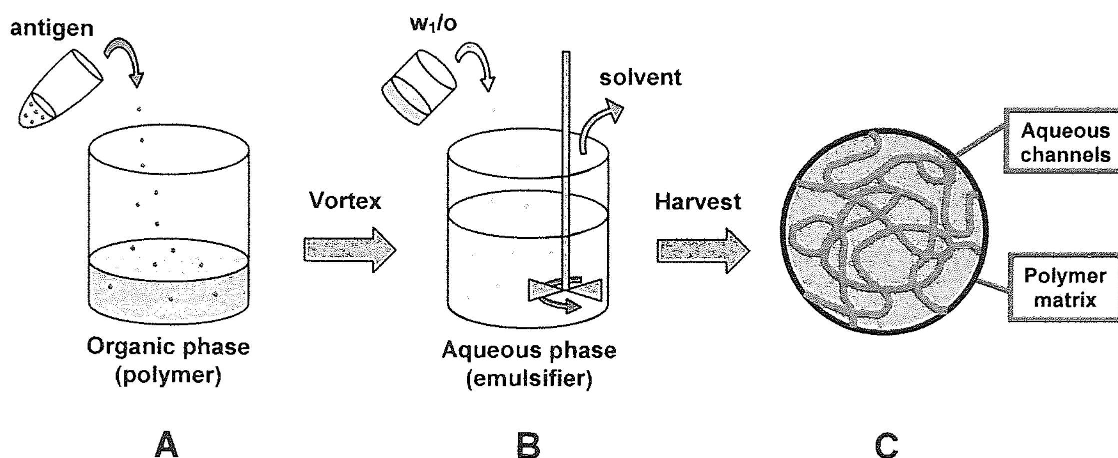
2.7. Preparation of PLGA (75:25) microspheres

2.7.1. Double emulsion solvent evaporation (w/o/w)

PLGA (75:25) microspheres were prepared using a modified w/o/w double emulsion solvent evaporation process, similar to that described elsewhere (Ogawa et al., 1988) (Fig 2.2a). Briefly, an aqueous solution of Ag85B-ESAT-6 (20.35 μL; 0.98 mg/mL) was emulsified with an organic solution of PLGA in chloroform (417 μL, 3% (w/v)) by vortex mixing for 1.5 minutes. In order to try and maintain protein integrity and reduce shear forces, vortex mixing, rather than the more commonly used high-speed homogenisation, was employed at this stage. The primary w/o emulsion was then transferred to 10 mL of an aqueous solution of either PVA (10%, w/v), Chitosan (0.75%, w/v in 3% (w/v) acetic acid) or CTAB (0.5%, w/v), and a secondary w/o/w emulsion was produced using high speed homogenisation (Silverson SL2 homogeniser at 6000 rpm), before being left under magnetic stirring for 12-18 hours at ambient conditions to allow for the evaporation of the organic solvent. The microspheres were then harvested by centrifugation (20 minutes at 10000 x *g*), and washed three times with 10 mL of double distilled water. Where relevant, the adjuvants DDA (20 %, w/w polymer) and TDB (4%, w/w polymer) were added to the oil phase, whereas MDP was adsorbed to pre-formed microspheres (4%, w/w polymer), facilitated by vortex mixing. Ag85B-ESAT-6 free microspheres were produced as described above, with PBS as the internal aqueous phase.

Harvested microspheres were either resuspended in ddH_2O for physico-chemical characterisation, or freeze-dried in the presence of 10% (w/v) sucrose for immunological investigation (Section 2.14.) and then resuspended in ddH_2O prior to immunisation.

A. water-in-oil-in-water double emulsion solvent evaporation process ($w_1/o/w_2$)



B. oil-in-water single emulsion solvent evaporation process (o/w)

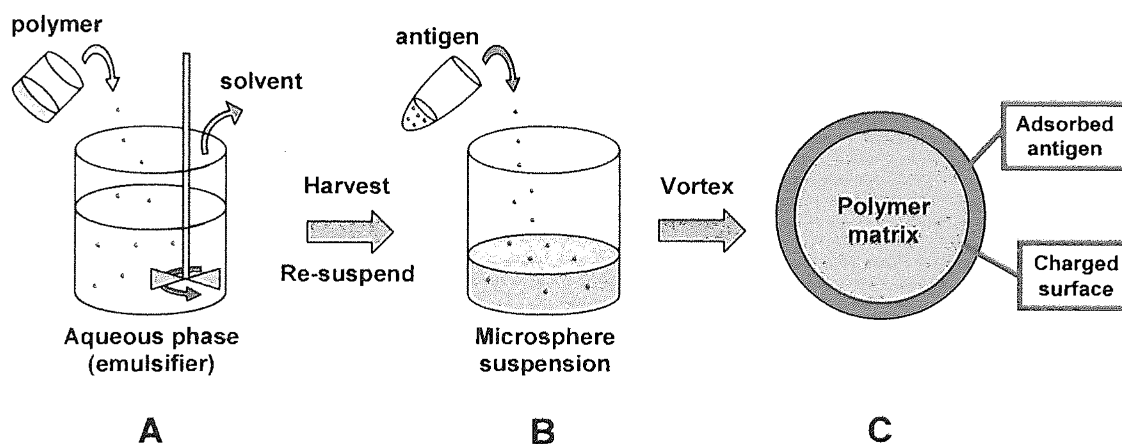


Fig. 2.2. Schematic representation of microsphere formulation by emulsion solvent evaporation processes.

A. water-in-oil-in-water double emulsion solvent evaporation process ($w_1/o/w_2$). Initially, an aqueous solution of antigen is emulsified with an organic polymer containing phase by vortex mixing to form a primary water-in-oil (w_1/o) emulsion (**A**). This is then transferred to an external surfactant containing aqueous phase (w_2) under homogenisation to yield the water-in-oil-in-water ($w_1/o/w_2$) emulsion (**B**). Solvent is then allowed to evaporate, and hardened microspheres are harvested by centrifugation (**C**).

B. oil-in-water single emulsion solvent evaporation process (o/w). A polymer containing organic phase is first emulsified with a surfactant containing aqueous phase under homogenisation, to yield an oil-in-water emulsion (o/w) (**A**). Solvent is then allowed to evaporate, and hardened microspheres harvested by centrifugation. Microspheres are then resuspended, and mixed with antigen solution by vortex mixing (**B**) to facilitate surface adsorption of antigen (**C**).

2.7.2. Single emulsion solvent evaporation (o/w)

For comparison, PLGA (75:25) microspheres were prepared using an o/w single emulsion solvent evaporation process (Fig 2.2b.). Briefly, an organic solution of PLGA (3%, w/v) and DDA (0.6%, w/v) in chloroform (417 μL) was emulsified with 10 mL of an aqueous solution of chitosan (0.75%, w/v) using high speed homogenisation (Silverson SL2 homogeniser at 6000 rpm), before being left under magnetic stirring for 12-18 hours at ambient conditions to allow for the evaporation of the organic solvent. The microspheres were then harvested by centrifugation (20 minutes at 10000 x *g*), and washed three times with 10 mL of double distilled water.

The resultant microspheres were then resuspended in 2 mL ddH_2O, and mixed with an aqueous solution of Ag85B-ESAT-6 (20.35 μL, 0.98 mg/mL) in order to facilitate surface adsorption of the antigen to the microspheres. For immunological investigations (section 2.14.), formulations were freeze-dried in the presence of 10% (w/v) sucrose, and then resuspended in ddH_2O prior to immunisation.

2.8. Particle size distribution analysis of microspheres

Laser diffraction ('low angle laser light scattering') was used to determine particle size and size distribution with either a Malvern Mastersizer S (He-Ne gas laser 633nm wavelength, Malvern Instruments, Malvern, UK) or a Sympatec Helos (Sympatec, Germany). This sizing technique relies on the assumption that diffraction angle is inversely proportional to particle size. Samples were added to a magnetically stirred cell containing filtered water until the desired obscuration was achieved (~15 %). The mean

particle size in this case represents the De Brouckere mean diameter, otherwise referred to as the volume or mass moment mean (D[4,3]), which avoids any need for particle counting. The distribution percentiles, d(10), d(50) and d(90) represent the volume of sample (10%, 50% and 90% respectively) with a diameter up to the value stated. Values denoted by s.d. (Mastersizer X) or Span (Sympatec) give an indication to the uniformity of the overall distribution of sizes.

2.9. Zeta potential analysis of microspheres

As for the liposome formulations (Section 2.3.) surface charge on the microspheres was measured indirectly as zeta potential. The measurements were performed at 25 °C using a *ZetaPlus* instrument (Brookhaven Instrument Corporation, NY) using 50 µL of the microsphere dispersion diluted to 2 mL with ddH_2O. The reported measurements were the mean values of three samples, each of which was the mean value of 10 readings.

2.10. Scanning Electron Microscopy (SEM)

The morphology of protein-loaded microspheres was studied by scanning electron microscopy using a Philips FEI XL30 SEM at 10kV (FEI, Eindhoven). Microspheres were prepared by the double emulsion solvent evaporation process (section 2.7.1.), harvested and then resuspended in ddH_2O. Microsphere suspensions were then loaded onto carbon coated aluminium stubs and air-dried overnight to remove water. The dried samples were then sputter-coated with gold before analysis in order to confer a conductive coating necessary for imaging.

2.11. Determination of Ag85B-ESAT-6 entrapment in microspheres

In order to determine Ag85B-ESAT-6 entrapment efficiency, microspheres were prepared by the w/o/w process as described above (section 2.7.1.), with the addition of 50 μL ^{125}I labelled antigen (prepared as in section 2.5.) to the internal aqueous phase in order to spike the non-radioactive Ag85B-ESAT-6. To harvest the radioactive microspheres, Beckman Quick-Seal™ centrifuge tubes (Beckman Instruments inc., Spinco division, Palo Alto, CA) were used, and entrapment efficiency was calculated from the difference of measured gamma radiation emitted from both supernatant and resuspended microspheres.

2.12. Determination of Ag85B-ESAT-6 adsorption to pre-formed microspheres

As with the determination of Ag8B-ESAT-6 entrapment, ^{125}I labelled antigen (section 2.5.) was used to determine Ag85B-ESAT-6 adsorption to microspheres prepared by the o/w process (Section 2.7.2.). Radiolabelled antigen (50 μL) was added with the non-radiolabelled antigen to pre-formed microspheres and vortexed briefly. The resulting mixture was then centrifuged using Beckman Quick-Seal™ centrifuge tubes (Beckman Instruments inc., Spinco division, Palo Alto, CA), and antigen adsorption efficiency determined from the difference of measured gamma radiation emitted from both supernatant and resuspended microspheres.

Although the use of ^{125}I labelled antigen does have drawbacks in terms of altering the volume of antigen solution entrapped or adsorbed, alternative methods (e.g. BCA assay) failed to yield consistent results, possibly through interference from the lipidic DDA.

2.13. In vitro release and retention of Ag85B-ESAT-6

PLGA microspheres with either entrapped (w/o/w) or adsorbed (o/w) ^{125}I labelled Ag85B-ESAT-6 were prepared as described above (section 2.7.) then incubated in 15 mL release medium (PBS, pH 7.4, 37°C) and maintained in a water bath under constant agitation. At regular time intervals, samples (1 mL) of release suspension were taken and counted for gamma radiation. Once counted, the aliquots were then passed through 0.22 μm PVDF filters (Kinesis, Beds., UK) to separate the free antigen, and the filtrate again counted for gamma radiation. One mL of fresh medium was added to the release suspensions in order to maintain sink conditions. Antigen release profiles were generated for each microsphere formulation in terms of cumulative antigen release (%, w/w) vs. time by calculating difference in gamma radiation between aliquots before and after filtration. For retention studies, ^{125}I labelled Ag85B-ESAT-6 loaded PLGA microspheres were prepared as above, resuspended in 2 mL PBS and then stored at 4°C or room temperature (25°C). At regular time intervals, 100 μL aliquots were taken and treated as for the release studies in order to determine percentage antigen retention.

2.14. Immunological analysis of microsphere formulations

Experimentation strictly adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to stringent ethical review and were carried out in a designated establishment. Groups of five female BALB/c mice, approximately six weeks old, received doses of microsphere vaccine formulations (as described in section 2.7.) containing 2 μg of Ag85B-ESAT-6 in a 50 μL volume. As a positive control, the liposomal formulation DDA-TDB, prepared as described in Section 2.2., was

administered due to its ability to initiate strong protective immune responses against *Mtb* (Holten-Andersen et al., 2004). Naïve groups received the appropriate volume of PBS. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks. Serum samples were taken at 12 days after the first administration and at two week intervals thereafter.

2.14.1. Analysis of Ag85B-ESAT-6 specific antibody isotypes

Sera samples obtained at different time intervals after immunisation were analysed for the presence of anti-Ag85B-ESAT-6 IgG1, IgG2a and IgG antibodies by enzyme-linked immunosorbent assay (ELISA) (Fig. 2.3.). ELISA plates (flat bottomed, high binding; Greiner Bio-One Ltd, Glos. UK) were coated with 60 μL of Ag85B-ESAT-6 per well (3 μg/ml) in PBS and incubated at 4°C overnight. Unbound antigen was aspirated and residual washings were removed by blotting firmly onto paper towel. Plates were blocked with 0.2 mL per well of 4% w/v Marvel (Premier Int. Foods Ltd, Lincs, UK) in PBS. Serially diluted serum samples (60 μL per well) were transferred to washed plates and incubated for 1 h at 37°C. Anti- Ag85B-ESAT-6 antibodies were detected by addition of horseradish peroxidase conjugated anti-mouse isotype specific immunoglobulin (goat anti-mouse IgG1, IgG2a or IgG; Oxford Biotechnology, Oxfordshire, UK), and subsequent addition of substrate solution, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma, Poole, Dorset, UK) in citrate buffer incorporating 5 μL of 30% H_2O_2/50 mL following repeated incubation and washing steps. Absorbance was measured at 405 nm (Bio-Rad, Herts, UK).

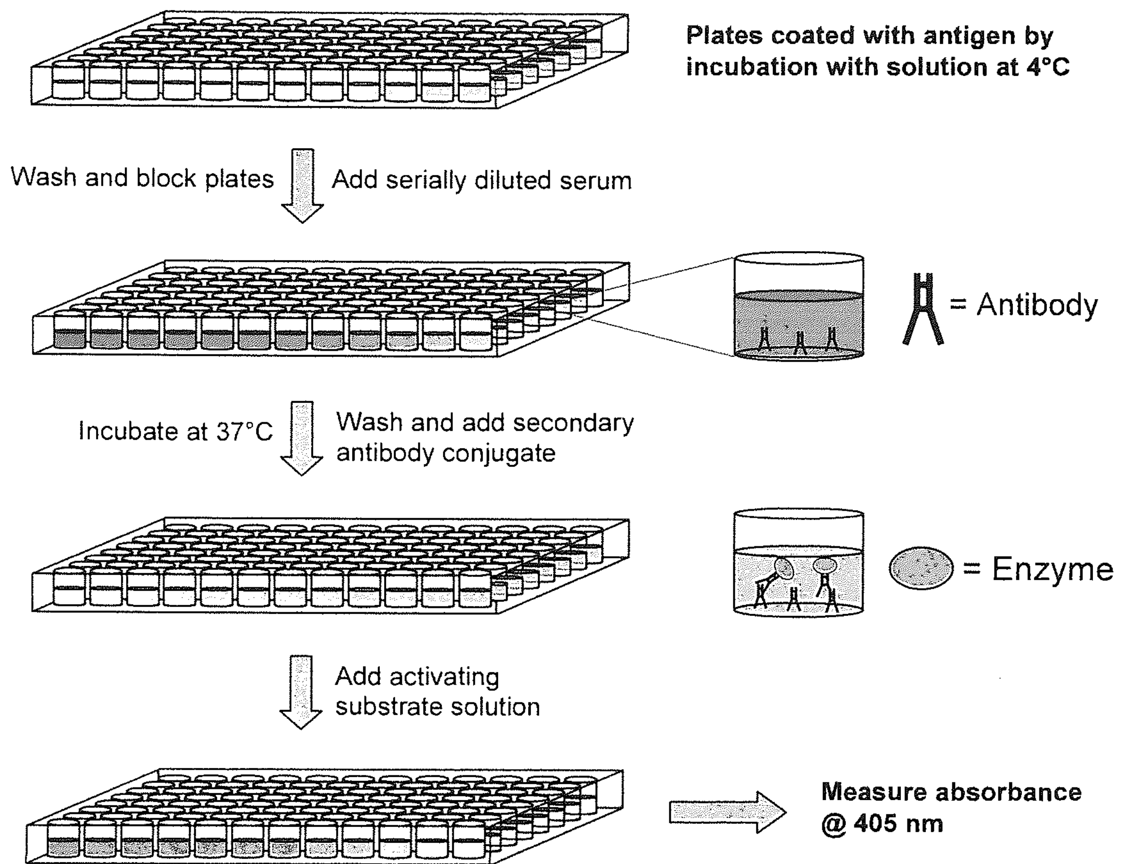


Fig. 2.3. Schematic representation of analysis of antibody production by Enzyme-linked immunosorbent assay (ELISA). Serially diluted serum samples are added to 96-well microplates coated with antigen and blocked with Marvel (4% (w/v)) in PBS. Serum antibodies recognise antigen adsorbed to wells. Antibodies are then detected using secondary antibody conjugate, which recognises antibody bound to antigen adsorbed to wells, following repeated incubation and washing steps.

2.14.2. Spleen cell culture preparation

Upon termination of experiments, mice were humanely culled and their spleens aseptically removed and placed into ice-cold sterile PBS. Spleens were treated as follows: A crude suspension of spleen cells in 10 mL working media (RPMI 1640 cell culture medium supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL) (Gibco-Invitrogen, Paisley, UK) was prepared by gently grinding the spleen on a fine wire screen. After allowing the cell suspension to settle for approximately 5 minutes the liquid was transferred to sterile 20

mL 'Falcon' tubes, without disturbing the cellular debris at the bottom. The cell suspension was centrifuged at 200 g for 10 min. After centrifugation the supernatant was removed, the cell pellet resuspended in 10 mL fresh working media and the centrifugation procedure was repeated. These single cell suspensions were used to assess antigen specific cytokine production and antigen specific recall responses.

2.14.2.1. Analysis of spleen cell proliferation

For study of antigen specific proliferative responses (Fig. 2.4.), aliquots of 150 μL volumes of sterile media or antigen in sterile media (at the concentrations stated (0.5 or 5 μg/mL)) were seeded onto 96 well suspension culture plates (Greiner Bio-One Ltd. Glos. UK) and 150 μL volumes of viable splenocytes (approximately 1×10^7 cells/mL) added to each well. As a positive control, cells were co-cultured with concanavalin A (Sigma, Poole, Dorset, UK) at a concentration of 3 μg/mL. Covered plates were incubated in a humid, 5% CO2 environment at 37°C for 72 h. After 72 h incubation, half a microcurie of [^{3}H] thymidine (Amersham, UK) in 40 μL volumes of freshly prepared sterile working media was added to each well, and the incubation continued for a further 24 h. The well contents were harvested onto plain filter mats (Molecular Devices Ltd., Wokingham, UK) using a cell harvester (Titertek). After drying, the discs representing each well were punched from the filter mats into 5 mL volumes of scintillation fluid (Optiphase Hisafe III, Fisher Scientific UK Ltd. Loughborough) and the incorporation of [^{3}H] thymidine into the cultured cells was measured using standard counting procedures.

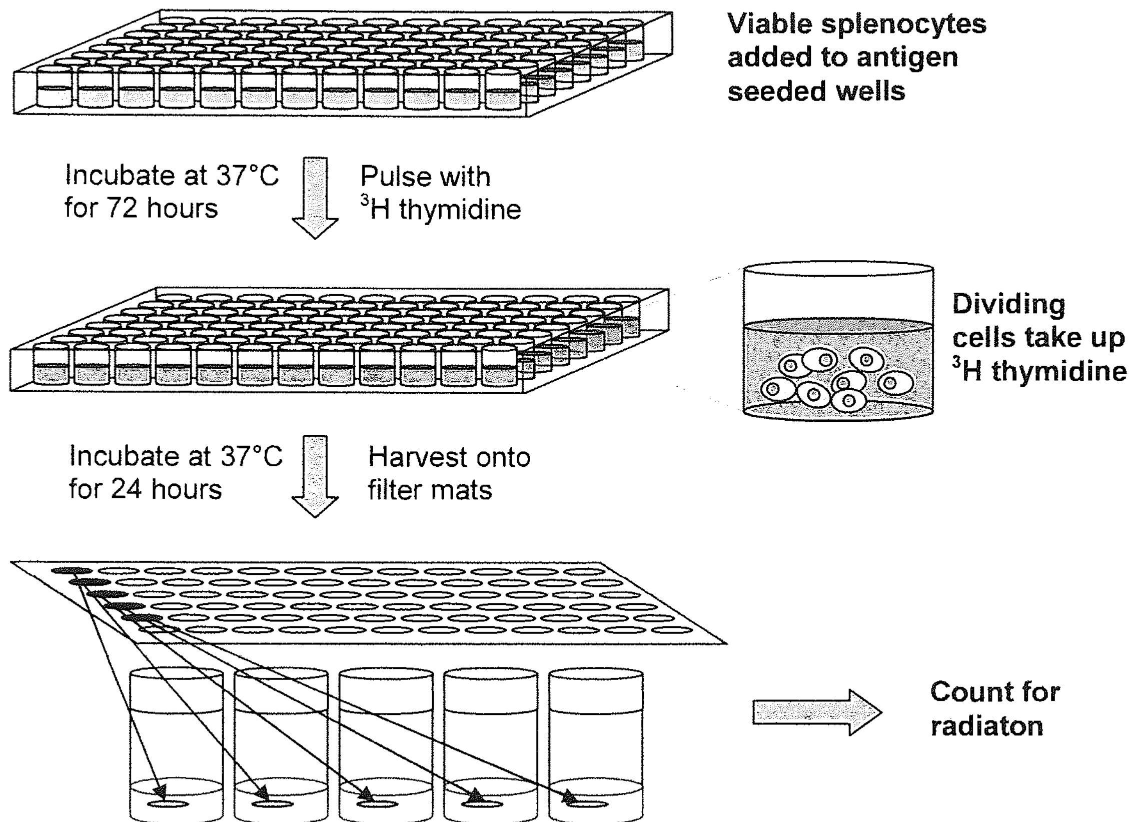


Fig. 2.4. Schematic representation spleen cell proliferation analysis. Antigen is seeded onto 96 well suspension culture plates and incubated with viable splenocytes at 37°C (5% CO_2) for 72 hours. Cells are then pulsed with ^3H thymidine, incubated, and harvested onto filter mats, which are counted for ^3H using standard counting procedures.

2.14.2.2. Analysis of cytokine production

Cytokines were detected by taking cell culture supernatants after 48 hours incubation with 2.5 μg/mL Ag85B-ESAT-6 fusion protein. The cell medium was separated by centrifugation, collected in eppendorfs and stored at -70°C until analysed using DuoSet® capture ELISA kits (mouse IFN-γ, IL-2, IL-5, IL-6) purchased from R&D systems, Abingdon, UK, according to the manufacturers instructions. Briefly, ELISA plates were first coated with capture antibody, followed by washing and blocking. Samples of cell culture supernatants were then added and cytokines detected by addition of detection

antibody, enzyme marker (Streptavidin-HRP) and substrate solution following repeated incubation and washing steps (Fig. 2.5.). Absorbance was measured at 405 nm (Bio-Rad, Herts, UK).

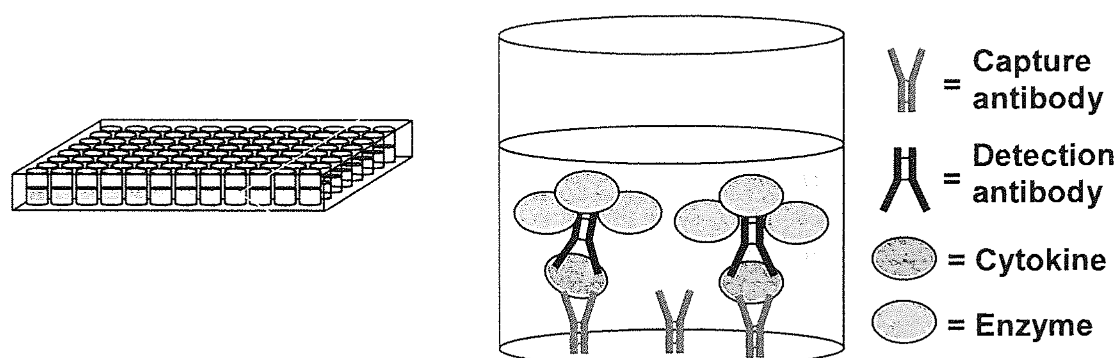


Fig. 2.5. Schematic representation of cytokine detection using DuoSet® capture ELISA kits. Spleen cell culture supernatants are added to 96 well microplates coated with capture antibody. Addition of detection antibody and enzyme marker, followed by activation with substrate solution, allows determination of cytokine secretion by optical density measurements.

2.15. Freeze-drying of microsphere formulations

Freeze-drying of microspheres was carried out using a Virtis Advantage freeze-drier (Virtis, USA). Microspheres were formulated as described in section 2.7., harvested and then resuspended in either ddH_2O (no cryoprotectant) or solutions of cryoprotectants based on either carbohydrates (sucrose and trehalose) or amino acids (lysine, arginine, histidine, glycine, valine or leucine) at various concentrations.

Microsphere suspensions were then transferred to an appropriate container and covered with paraffin film, which was ventilated (uniform punctures) to facilitate the removal of water during freeze-drying. The freeze-drying process consisted of initial pre-freezing at -70°C followed by drying at -40°C at reduced pressure for 48 hours.

2.15.1. Analysis of freeze-dried microspheres

Following freeze-drying, the resulting dried microsphere 'cake' was rehydrated with ddH_2O and analysed for physico-chemical characteristics as for non-freeze-dried microspheres (section 2.8. and 2.9.).

2.16. Environmental Scanning Electron Microscopy (ESEM) of microspheres

ESEM analysis was performed using a Philips XL30 ESEM-FEG (Philips Electron Optics (FEI), Eindhoven). PLGA microspheres incorporating DDA were prepared by the w/o/w or o/w process (section 2.7.1. and 2.7.2. respectively), employing either PVA (10%, w/v) or Chitosan (0.75%, w/v) as emulsion stabiliser. Following harvesting and resuspension, microsphere suspensions were loaded onto gold-sputtered mica plates in order to yield high resolution ESEM images. Gradual reduction of pressure in the sample chamber of the ESEM instrument resulted in the controlled dehydration of the sample environment. In addition, for analysis of freeze-dried microspheres, freeze-drying of w/o/w microspheres stabilised by PVA (10%, w/v) was carried out without the addition of cryoprotectants (section 2.15). The dried microsphere 'cake' was then rehydrated with ddH_2O, and analysed by ESEM as for the other formulations.

2.16. Statistical Analysis

Physico-chemical characterisation results represent the means of triplicate assays (n=3) ± standard deviations of the means, whereas immunological analyses represent n=5 ± standard deviations.

Statistical analyses were performed using GraphPad Instat 3 software (Version 3.06, GraphPad Software). For *in vitro* investigations, differences between means were assessed by Student's t test, with P values of <0.05 being considered significant. Analysis of variance (ANOVA) followed by Tukey test was performed to compare the mean values of different groups. For *in vivo* data, Kruskall-Wallis' non-parametric rank sum test followed by Dunn's post test was used for differences in humoural and cellular immune responses. Statistical significance was considered at $P < 0.05$ in all the studies.

Chapter 3

Formulation and characterisation of cationic liposomes based on dimethyldioctadecyl ammonium and trehalose 6,6'-dibehenate

3.1. Introduction

Liposomes were first shown to act as immunological adjuvants over 30 years ago (Allison & Gregoriadis, 1974), and have since received much attention for their potential role in drug delivery due to their ability to reduce adverse effects (Allison & Gregoriadis, 1974; Gregoriadis & Allison, 1974; Manesis et al., 1978), provide protection from *in vivo* degradation, and promote sustained release, intracellular delivery and targeting of APCs (Gregoriadis, 1990). In addition, the versatile formulation methods available are able to produce vesicles of varying physical characteristics (e.g. size, charge, lamellarity) able to deliver a wide range of molecules, including both protein and DNA vaccines (Gregoriadis et al., 1999; Perrie et al., 2001; Perrie et al., 2002; Perrie et al., 2003). Although generally composed of phospholipids, there is an increasing interest in non-phospholipid "membrane mimetic" amphiphiles that may enable more cost effective and scalable production (Gupta et al., 1996).

A lipid of particular interest is the quaternary ammonium compound, dimethyldioctadecylammonium (DDA), a synthetic amphiphilic lipid compound comprising a hydrophilic positively charged dimethylammonium head-group attached to two hydrophobic 18-carbon alkyl chains (tail). In an aqueous environment, DDA self-assembles into liposomes that have been used as an adjuvant for various vaccines (Lindblad et al., 1997; Brandt et al., 2000; Olsen et al., 2001; Holten-Andersen et al., 2004). However, DDA liposomes are physically unstable (Davidsen et al., 2005; Vangala et al., 2006), and the initial focus of this work was to resolve these stability issues and fully characterise the resulting formulation. Furthermore, the addition of the

immunomodulator trehalose 6,6'-dibehenate (TDB) to the liposome formulation was investigated for its effect on physico-chemical and immunological characteristics.

The aim of the work in this chapter was to: formulate and fully characterise, both physico-chemically and immunologically, cationic liposomes based on DDA; attempt to stabilise the formulation; determine the effect of the addition of TDB and antigen; and evaluate the adjuvanticity of such formulations for the delivery of sub-unit TB vaccines.

3.2. Preparation and characterisation of DDA vesicles

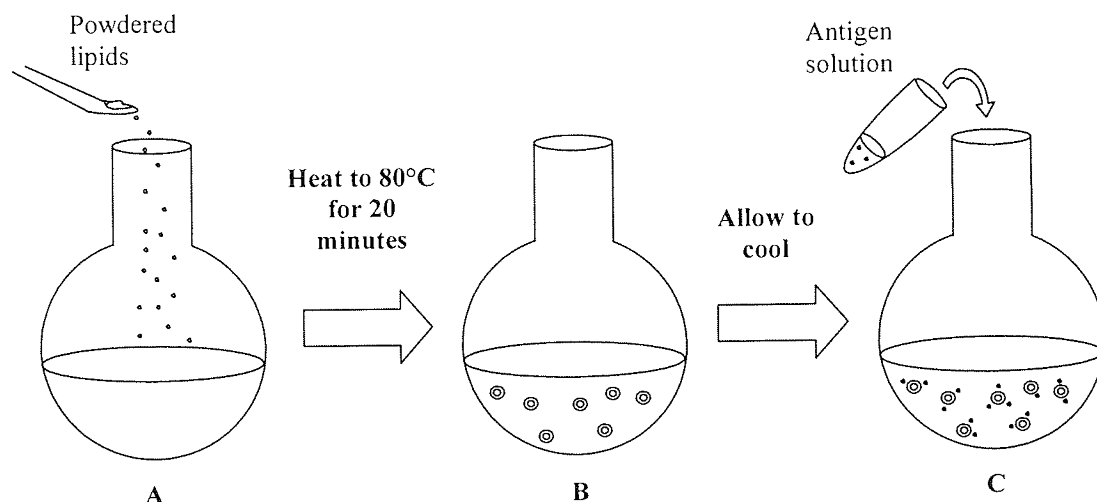
3.2.1. Aqueous heat stability

Initially, DDA vesicles were prepared by the aqueous heat method in various media: (1) ddH_2O; (2) 0.9% (w/v) NaCl; (3) 5% (w/v) Dextrose; (4) 10mM Tris-HCl as described in section 2.2.2. and outlined in Fig. 3.1. The physico-chemical characteristics of the resulting formulations were then investigated (Table 3.1.).

Formulation medium	Day 0		Day 14	
	Size (nm)	ZP (mV)	Size (nm)	ZP (mV)
0.9% NaCl	2425 ± 127	45 ± 4.3	5589 ± 1555	29 ± 2.0
ddH_2O	1095 ± 35	57 ± 4.5	1256 ± 36	56 ± 4.0
5% Dextrose	982 ± 43	47 ± 2.9	1071 ± 119	54 ± 1.6
10mM Tris-HCl	846 ± 130	52 ± 2.6	945 ± 117	52 ± 2.1

Table 3.1. Vesicle size and zeta potential of DDA liposomes prepared by the aqueous heat method in various media. All preparations were stored at 4°C. Vesicle size and zeta potential (ZP) were measured using a Brookhaven ZetaPlus with zeta potential being measured in 1 mM Tris buffer pH 7.4. Results denote mean ± S.D. from at least 3 independent batches.

A. Aqueous Heat Method



B. Lipid Hydration Method

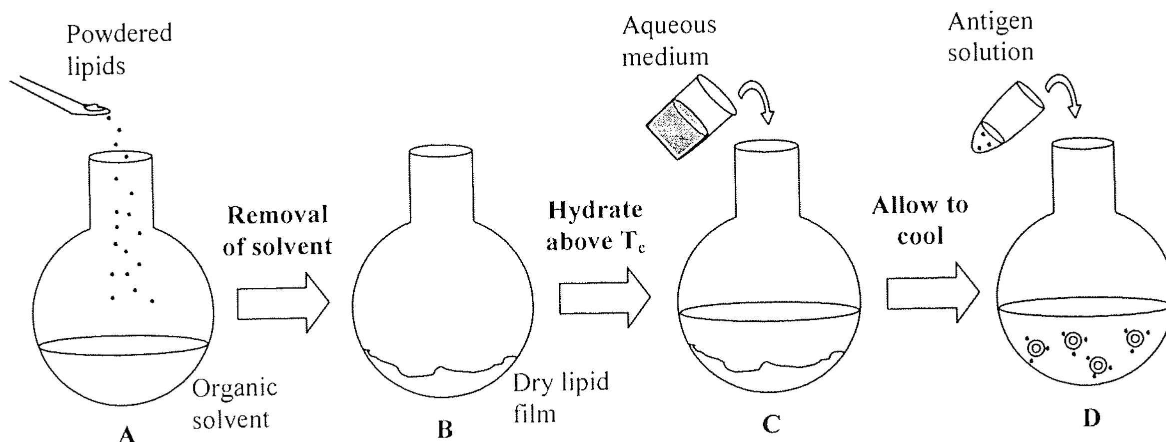


Figure 3.1. Schematic representation of DDA based liposome preparation.
A. Aqueous Heat Method: Initially, DDA was mixed with an aqueous phase to a concentration of 1.25 mg/mL (**A**), and then heated to 80°C for 20 minutes to facilitate formation of the DDA vesicles (**B**). This solution was then allowed to cool to room temperature before addition of Ag85B-ESAT-6 at a concentration of 0.01 mg/mL, followed by vortex mixing.
B. Lipid Hydration Method: Weighed amounts of DDA, and TDB where appropriate, were first dissolved in chloroform/methanol (9:1, by volume) (**A**), followed by removal of the solvent under vacuum to leave a thin lipid film (**B**), which was then purged with a gentle stream of N_2 to remove any residual solvent. Vesicles were subsequently formed by simply hydrating the lipid film with an aqueous phase for 20 minutes, at a temperature 10°C above the main phase transition of DDA ($T_m \approx 47$°C) (**C**), with the final concentrations of DDA and TDB corresponding to 1.25 mg/mL and 0.25 mg/mL respectively. Finally, the solution was allowed to cool before addition of Ag85B-ESAT-6 at a concentration of 0.01 mg/mL, followed by vortex mixing (**D**).

As previously reported, small amounts of salt results in instantaneous vesicle aggregation, possibly due to reduced long range electrostatic repulsion between the positively charged DDA liposomes (Tran et al., 1978; Carmona-Ribeiro & Chaimovich, 1986; Tsuruta et al., 1995; Tsuruta & Carmona-Ribeiro, 1996). Indeed, those vesicles prepared in 0.9% (w/v) NaCl were of a significantly larger size (Table 3.1.), with aggregates beginning to form and precipitate out of solution within 3-5 days of storage (Fig. 3.2.). This salt-induced aggregation is to some extent reversible (Tran et al., 1978; Carmona-Ribeiro et al., 1985; Tsuruta et al., 1995; Tsuruta & Carmona-Ribeiro, 1996), although the results show that even in pure water there is some aggregation over time (Table 3.1.), indicating that electrostatic repulsive forces between the cationic liposomes may be insufficient to stabilise the vesicles (Hilgers & Weststrate, 1991).

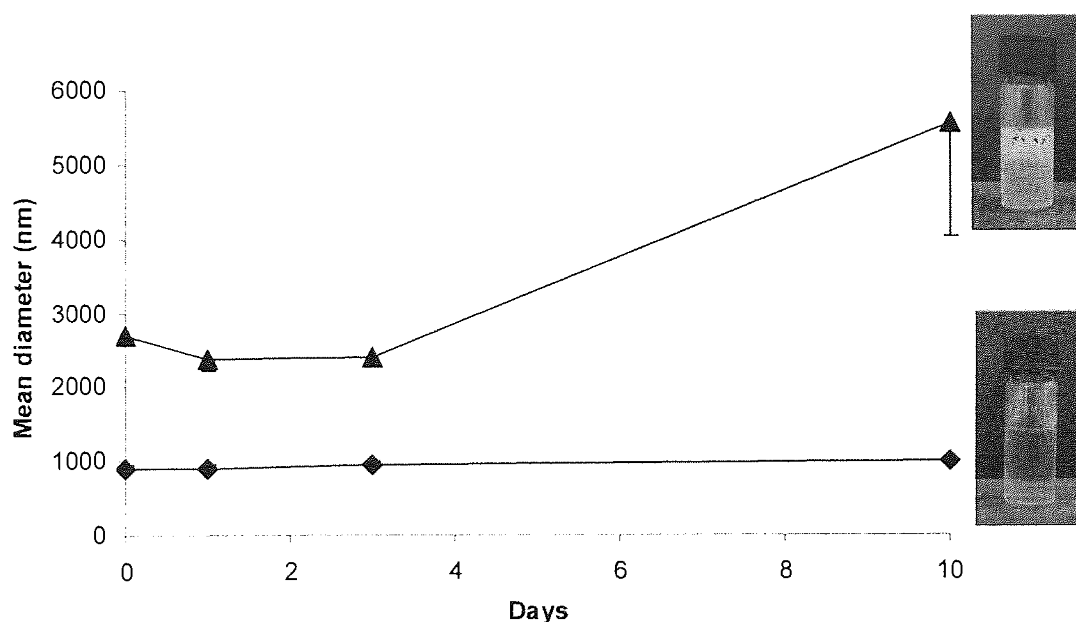


Fig. 3.2. Time development of average particle size of DDA liposomes prepared by the aqueous heat method. A significant increase is observed after 3 days storage in 0.9% (w/v) NaCl (-▲-), whereas no significant change is observed over the time period studied for those liposomes prepared and stored in 5% (w/v) Dextrose (-◆-). Results denote mean ± S.D. from at least 3 independent batches.

Formulation medium	Size (nm)	ZP (mV)	Ag85B-ESAT-6 adsorption efficiency (%)
5% Dextrose	982 ± 43	47 ± 2.9	44 ± 5.3
10mM Tris-HCl	846 ± 130	52 ± 2.6	74 ± 3.2

Table 3.2. Vesicle size, zeta potential and Ag85B-ESAT-6 adsorption efficiency of DDA liposomes prepared by the aqueous heat method in either 5% (w/v) Dextrose or 10 mM Tris-buffer (pH 7.4). Vesicle size and zeta potential (ZP) were measured using a Brookhaven ZetaPlus, with zeta potential being measured in 1 mM Tris buffer pH 7.4. For antigen adsorption, ^{125}I-labelled Ag85B-ESAT-6 was adsorbed onto liposomes, and non-associated antigen removed by ultracentrifugation. Results denote mean ± S.D. from at least 3 independent batches.

Nevertheless, although the zeta potential of the vesicles decreases over time when formulated in 0.9% NaCl, the charge density of the systems remains approximately equivalent considering the increased vesicle size. Therefore, the relatively large size of the vesicles is likely to be a greater contributory factor in the reduced stability, since larger vesicles have a greater tendency to aggregate.

Indeed, this aggregation is reduced when the liposomes are formulated in either 5% (w/v) dextrose (Fig. 3.2.) or 10mM Tris-HCl (pH 7.4) and stored at 4°C (Table 3.1.), with the vesicles exhibiting significantly smaller sizes than when formulated in 0.9% NaCl. Consequently, 10mM Tris-HCl (pH 7.4) was carried forward as the medium of choice due to the smaller size of DDA vesicles, much improved Ag85B-ESAT-6 adsorption efficiency (74 ± 3.2 % as compared to 44 ± 5.3 %; Table 3.2.), and ability to maintain pH at an acceptable level (results not shown).

Following on from this initial work, the lipid hydration method (see section 2.2.1. and Fig. 3.1.) was then proposed as an alternative preparation technique, leading to a

comparison between the methods in formulating vesicles possessing the desired physico-chemical attributes, the results of which have been recently published (Davidsen et al., 2005).

3.2.2. Dynamic light scattering: Particle size and zeta potential

The physico-chemical characteristics of DDA and DDA-TDB vesicles prepared by either the lipid film hydration method or the aqueous hydration method were studied. Results in Table 3.3. demonstrate that liposomes prepared by lipid hydration were significantly smaller than liposomes of the same composition prepared by the aqueous heat method (400-500nm vs. 800-1300nm respectively; Table 3.3.). Further, whilst supplementation of DDA liposomes with TDB resulted in no significant difference in vesicle size when prepared by the lipid hydration method, the opposite was apparent when liposomes were prepared by the aqueous heat method, with DDA-TDB vesicles being larger than their DDA counter-parts (Table 3.3.).

Formulation	Aqueous heat method		Lipid hydration method	
	Size (nm)	ZP (mV)	Size (nm)	ZP (mV)
DDA	846 ± 130	52 ± 2.6	488 ± 124	46 ± 1.6
DDA+H1	929 ± 113	47 ± 2.6	421 ± 26	52 ± 3.1
DDA-TDB	1281 ± 134	47 ± 0.6	416 ± 40	48 ± 5.1
DDA-TDB+H1	1256 ± 150	44 ± 0.3	486 ± 110	60 ± 4.7

Table 3.3. Vesicle size and zeta potential of DDA liposomes prepared from DDA with and without TDB (11 mol%) by either the aqueous heat or lipid hydration method. In both cases the liposomes were dispersed in 10 mM Tris buffer with pH 7.4. The effect of Ag85B-ESAT-6 (H1) adsorption on vesicle size and zeta potential was measured by adding H1 (0.01 mg/ml). Vesicle size and zeta potential (ZP) were measured using a Brookhaven ZetaPlus, with zeta potential being measured in 1 mM Tris buffer pH 7.4. Results denote mean ± S.D. from at least 3 independent batches.

This result suggests that the TDB is potentially less effectively incorporated/packaged within the lipid membranes when the aqueous heat method was adopted, whereas the concentration of the lipids as a film in the alternative lipid hydration method is likely to lead to improved packing of TDB within the lipid membrane.

The zeta potential of liposomes can both directly influence liposome suspension stabilities and indirectly reflect vesicle surface net charge – a factor which can be used to evaluate the extent of charged lipid incorporation and head-group interaction. Zeta potential analysis of the DDA and DDA-TDB formulations show that all liposomes tested have a high net positive charge (46-52 mV; Table 3.3.), clearly resulting from the cationic nature of DDA. Neither the method of preparation nor the inclusion of TDB within the liposome membranes was shown to make a significant difference to this positive surface charge, suggesting that no marked electrostatic interactions between the DDA and TDB lipids occurred at the surface of the vesicle (Table 3.3.). In addition, the adsorption of Ag85B-ESAT-6 antigen (0.1 mg/ml) to both DDA and DDA-TDB liposomes made no significant difference to the measured size or zeta potential of these vesicles (Table 3.3.), suggesting that, at these concentrations, adsorption of antigen on the vesicle surface made no significant impact on the physical attributes of the liposomes.

Unfortunately, dispersions of DDA liposomes are physically unstable, and prolonged storage at 4°C is not possible without the visible occurrence of aggregation and precipitates. This is supported by the particle size data shown in Fig. 3.3. The average particle size of pure DDA liposomes increases rapidly during the first 10 days of storage

due to aggregation or fusion of the liposomes, and after 14 days of storage it becomes increasingly difficult to determine the size of the liposomes using dynamic light scattering.

In contrast, the liposomes containing small amounts of the glycolipid TDB showed little or no change in particle size, indicating that the presence of TDB in the DDA liposome bilayers leads to short- as well as long-term stabilisation of the size of the liposomes. Similar results were also shown at room temperature (25°C; Fig. 3.3.), with the particle size of DDA liposomes increasing approximately four-fold after only 7 days to 2033 nm, compared to DDA-TDB (11 mol%) which showed no significant increase in size after 14 days.

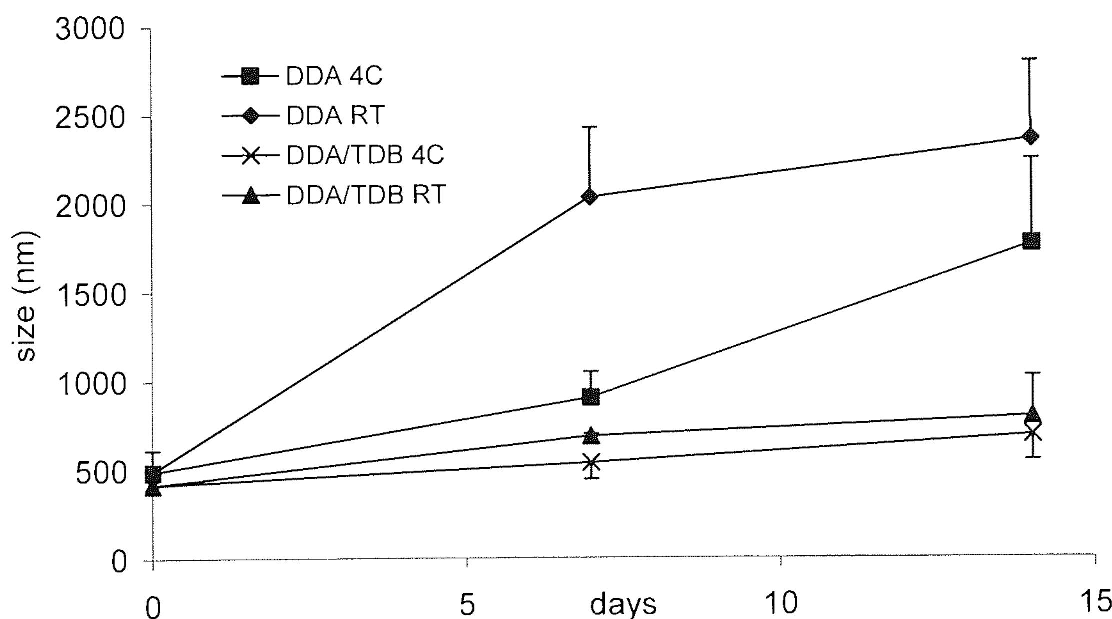


Fig. 3.3. Time development of average particle size of DDA and DDA-TDB (11 mol%) liposomes stored at 4°C and room temperature (RT). The liposomes were dispersed in 10 mM Tris buffer adjusted to pH 7.4. A significant increase is observed for DDA liposomes without TDB after 14 days. Vesicle size and zeta potential (ZP) were measured using a Brookhaven ZetaPlus, with zeta potential being measured in 1 mM Tris buffer pH 7.4. Results denote mean ± S.D. from at least 3 independent batches.

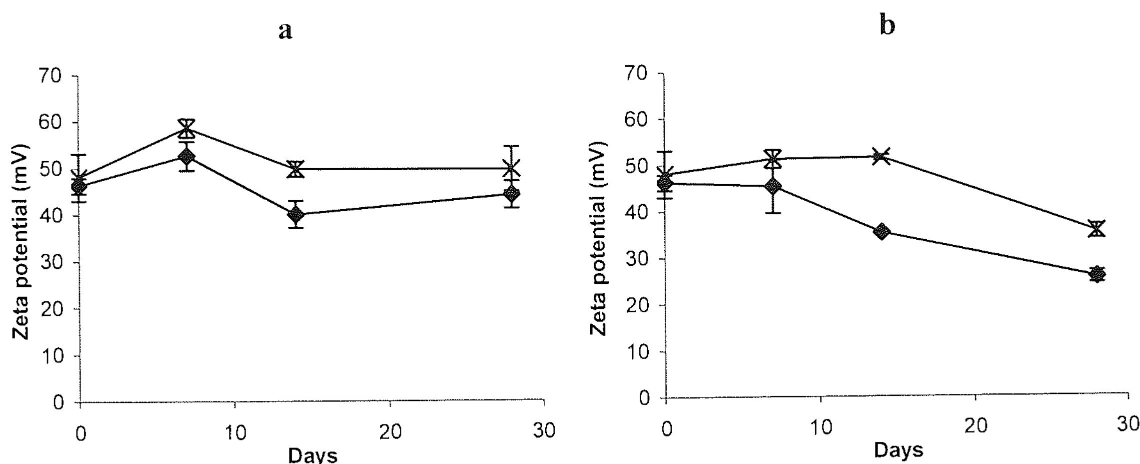


Fig. 3.4. Time development of average zeta potential of DDA (-♦-) and DDA/TDB (11 mol %) (-*-) liposomes dispersed in 10 mM Tris-buffer (pH 7.4) and stored at (a) 4°C and (b) 25°C. Zeta potential (ZP) was measured using a Brookhaven ZetaPlus, with zeta potential being measured in 1 mM Tris buffer pH 7.4. Results denote mean ± S.D. from at least 3 batches.

Zeta potential analysis of these formulations reveals no major changes in the surface characteristics of liposomes stored at 4°C for up to 28 days (Fig. 3.4a.), with the zeta potential remaining at approximately 45 mV, although storage at room temperature conditions resulted in a marked reduction in the zeta potential of the DDA and DDA-TDB liposomes to around 35 mV at day 14 and 28 respectively (Fig. 3.4b.).

It has previously been shown that double chain glycolipids, such as TDB and TDM, inhibit fusion between phospholipid vesicles (Spargo et al., 1991; Crowe et al., 1994b). The stabilising effect is presumably caused by the relatively large hydrophilic trehalose head-group increasing the overall hydration of the liposomal surface, preventing dehydration of the quaternary ammonium head-groups and aggregation caused by reduced charge repulsion. Alternatively, the trehalose moiety might act as a steric barrier that can inhibit electrostatic repulsion between DDA headgroups, thus stabilising the membrane structure, whilst also preventing close contact between opposing liposomes,

which is a prerequisite for the formation of aggregation or fusion of the liposomes (Spargo et al., 1991; Crowe et al., 1994b).

3.2.3. Adsorption of antigen

To characterise the antigen adsorption to DDA-TDB liposomes, the mycobacterial Ag85B-ESAT-6 fusion protein was used. This protein has been identified as a promising vaccine antigen against tuberculosis in several studies (Olsen et al., 2001; Olsen et al., 2004; Langermans et al., 2005), a disease for which a Th1 type immune response is required for protection (Flynn et al., 1993; Ellner et al., 2000).

The degree of adsorption of antigen (% of total used; 0.01 mg) to both DDA and DDA-TDB liposomes was determined using ^{125}I-labelled Ag85B-ESAT-6 (Section 2.5.). The addition of TDB had no detrimental effect on protein adsorption, with both DDA and DDA-TDB formulations adsorbing approximately 65 – 80% of the total antigen initially added (Table 3.4.). The adsorption of antigen to the formulation is most probably electrostatic in nature, since the antigen is highly negatively charged and has a theoretical pI value of 4.80, whereas the zeta potential analysis of the liposomes demonstrates that all DDA-TDB formulations had a high net positive charge of 46 to 52 mV (Table 3.3.). The method of preparation was shown to impact on antigen adsorption, with slightly lower adsorption for liposomes prepared by the lipid hydration method (Table 3.4.); however, this could be related to the small vesicle size associated with this method, which could result in some small vesicle populations failing to pellet effectively during the centrifugation process.

Formulation	Aqueous heat method	Lipid hydration method
DDA	74.3 ± 3.2 %	69.9 ± 10.2 %
DDA-TDB	78.7 ± 2.1 %	67.0 ± 2.8 %

Table 3.4. Adsorption of antigen to adjuvant liposomes. ^{125}I-labelled Ag85B-ESAT-6 was adsorbed onto DDA or DDA-TDB (11 mol%) liposomes prepared by either the aqueous heat or lipid hydration method as in Table 3.3, and non-associated antigen removed by ultracentrifugation. Values denote mean ± S.D. from at least 3 independent experiments.

3.2.3.1. Antigen retention

After removal of non-adsorbed Ag85B-ESAT-6 via centrifugation, antigen retention to the liposomes after storage at both 4 °C and 25°C was measured (Fig. 3.5.). After 7 days storage at 4°C (Fig. 3.5a.), antigen retention in both formulations dropped to approximately 80% of the initial antigen adsorption. Subsequently at time points thereafter, retention values for DDA-TDB liposomes remained constant at ~80% with no further losses of antigen being measured (Fig. 3.5a.).

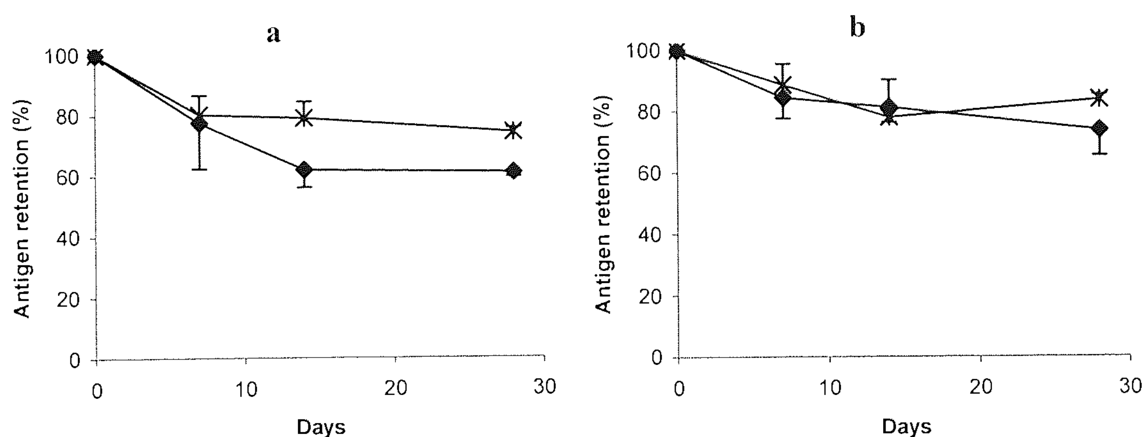


Fig. 3.5. Adsorbed antigen retention over time. DDA (-♦-) and DDA-TDB (11 mol %) (-*-) liposomes were prepared by the lipid hydration method, dispersed in 10 mM Tris buffer (pH 7.4) and stored at 4°C (a) and 25°C (b). ^{125}I-labelled Ag85B-ESAT-6 was adsorbed to liposomes and antigen retention of Ag85B-ESAT-6 was determined on the basis of ^{125}I radioactivity recovered in the suspended pellets after ultracentrifugation. Results represent percentage retention of initial adsorbed antigen (Table 3.4.) expressed as mean ± SD from at least 3 independent batches.

In contrast, further loss of antigen was associated with the DDA formulation, with retention values continuing to drop to approximately 65% (of initial adsorption values) at day 14 and subsequently levelling around this value thereafter (Fig. 3.5a.). Storage of the formulations at 25°C had no major impact on antigen retention compared to refrigerated samples over the period of analysis, however at this temperature significant differences ($p<0.05$) in antigen retention between liposomes with and without TDB were only apparent at day 28 (Fig. 3.5b).

3.2.3.2. Antigen release

^{125}I-labelled Ag85B-ESAT-6 was also employed to investigate the *in vitro* release profiles of DDA and DDA-TDB liposomes by incubating the samples at 37°C in physiological pH conditions in a shaking water bath (Fig. 3.6.).

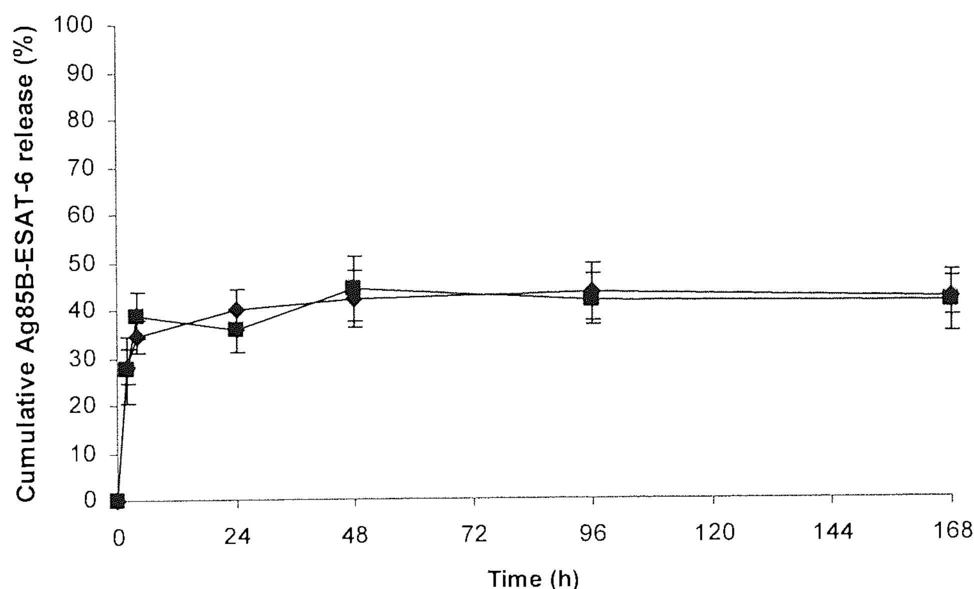


Fig. 3.6. Cumulative antigen release (%, w/w) vs time. DDA (♦) and DDA-TDB (11 mol%) (■) liposomes were prepared by the lipid hydration method, dispersed in 10 mM Tris buffer (pH 7.4) and incubated in simulated physiological conditions (pH 7.4, 37°C) in a shaking water bath. Ag85B-ESAT-6 release was determined on the basis of radioactivity of ^{125}I-labelled Ag85B-ESAT-6 recovered in the suspended pellets after ultracentrifugation. Results represent percentage release of initially loaded antigen expressed as mean ± s.d., n= 3.

The inclusion of TDB appeared to have no effect on the observed release profiles of the liposomes, with both formulations showing an initial burst release of around 40% of the adsorbed antigen, followed by a prolonged plateau. This result suggests that the remaining 60% of antigen is still associated with the liposomes, which is potentially beneficial for its delivery within the antigen presenting cells.

3.2.4. Morphology studies using Transmission Electron Microscopy

Initially Transmission Electron Microscopy (TEM) was used to investigate the morphological characteristics of liposomes prepared by either the aqueous heat or lipid hydration method. Fig. 3.7a shows that DDA liposomes prepared by the aqueous heat method are angular and relatively large in size (> 1 μm), with notable signs of vesicle aggregates. However, the angular morphology of the liposomes prepared by the aqueous heat method may simply be an artefact of the TEM sample preparation, since the staining and drying of the sample prior to imaging could cause the vesicle to crinkle up, thus appearing angular as in Fig. 3.7a. The larger vesicle size of liposomes produced by aqueous heat compared to lipid hydration is also apparent when comparing the DDA-TDB (Fig. 3.7b. vs. 3.7e.) and DDA-TDB/protein (Fig. 3.7c. vs. 3.7f.) liposome formulations. The formulation of the liposomes had less of an impact on the morphological characteristics of the aqueous heat preparations, with no notable morphological changes seen upon addition of 11 mol% TDB (Fig. 3.7b.) and protein (Fig. 3.7c.), with vesicles remaining around 1 micron in size. However, the extent of aggregation appears reduced when TDB is incorporated within the liposomal membranes, again suggesting the stabilising role TDB may play in the vesicle construct.

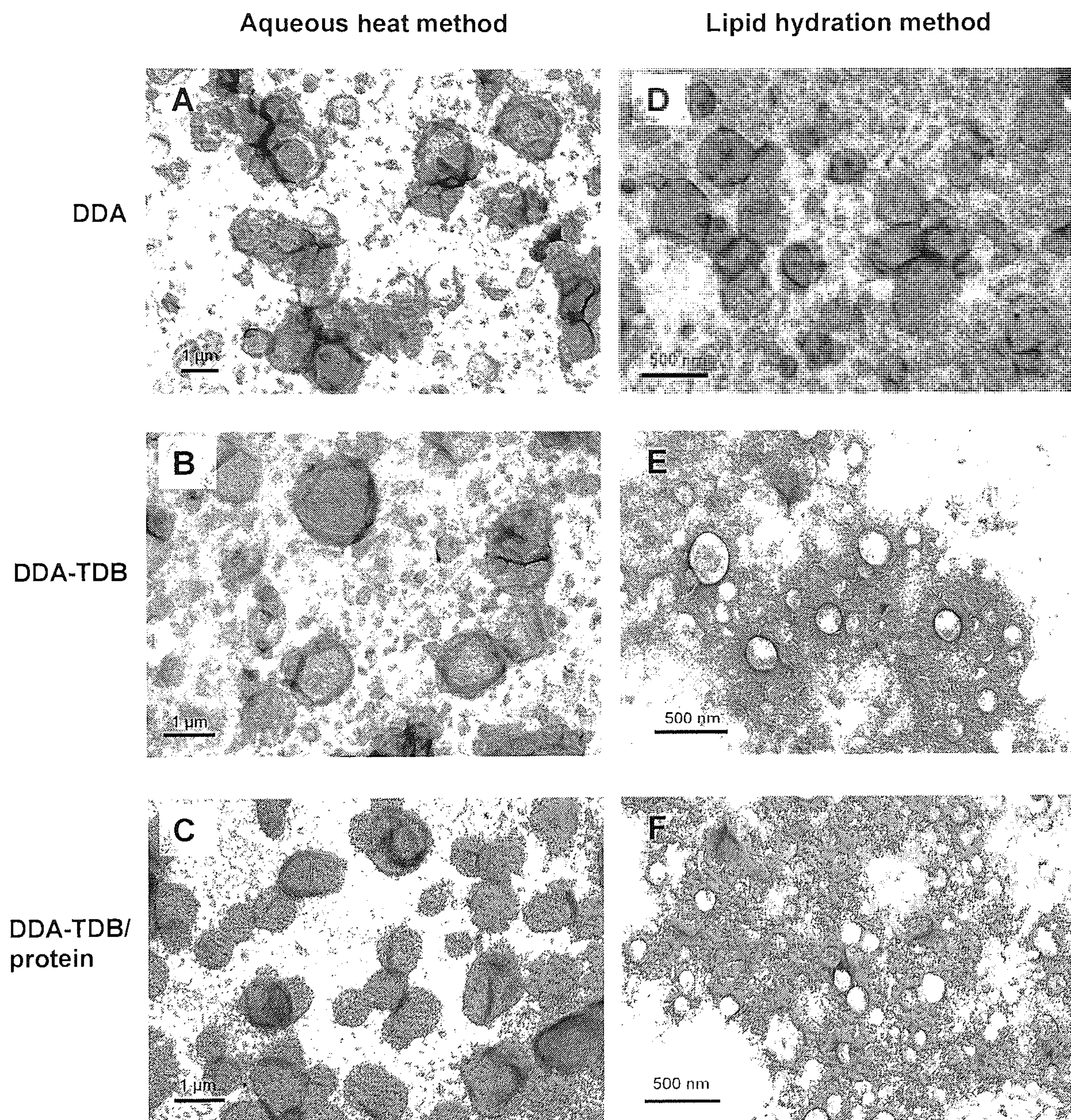


Fig. 3.7. Morphological analysis by TEM. DDA Liposomes in Tris-buffer prepared by aqueous heat method (a) were angular, relatively large in size and show significant vesicle aggregation. However, the angular morphology may be a consequence of the staining and drying involved n the sample preparation prior to imaging. No morphological changes are seen upon addition of 11 mol% TDB (b) or protein (c). When prepared by lipid hydration method, DDA liposomes (d) were smaller than those prepared by aqueous heat (a), although some large vesicles are present. Addition of 11 mol% TDB to these DDA liposomes gave more spherical vesicles which display less aggregation (e). Similar to aqueous heat preparations, no obvious morphological change was apparent upon addition of protein to lipid hydration liposomes (f). In all formulations, liposomes prepared by the aqueous heat method are larger than the lipid hydration counter-parts.

In agreement with the particle size analysis, when prepared by lipid hydration method, DDA liposomes (Fig. 3.7d.) were smaller (~ 500nm) than those prepared by aqueous heat (Fig. 3.7a.), although some large vesicles and aggregates are present.

Addition of 11 mol% TDB to the liposome formulation (Fig. 3.7e.) gave more spherical vesicles, which display less aggregation compared to their DDA counterparts, possibly again due to the bilayer stabilising effect of the longer alkyl chain TDB. Furthermore, the absence of angular vesicles, as seen previously for the aqueous heat preparations, once again suggests that the TDB is more effectively incorporated into the bilayer when prepared by the lipid hydration method, thereby enhancing rigidity and avoiding the crinkling up of the liposomes following staining and drying. Similar to aqueous heat preparations, no obvious morphological change was apparent upon addition of protein to lipid hydration liposomes (Fig. 3.7f.).

3.2.5. Immunological characterisation of the DDA-TDB Ag85B-ESAT-6 vaccine

The adjuvant effect of the DDA-TDB formulation for the delivery of the Ag85B-ESAT-6 fusion protein was subsequently tested in mice in collaboration with the Statens Serum Institute (SSI), Copenhagen. Mice were immunised three times with the vaccine antigen mixed with DDA liposome dispersions either alone or incorporating TDB (11 mol%) (for immunisation schedule, see Appendix 2). One week after the third immunisation, the specific immune response of the blood cells was investigated by re-stimulation with Ag85B-ESAT-6 *in vitro* (Fig. 3.8a.) and subsequently measuring secretion of the cytokines IFN-γ and IL-5 as indicators of a Th1 and Th2 response, respectively.

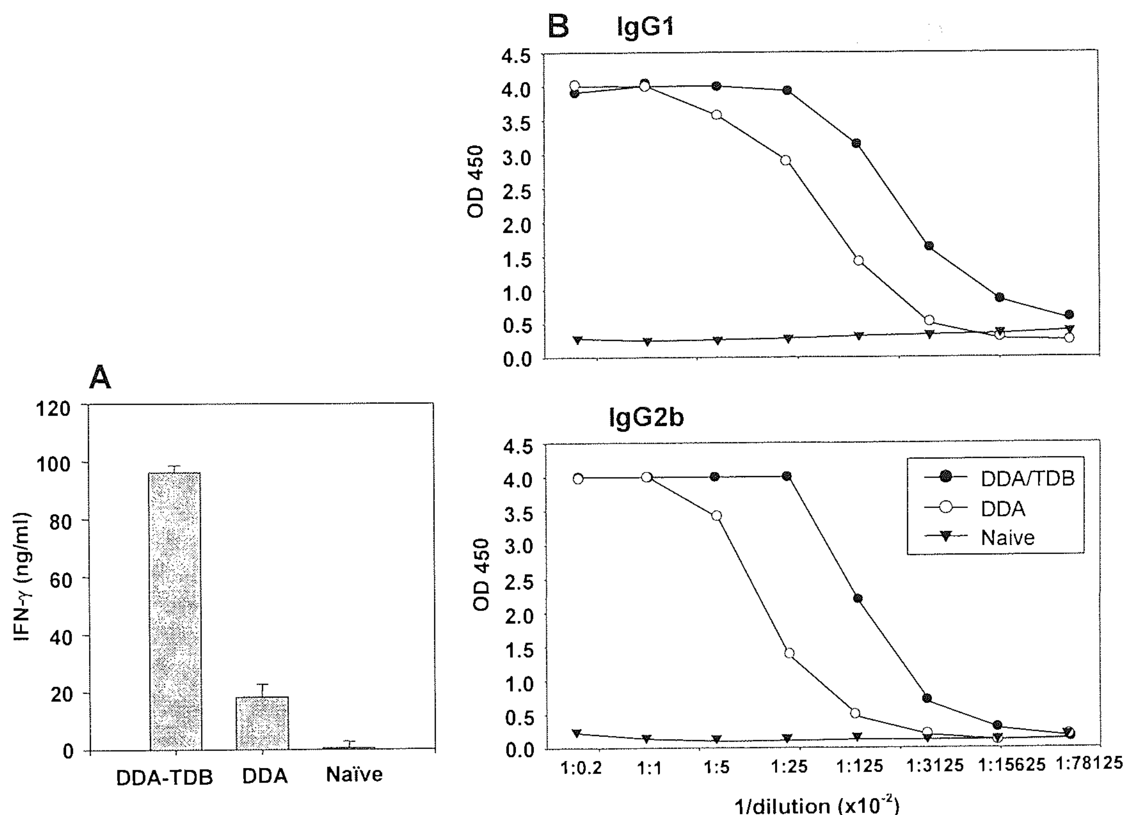


Fig. 3.8. Induction of immune response using DDA-TDB liposomes as adjuvant. Release of IFN-γ from blood lymphocytes isolated from C57Bl/6j mice immunised with 2 μg of Ag85B-ESAT-6 administered in DDA liposomes incorporating 11 mol% TDB (**A**). Blood lymphocytes were isolated 5 weeks after the first immunisation and re-stimulated in vitro with the Ag85B-ESAT-6 (5μg/ml). IgG1 and IgG2b Ag85B-ESAT-6-specific antibody dilution curves (**B**). Experiments performed by SSI. For immunisation schedule, see Appendix 2.

The formulation with TDB at 11 mol% was able to induce high levels of IFN-γ (96.2 ± 1.6 ng/ml) (Fig. 3.8a.) (Davidsen et al., 2005), which can be expected since the lipid components of the formulation have previously been reported to be potent Th1 adjuvants (Holten-Andersen et al., 2004). Furthermore, the ability of DDA-TDB to induce antibody responses after immunisation was investigated by immunising with Ag85B-ESAT-6 in the DDA and DDA-TDB formulations three times, and measuring the levels of antigen-specific antibodies of the IgG1 and IgG2b isotypes by ELISA (Fig. 3.8b.). The IgG2a

titre, which is widely used as an indicator of a Th1-type of immune response, was not evaluated since the gene is deleted in C57Bl/6 mice (Martin & Lew, 1998).

As shown in Figure 3.8b., the addition of TDB to DDA causes an elevation of the antibody levels, with a level of IgG2b 9-fold higher compared to DDA alone. The groups receiving the DDA-TDB formulation exhibited a high ratio of IgG2b compared to IgG1 (ratio 0.2), which is a characteristic seen with the induction of a Th1-immune response, further indicating a potential role in inducing anti-mycobacterial immunity.

In order to evaluate the adjuvant activity of DDA-TDB compared to an adjuvant already approved for human use, our collaborators at the SSI also immunised mice with Ag85B-ESAT-6 in either DDA-TDB or Alum and the immune responses post-immunisation evaluated (Davidsen et al., 2005).

As shown in Fig. 3.9a., immunisation with DDA-TDB leads to high levels of IFN-γ release and low levels of IL-5 whereas Alum-immunised mice exhibited the opposite pattern with a negligible IFN-γ secretion and higher levels of IL-5. DDA-TDB gives rise to the same high levels of IgG1 antibody titres as seen after immunisation with alum, whereas the level of Ig2b antibodies were 8-fold higher after immunisation with DDA-TDB compared to alum, indicating a dominant Th1-type of immune response obtained with DDA-TDB (Fig. 3.9b).

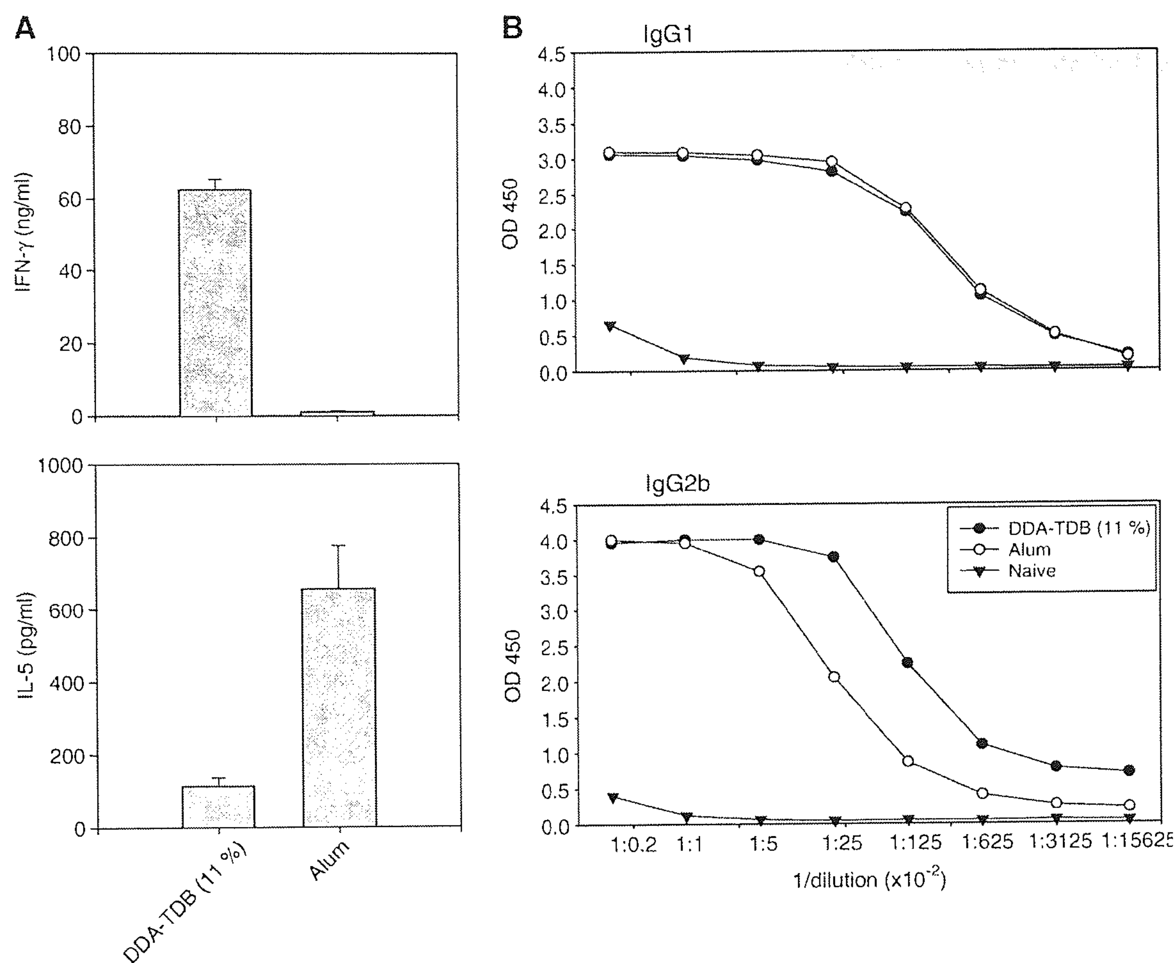


Fig. 3.9. Immune responses generated by DDA-TDB liposomes and alum. Release of IFN-γ and IL-5 from blood lymphocytes isolated from C57Bl/6j mice immunised with 2 μg of Ag85B-ESAT-6 administered in DDA-TDB with 11 mol% TDB incorporated or alum (**A**). IgG1 and IgG2b Ag85B-ESAT-6-specific antibody dilution curves (**B**). Experiments performed by SSI. For immunisation schedule, see Appendix 2.

The Th1 polarisation and the robust immune response induced by the DDA-TDB formulation is likely due to several factors, including: the particulate nature of the formulation, which lends an inherent adjuvanticity to the system (Storni et al., 2005; Perrie et al., 2007a); the relatively high cationic surface charge, which will facilitate interaction with the negatively charged surface of the APCs (Denis-Mize et al., 2003; Smith Korsholm et al., 2007); and also the chemical composition, since both components are potent immunomodulators with a tendency for Th1 type immune responses (Gall, 1966; Lindblad et al., 1997; Holten-Andersen et al., 2004).

3.3. Conclusions

The adjuvant liposomes presented here show the commendable ability to stimulate both a cell mediated Th1 immune response and an antibody response. This broad stimulation of the immune response is a unique property of DDA-TDB and makes the adjuvant advantageous for a high number of disease targets including infectious diseases, such as TB, and cancers. The testing of DDA-TDB as an adjuvant for vaccines with different requirements is presently ongoing and would provide important information on the applicability of the adjuvant. Besides potentiating the immune response of DDA, incorporation of TDB was shown to effectively stabilise the DDA liposomes.

This is, to our knowledge, the first time it has been demonstrated that cationic adjuvant liposomes are stabilised by incorporating synthetic glycolipid analogues of TDM into the liposome bilayers, thus overcoming the major obstacle for using DDA as an efficient adjuvant system, whilst simultaneously improving the immunogenicity of the adjuvant considerably.

Chapter 4

Optimisation of formulation parameters involved in the production of biodegradable PLGA microspheres for the delivery of the novel sub-unit TB vaccine antigen, Ag85B-ESAT-6

4.1. Introduction

Biodegradable poly(DL-lactide-co-glycolide) (PLGA) microspheres appear to be an ideal candidate for the delivery of the sub-unit vaccine Ag85B-ESAT-6 for several reasons, including their relative biocompatibility, adjuvanticity and prolonged drug release profile.

It has been reported that PLGA microparticles exhibit an adjuvant effect for both humoural (Eldridge et al., 1991; O'Hagan et al., 1991) and cell-mediated immunity. Indeed, sub-10 μm PLGA microspheres are readily recognised and ingested by macrophages and dendritic cells, an important property for stimulating the immune response (Audran et al., 2003).

In addition, the possibility to encapsulate antigens into the polymer matrix not only acts to facilitate more efficient delivery by acting as a shield from the hostile external environment (Jilek et al., 2005), but also has the potential to reduce adverse reactions and abrogate problems caused by the vaccine strain in immunocompromised individuals (Bramwell & Perrie, 2005a). Furthermore, the controlled, sustained release of the encapsulated antigen from such formulations suggests they have the potential to eliminate the need for multiple vaccination doses (Langer & Folkman, 1976; Preis & Langer, 1979; Jiang et al., 2005).

The work outlined in this chapter describes the optimisation of formulation parameters involved in production of polymeric microspheres for the delivery of the aforementioned

fusion protein Ag85B–ESAT-6 by the modified water-in-oil-in-water ($w_1/o/w_2$) double emulsion-solvent evaporation method (described in section 2.7.1.).

In order to achieve an optimum formulation, the following parameters involved in the double emulsion solvent evaporation process were investigated for their effect on size of empty (Ag85B-ESAT-6 free) PLGA (75:25) microspheres:

1. Time of homogenisation during formation of the secondary w/o/w emulsion: investigated by varying the duration of homogenisation (1, 3, 5, 10, 15 or 30 minutes) at a constant stirring speed of 6000 rpm.
2. Concentration of polymer in the organic phase: investigated by preparing empty PLGA microspheres with varying amounts (0.5, 1, 3, 5 or 6 % (w/v)) of polymer in a fixed volume of organic phase (417 μl $CHCl_3$).
3. Concentration of emulsion stabiliser in the external aqueous phase: investigated by varying the amount of PVA (0.5, 1.5, 3, 5 or 10 % (w/v)) while maintaining a constant volume for the external aqueous phase (10 ml double distilled water).

Additionally, the effect of the addition of immunomodulators DDA and TDB to the organic phase of the double emulsion, either alone or in combination, was investigated in terms of physico-chemical characteristics. DDA was added at a concentration of 20% (w/w) of polymer, whilst TDB was added at a concentration of 4% (w/w/) of polymer in order to standardise the amount of each adjuvant for comparison with the previously studied DDA-TDB liposomal system (see Chapter 3). Finally, the entrapment of Ag85B-ESAT-6 into the microspheres was also investigated in terms of physico-chemical and immunological characteristics.

4.2. Effect of formulation parameters on mean particle size and size distribution

4.2.1. Duration of homogenisation

Initially, the time of homogenisation of the secondary w/o/w emulsion was investigated with regard to microsphere particle size characteristics. For a constant speed of 6000 rpm, an increase in duration of homogenisation time from 1 to 10 minutes led to a general decrease in particle size (Table 4.1.). However, once ten minutes was exceeded, the mean particle size tended to increase once more, suggesting fusion of semi-formed particles, or possibly instability in the emulsion, due to prolonged shear forces.

Although all microspheres produced were well within the desired sub-10 μm range, a homogenisation time of 3 minutes was chosen for formulating the vaccines. Whilst there is no significant difference between 1 and 3 minutes in terms of sizes produced, further investigation is necessary to determine the effect of this parameter on protein entrapment and long-term stability. Also, despite statistics suggesting significantly smaller particles are produced when the homogenisation time is increased from 3 minutes to 5, 10 or 15 minutes ($p<0.05$, ANOVA followed by Tukey test), excessive shear forces should be avoided in order to limit denaturation of the protein.

Therefore, in an attempt to reduce the deviation on the particle size, whilst also reducing the shear stresses experienced by the protein and ensuring efficient emulsification, the homogenisation time was standardised to 3 minutes per outlined batch.

4.2.2. Concentration of polymer in the organic phase

Similarly, varying polymer concentrations (from 0.5 to 6%, w/v) in the organic phase were investigated for their effect on particle size (Table 4.1.). Although no significant change in mean particle size was observed with an increase in PLGA content from 0.5 to 3% (w/v), this did result in a significant increase in uniformity ($p<0.05$, ANOVA followed by Tukey test), as derived from the standard deviation value (s.d.) calculated by the Mastersizer software that is an indication of the distribution of sizes. This observation can be explained by the increase in viscosity, and hence stability, of the emulsion produced. As polymer concentration exceeded 3% (w/v), an overall increase in particle size was seen; suggesting that an increase in the frequency of collisions may result in fusion of semi-formed particles, as previously reported (Benoit et al., 1999). In addition, high viscosity solutions, such as the 5% and 6% (w/v) polymer solution, may reduce the efficiency of homogenisation, possibly explaining the production of significantly larger particles as compared to those produced by the 3% (w/v) polymer solution (Table 4.1.).

As regards antigen entrapment, migration of the internal aqueous phase to the external aqueous phase may be restricted with increased viscosity of the organic phase (Rafati et al., 1997), although, once again, reduced emulsion efficiency caused by high viscosity can lead to a reduction in antigen entrapment.

As a result, a polymer concentration of 3% (w/v) was chosen for the organic phase of the final preparations, since the microspheres produced exhibit the smallest and most uniform size of the concentrations investigated.

	Distribution percentiles (μm)				
	d (10)	d (50)	d (90)	Mean D[4,3]	s.d.[a]
Homogenisation time					
1 min	0.76 ± 0.01	1.27 ± 0.01	2.16 ± 0.06	1.40 ± 0.02	0.64 ± 0.08
3 min[b]	0.81 ± 0.03	1.34 ± 0.05	2.18 ± 0.10	1.43 ± 0.06	0.55 ± 0.03
5 min	0.67 ± 0.02	1.04 ± 0.03	1.59 ± 0.04	1.10 ± 0.03	0.37 ± 0.01
10 min	0.59 ± 0.02	0.93 ± 0.03	1.45 ± 0.03	0.99 ± 0.03	0.34 ± 0.01
15 min	0.64 ± 0.03	1.00 ± 0.06	1.55 ± 0.10	1.06 ± 0.06	0.36 ± 0.03
30 min	0.83 ± 0.02	1.35 ± 0.03	2.17 ± 0.06	1.44 ± 0.03	0.54 ± 0.01
Polymer concentration					
0.50%	0.25 ± 0.03	0.62 ± 0.05	5.24 ± 1.74	1.65 ± 0.26	2.99 ± 0.54
1%	0.32 ± 0.02	0.72 ± 0.07	2.55 ± 0.58	1.45 ± 0.16	2.48 ± 0.16
3%[c]	0.81 ± 0.03	1.34 ± 0.05	2.18 ± 0.10	1.43 ± 0.06	0.55 ± 0.03
5%	0.88 ± 0.02	1.49 ± 0.03	2.42 ± 0.03	1.59 ± 0.03	0.61 ± 0.01
6%	1.04 ± 0.05	1.85 ± 0.09	3.09 ± 0.14	1.98 ± 0.09	0.81 ± 0.04
PVA concentration					
0.50%	1.31 ± 0.02	3.05 ± 0.05	5.66 ± 0.13	3.35 ± 0.06	1.89 ± 0.07
1.50%	1.42 ± 0.02	2.83 ± 0.07	5.05 ± 0.13	3.12 ± 0.07	1.65 ± 0.03
3%	1.36 ± 0.02	2.44 ± 0.03	4.04 ± 0.03	2.60 ± 0.03	1.07 ± 0.01
5%	1.30 ± 0.02	2.34 ± 0.06	3.99 ± 0.12	2.52 ± 0.06	1.09 ± 0.04
10%[d]	0.81 ± 0.03	1.34 ± 0.05	2.18 ± 0.10	1.43 ± 0.06	0.55 ± 0.03

Table 4.1. Size distributions of empty (Ag85B-ESAT-6 free) PLGA (75:25) microspheres prepared by the w/o/w double emulsion solvent evaporation method with varying formulation parameters. Values represent mean ± s.d., n=3.

a – denotes standard deviation as calculated by the Mastersizer software, an indication of the overall distribution of sizes of microspheres in the sample.

b – chosen homogenisation time to ensure effective emulsification despite significantly larger particles than increased times ($p<0.05$) in order to minimise shear forces.

c – chosen polymer concentration since significant increase in uniformity than at lower concentrations and significantly smaller particles than at higher concentrations ($p<0.05$ in both cases).

d – chosen PVA concentration since significantly smaller and more uniform sizes than at any other concentration studied ($p<0.05$).

4.2.3. Concentration of emulsion stabiliser in the external aqueous phase

In accordance with the empirical relationship between particle size and stabiliser concentration/viscosity (Arshady, 1991), an increase in PVA concentration in the external aqueous phase resulted in a decrease of mean particle size and size distribution (Table 4.1.). Indeed, this relationship appears to be relatvely linear, corresponding well with similar trends reported elsewhere (Rafati et al., 1997; Capan et al., 1999). Furthermore, regardless of the concentration of PVA present in the external aqueous phase, the emulsion droplets formed were stable enough to produce microspheres of the desired size following solvent evaporation, again in agreement with previous studies (Benoit et al., 1999).

As a consequence of these results (Table 4.1.), a PVA concentration of 10% (w/v) was chosen for the external aqueous phase in the view that the particles produced were of a significantly smaller and more uniform size than at any other stabiliser concentration investigated ($p<0.05$, ANOVA followed by Tukey test).

4.3. Effect of addition of immunomodulators

4.3.1. Effect on size of PLGA microspheres

Initially, empty microspheres were studied for the effect of addition of adjuvants on mean particle size (Table 4.2.). Systems with no adjuvant present showed a mono-modal distribution of sizes (Fig. 4.1a.) with a mean microsphere size of 1.43 ± 0.06 μm, whilst the addition of DDA alone led to a mean particle size increase of about three-fold (4.22 ± 0.33 μm), with a multi-modal distribution being seen (Fig. 4.1b.). Increases in size can be

expected when substances are added to the polymer matrix, and charged components, such as DDA, are likely to have a profound effect on the formulation of particulate delivery systems. It is conceivable that the positive charge associated with DDA could bring about electrostatic repulsion within the matrix, leading to larger particles following the solvent evaporation process. Likewise, the presence of DDA may contribute to a less homogenous coalescence of emulsion droplets, thereby resulting in a wider distribution of sizes. Alternatively, the cationic headgroup may encourage aggregation or fusion of the microspheres produced through dipolar interaction with the polymer, which may explain the wider distribution of sizes.

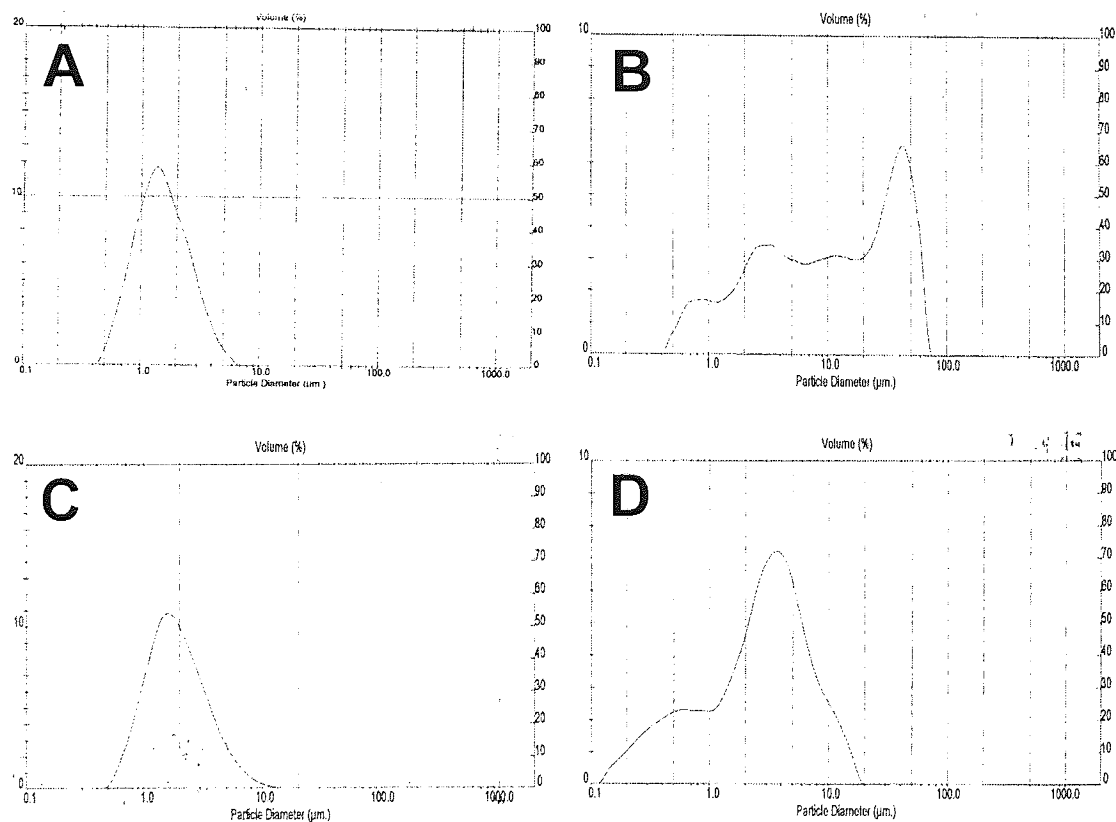


Fig. 4.1. Size distributions of microspheres as determined by laser diffraction. Microspheres consisting of PLGA (**A**), PLGA+DDA (**B**), PLGA+TDB (**C**) and PLGA+DDA+TDB (**D**) were prepared by the w/o/w method (Section 2.7.1.) and then resuspended in solution. Figures represent distribution diagram outputs following laser diffraction analysis using a Mastersizer X (Malvern, UK).

In contrast, the addition of TDB alone resulted in no significant change in mean particle size (2.16 ± 0.35 μm; Table 4.2.) or distribution (Fig. 4.1c.), although this could be attributable to the relatively low content of TDB within the polymer matrix (4 % (w/w) of polymer). However, the addition of both DDA and TDB led to an increase of only about two-fold in mean particle size (2.98 ± 0.08 μm; Table 4.2.), which is significantly smaller ($p < 0.005$) than those microspheres produced with the addition of DDA alone, suggesting that TDB has a stabilising role in the formation of the polymer matrix. In addition, the particle size distribution remained relatively mono-modal (Fig. 4.1d.), further implicating the stabilising effect of TDB.

It is likely that the presence of the TDB molecules will affect the interfacial tension of the emulsion droplets due to the large trehalose head-group, therefore leading to a more homogeneous coalescence of droplets and, subsequently, a more homogeneous size distribution. However, further studies with varying amounts of TDB would be required to establish this hypothesis, although similar work investigating the effect of TDB on surface pressure of DDA monolayers is currently being carried out by our collaborators (Christensen et al, unpublished data), which may lend weight to this theory.

4.4. Antigen loaded Microspheres

4.4.1. Antigen entrapment efficiencies

^{125}I radiolabelled Ag85B-ESAT-6 was employed to investigate entrapment efficiency in each of the microsphere formulations (Table 4.2.; Section 2.7.1.). Microspheres prepared with no immunomodulator present (i.e. PLGA only) showed a high antigen entrapment of

95 ± 1.2%, possibly attributable to the relatively low antigen concentrations used in the systems (0.2% (w/w) of polymer). However, this entrapment efficiency is greatly reduced to 57 ± 0.8% with the addition of DDA. As stated previously, the addition of the cationic DDA to the formulation may lead to a more porous or expanded polymer matrix, and hence an increase in the migration of the internal aqueous phase to the external phase, resulting in a decrease in antigen entrapment, although further investigations, with cryo-SEM for instance, would be required to confirm this.

Indeed, microscopic investigations of the primary emulsion (Figs. 4.2a-d.) reveal that, in contrast to the fairly homogeneously populated droplets formed in the absence of DDA (Fig 4.2a.), the presence of DDA within the organic phase leads to a more heterogeneous population of droplets in terms of size (Fig. 4.2b.), as hypothesised earlier, with droplets in droplets also being seen (Fig. 4.2c.). This instability within the primary emulsion evidently leads to a reduction in Ag85B-ESAT-6 entrapment efficiency.

The addition of TDB also reduced antigen loading efficiency, down to 80 ± 0.7% (Table 4.2.). This reduction was less marked than the effect of DDA addition, which again suggests that TDB is less destabilising than DDA. However, the addition of both immunomodulators led to the greatest reduction in entrapment efficiency, down to 55 ± 2.3%, despite the relative stability of the emulsion droplets (Fig. 4.2d.). Nevertheless, the differences in antigen entrapment are not as profound as the results suggest when the relatively low antigen loading of the microsphere formulations (0.2% w/w of polymer) is considered.

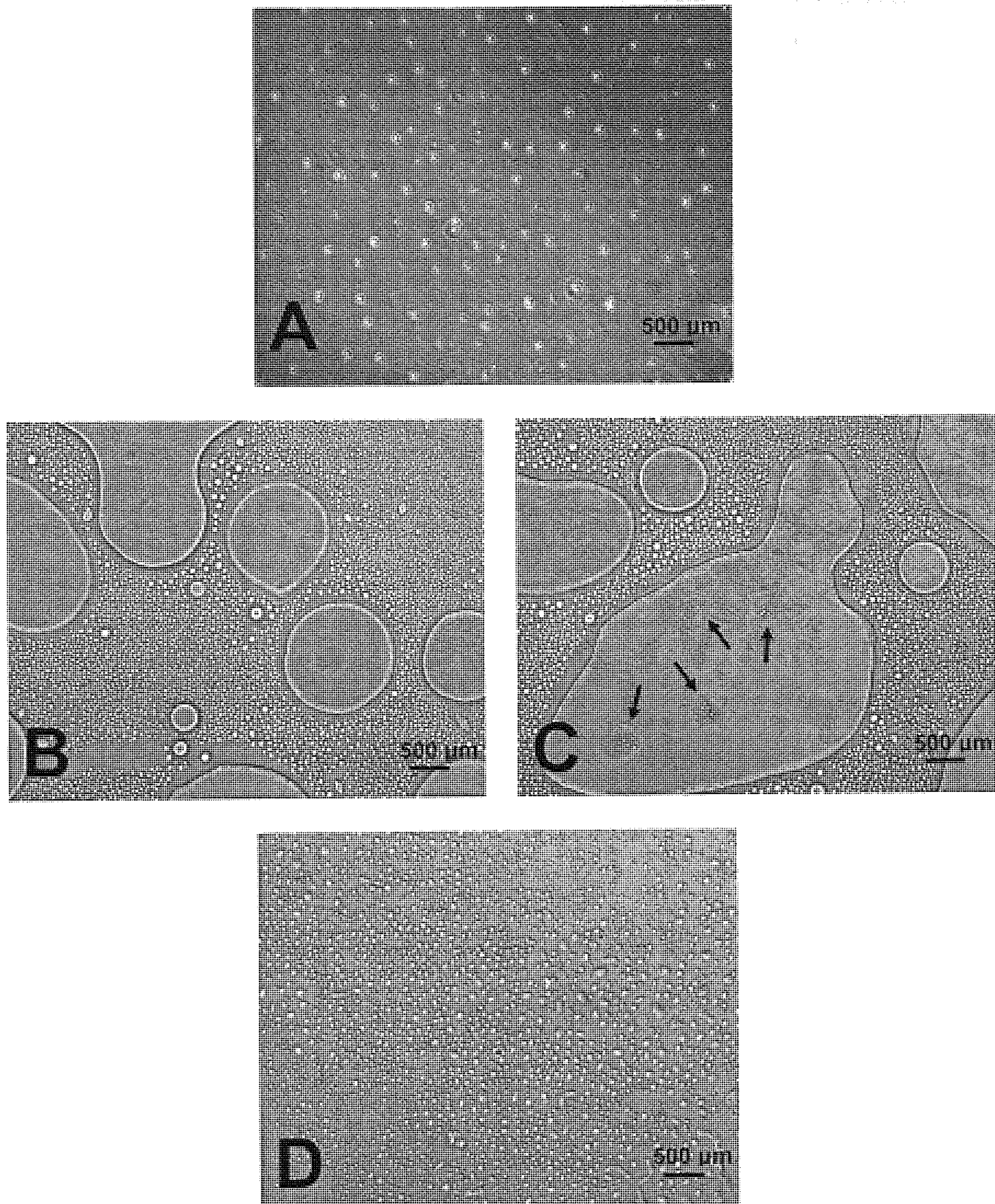


Fig. 4.2. Micrographs of primary emulsions in the w/o/w process. An aqueous solution of Ag85B-ESAT-6 was emulsified, by vortex mixing, with an organic phase consisting of chloroform and PLGA (**A**), PLGA+DDA (**B-C**) and PLGA+DDA+TDB (**D**). Images are at 40x magnification. Arrows indicate presence of droplets in droplets (**C**), although this may simply be droplets of aqueous phase within the chloroform, whereas the chloroform has evaporated off in the other samples.

Formulation	Volume mean diameter of empty microspheres (μm)	Volume mean diameter of Ag85B-ESAT-6 loaded microspheres (μm)	Ag85B-ESAT-6 entrapment efficiency (%)
PLGA	1.43 ± 0.06	1.50 ± 0.22	94.8 ± 1.16
PLGA + DDA	4.22 ± 0.33	20.26 ± 1.85[b]	57.4 ± 0.82
PLGA + TDB	2.16 ± 0.35	2.37 ± 0.34	79.6 ± 0.74
PLGA + DDA + TDB	2.98 ± 0.08[a]	3.16 ± 0.47[a]	55.3 ± 2.29

Table 4.2. Microsphere size following addition of immunomodulators to the organic phase, and subsequent Ag85B-ESAT-6 entrapment efficiency. Microspheres were produced by the w/o/w double-emulsion solvent evaporation technique outlined above. Size was measured using a Mastersizer X (Malvern, UK). For entrapment efficiency, ^{125}I-labelled Ag85B-ESAT-6 was added to the internal aqueous phase, and non-entrapped antigen removed by ultracentrifugation. Results denote mean ± S.D. from at least 3 independent batches.
a – denotes significantly smaller particles produced than with PLGA+DDA alone ($p<0.005$).
b – denotes significant increase in mean diameter following addition of Ag85B-ESAT-6 ($p<0.05$).

4.4.2. Microsphere size

The effect of Ag85B-ESAT-6 entrapment on mean particle size was also investigated (Table 4.2.). The results once again confirm the stabilising influence, in terms of microsphere size, of TDB on the formulations, since the only instance of a significant increase in size occurred when DDA alone was added to the formulation. This size increase may be expected when incorporating further materials to an already destabilised polymer matrix, as previously shown for the formulation with DDA alone, and this increase in diameter may also be as a consequence of aggregation as outlined by the SEM images (Fig. 4.3.).

Furthermore, when TDB was added in combination with DDA, the microspheres produced were of a significantly smaller size to those produced with addition of DDA

alone (3.16 ± 0.47 μm as compared to 20.3 ± 1.85 μm ($p < 0.005$)). This result once again provides evidence of the stabilising role of TDB within the polymer matrix similar to work previously shown with liposome systems (Davidsen et al., 2005; Vangala et al., 2006).

4.4.3. Morphology of microspheres

The morphology of these microspheres was then investigated by scanning electron microscopy (Figs. 4.3a-d.). With all four formulations, it appears that the microspheres are mainly spherical with smooth surfaces, with the addition of immunomodulators showing no detrimental effect in terms of surface porosity. Interestingly, it appears from the SEM images that the microspheres are in fact smaller in diameter than the sizing data suggests (Table 4.2.).

However, it is also evident from the SEM images that there is aggregation present in the formulations, particularly those containing DDA (Fig. 4.3b.), which may explain the larger sizes calculated by the Mastersizer software as well as the multi-modal distribution seen (Fig. 4.1b.). Further, the discrepancy in microsphere diameter between the SEM and the sizing data may be as a consequence of the agitation within the sample cell of the sizing equipment leading to inefficient dispersion of aggregates within the microsphere suspension. In addition, the SEM images are merely a small proportion of the sample, whereas the sizing data takes into account a much greater proportion of the population and calculates the volume mean diameter, suggesting that aggregates may lead to larger sizes being reported.

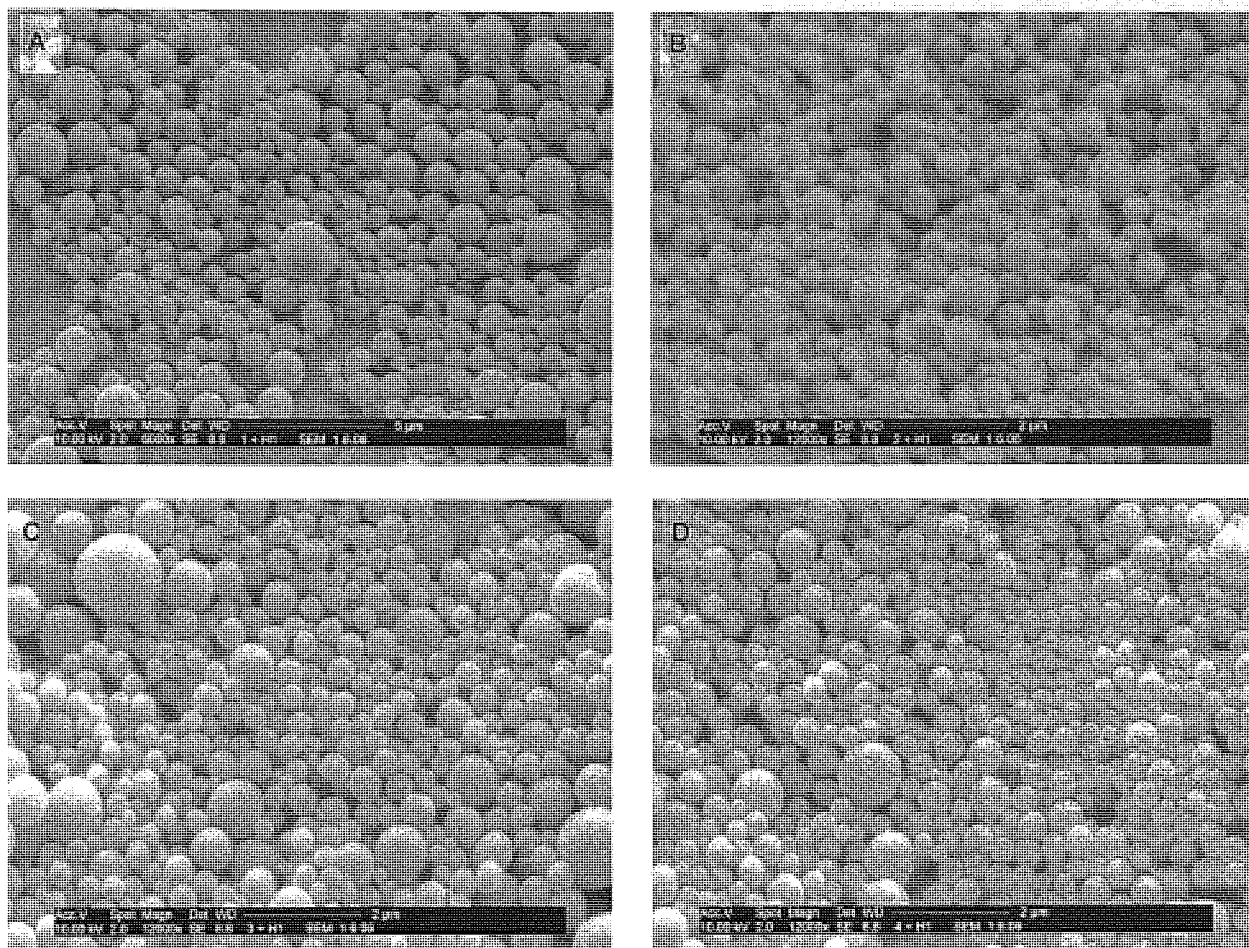


Figure 4.3. Scanning electron micrographs of Ag85B-ESAT-6 loaded microspheres consisting of (A) PLGA, (B) PLGA + DDA, (C) PLGA + TDB and (D) PLGA + DDA + TDB. Microspheres were prepared by the double emulsion solvent evaporation method (Section 2.7.1.), harvested and resuspended in ddH_2O. Microsphere suspensions were then loaded onto carbon coated aluminium stubs, and air-dried. Analysis was then carried out using a Philips FEI XL30 SEM at 10kV.

4.4.4. Stability of Microspheres

In order to investigate the storage stability of microspheres when stored at 4°C and 25°C, antigen loaded microspheres were prepared as described above, harvested and then resuspended in 2 ml sterile PBS. At regular time intervals, samples were measured for the effect of storage conditions on mean particle size (Figs. 4.4a-b.). All formulations showed no significant increase in size over a period of over 3 months, with no change being notable in either zeta potential (Figs. 4.5a-b.) or pH (Figs. 4.6a-b.) for the same period.

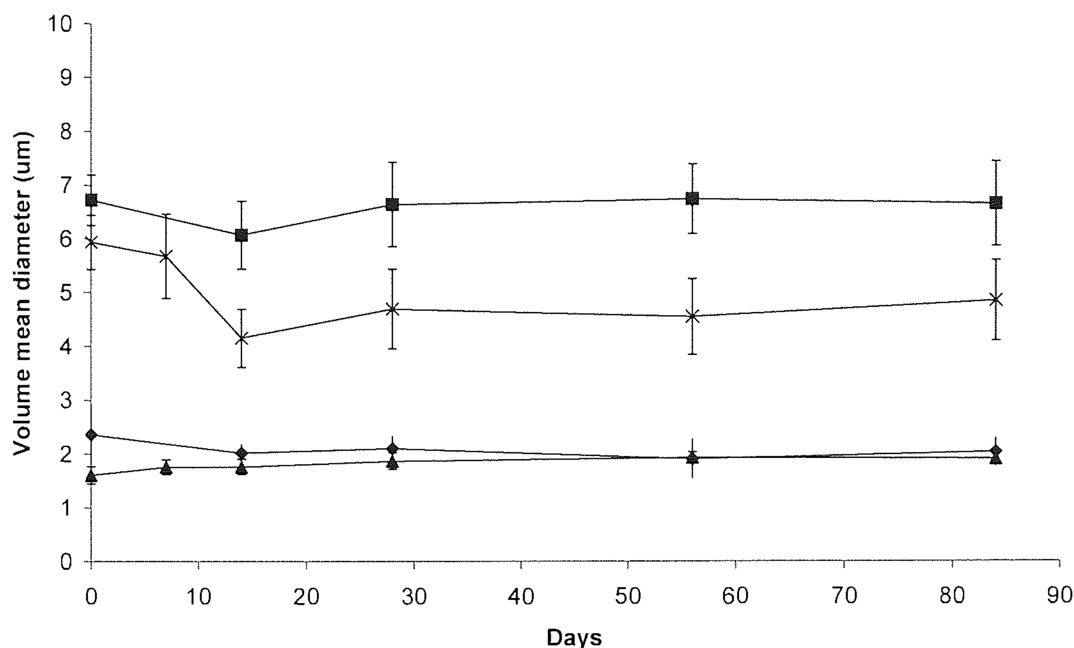


Figure 4.4a. Stability of mean diameter over time of PLGA (♦), PLGA + DDA (■), PLGA + TDB (▲) and PLGA + DDA + TDB (×) microspheres prepared by the w/o/w double emulsion solvent evaporation method, when stored at 4°C in sterile PBS, pH 7.4. Sizes were measured with a Mastersizer X (Malvern, UK). Results represent volume mean diameter for 3 independently synthesised batches, expressed as mean ± s.d.

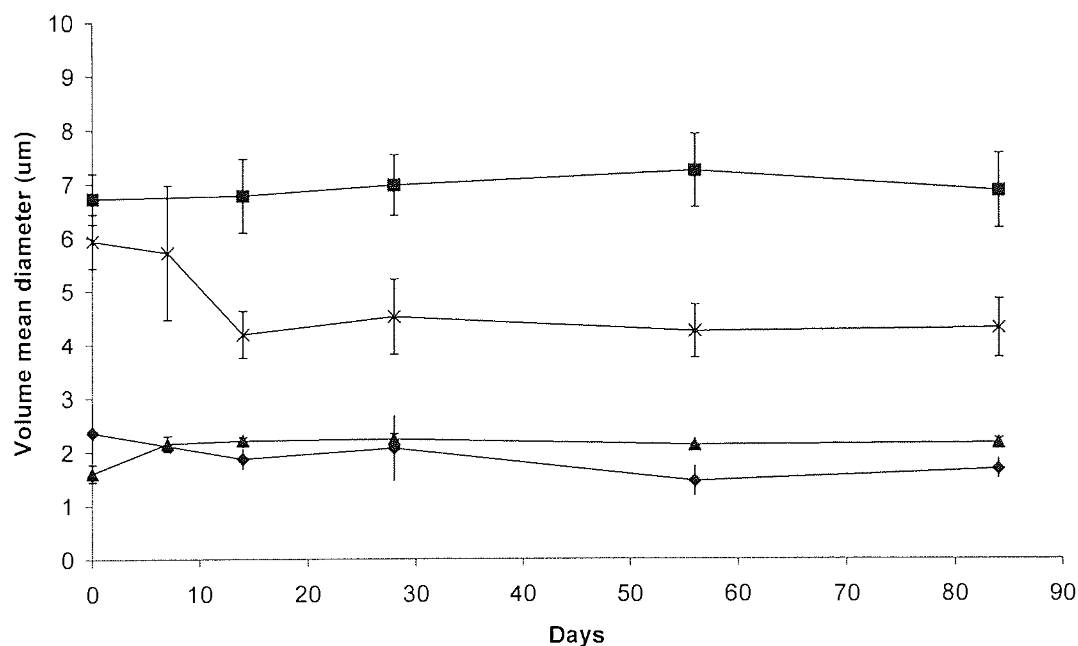


Figure 4.4b. Stability of mean diameter over time of PLGA (♦), PLGA + DDA (■), PLGA + TDB (▲) and PLGA + DDA + TDB (×) microspheres prepared by the w/o/w double emulsion solvent evaporation method, when stored at room temperature in sterile PBS, pH 7.4. Sizes were measured with a Mastersizer X (Malvern, UK). Results represent volume mean diameter for 3 independently synthesised batches, expressed as mean ± s.d.

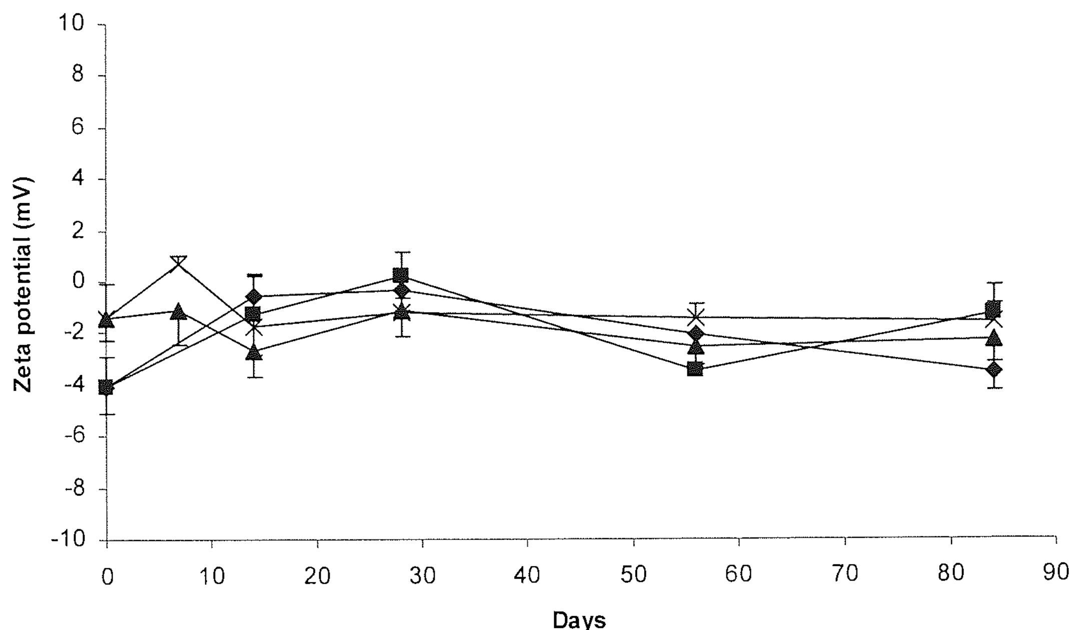


Figure 4.5a. Stability of zeta potential over time of PLGA (♦), PLGA + DDA (■), PLGA + TDB (▲) and PLGA + DDA + TDB (×) microspheres prepared by the w/o/w double emulsion solvent evaporation method, when stored at 4°C in sterile PBS, pH 7.4. Zeta potential was measured with a Zetasizer (Brookhaven, NY). Results represent volume mean diameter for 3 independently synthesised batches, expressed as mean ± s.d.

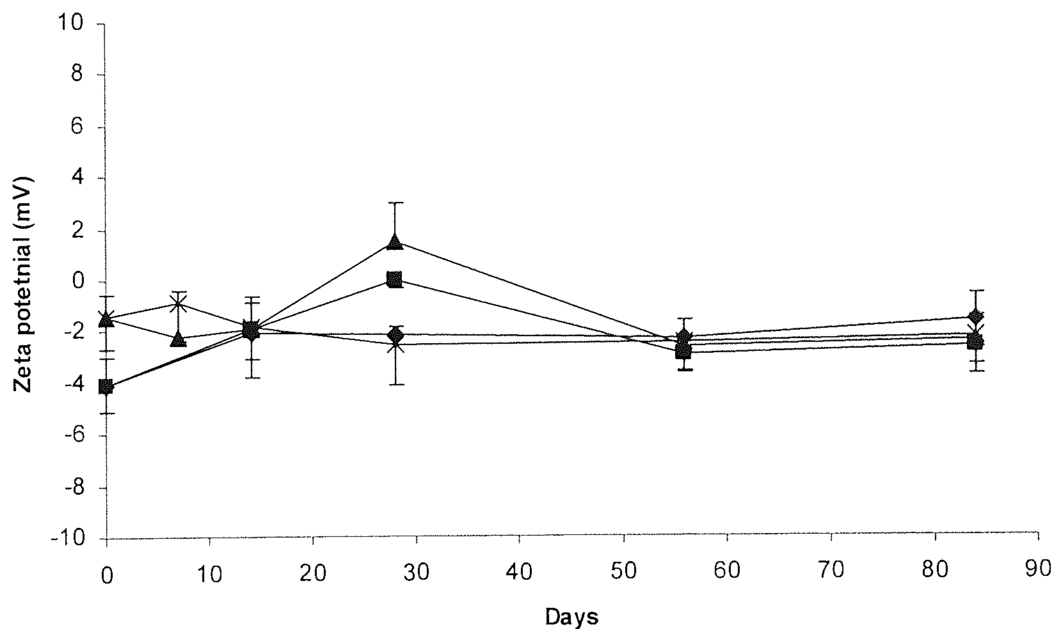


Figure 4.5b. Stability of zeta potential over time of PLGA (♦), PLGA + DDA (■), PLGA + TDB (▲) and PLGA + DDA + TDB (×) microspheres prepared by the w/o/w double emulsion solvent evaporation method, when stored at room temperature in sterile PBS, pH 7.4. Zeta potential was measured with a Zetasizer (Brookhaven, NY). Results represent volume mean diameter for 3 independently synthesised batches, expressed as mean ± s.d.

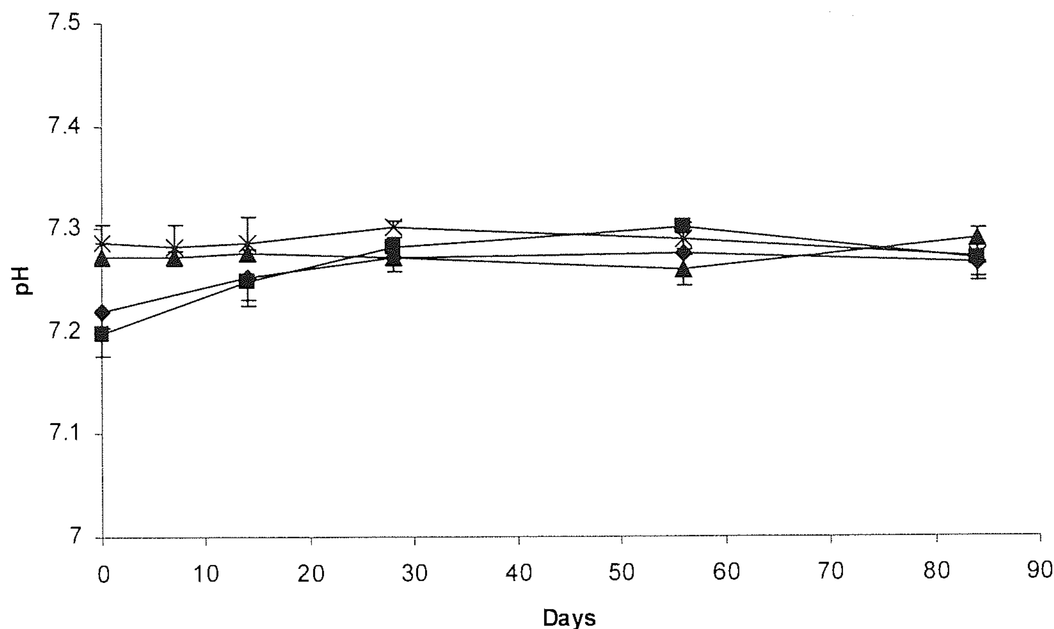


Figure 4.6a. Stability of pH over time of PLGA (♦), PLGA + DDA (■), PLGA + TDB (▲) and PLGA + DDA + TDB (×) microspheres prepared by the w/o/w double emulsion solvent evaporation method, when stored at 4°C in sterile PBS, pH 7.4. Results represent volume mean diameter for 3 independently synthesised batches, expressed as mean ± s.d.

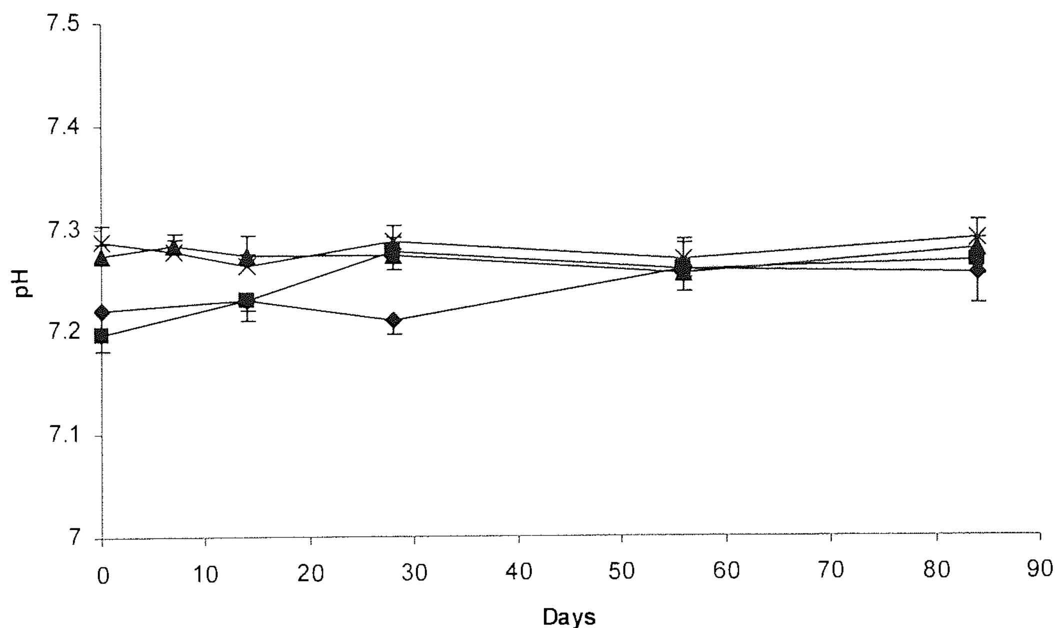


Figure 4.6b. Stability of pH over time of PLGA (♦), PLGA + DDA (■), PLGA + TDB (▲) and PLGA + DDA + TDB (×) microspheres prepared by the w/o/w double emulsion solvent evaporation method, when stored at room temperature in sterile PBS, pH 7.4. Results represent volume mean diameter for 3 independently synthesised batches, expressed as mean ± s.d.

However, the presence of PBS may have affected both the zeta potential measurements, due to the presence of ions, and more notably the stability in pH, since this would be expected in the presence of a buffer, such as PBS, whereas a drop in pH would be expected following degradation of the PLGA into lactic and glycolic acid. Further results would, therefore, be required to substantiate the level of degradation of the microspheres.

4.4.5. Antigen retention

It appears that all formulations exhibit good antigen retention (~90%) for up to a month when stored at 4°C (Fig. 4.7a.), as shown elsewhere for similar formulations (Guterres et al., 1995; Chacon et al., 1999). However, beyond this time there is a decrease in retention for all formulations, with a considerable variation in the results, particularly with the formulations where TDB is present. A possible explanation for this may be the proven ability of TDB to lower the phase transition temperature of particulate carriers (Davidsen et al., 2005), hence leading to a disruption of the polymer matrix upon storage.

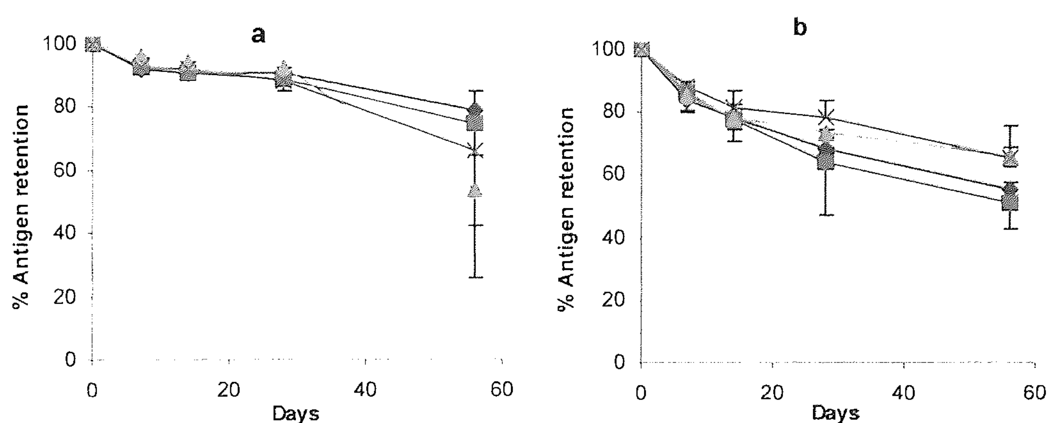


Figure 4.7. Retention of Ag85B-ESAT-6 over time of PLGA (♦), PLGA + DDA (■), PLGA + TDB (▲) and PLGA + DDA + TDB (×) microspheres prepared by the w/o/w double emulsion solvent evaporation method, when stored at (a) 4°C and (b) 25°C in sterile PBS, pH 7.4. ^{125}I-labelled Ag85B-ESAT-6 was entrapped and antigen retention was determined from the difference in ^{125}I radioactivity in supernatant and pellet following filtration. Results represent percentage retention of initially loaded antigen, expressed as mean ± s.d., n= 3. Recovery of initial radioactivity was consistently within acceptable limits of 85-115%.

Formulations stored at room temperature (Fig. 4.7b.) showed an immediate drop-off in antigen retention, with an almost linear decrease over a two month period. Once again, all formulations follow a similar pattern for antigen retention. Evidently, the increased temperature will only accelerate the degradation of the polymer chains, thus leading to a leeching of antigen. Increasing the molecular weight of the polymer may reduce this antigen leakage by altering the glass transition temperature of the polymer (Park, 1994), although previous reports suggest that an increase in molecular weight yields more potential sites of degradation, thereby leading to an increase in degradation rate (Delgado et al., 1996). Therefore, the formulation of a freeze-dried product is likely to be the most appropriate solution to any potential stability issues, which will be discussed further in Chapter 6.

4.4.6. In vitro release

Microsphere formulations were also investigated for their *in vitro* release of Ag85B-ESAT-6 by incubating the samples at 37°C in physiological pH conditions in a shaking water bath (Fig. 4.8.). Over the first 10 days, all formulations showed very similar release patterns, regardless of presence or absence of either of the immunomodulators, with a cumulative release of about 20% of the initial Ag85B-ESAT-6 load. After this time, the microsphere formulation with no immunomodulator present displayed the most rapid antigen release, and a plateau is reached before any other formulation (approximately 35 days), with an accumulated release of around 85%. As for the other formulations, a similar plateau is still not achieved after up to 9 weeks. Indeed, the formulations with the most sustained, prolonged release appear to be those with DDA present, potentially due to

partial adsorption of the antigen to the cationic head group of the DDA molecule. This phenomenon may actually add to the potential of these formulations as vaccine delivery systems, since they may have greater ability to act as sustained depot systems for the antigen over a period of time (Katz et al., 1996; Rosenkrands et al., 2005).

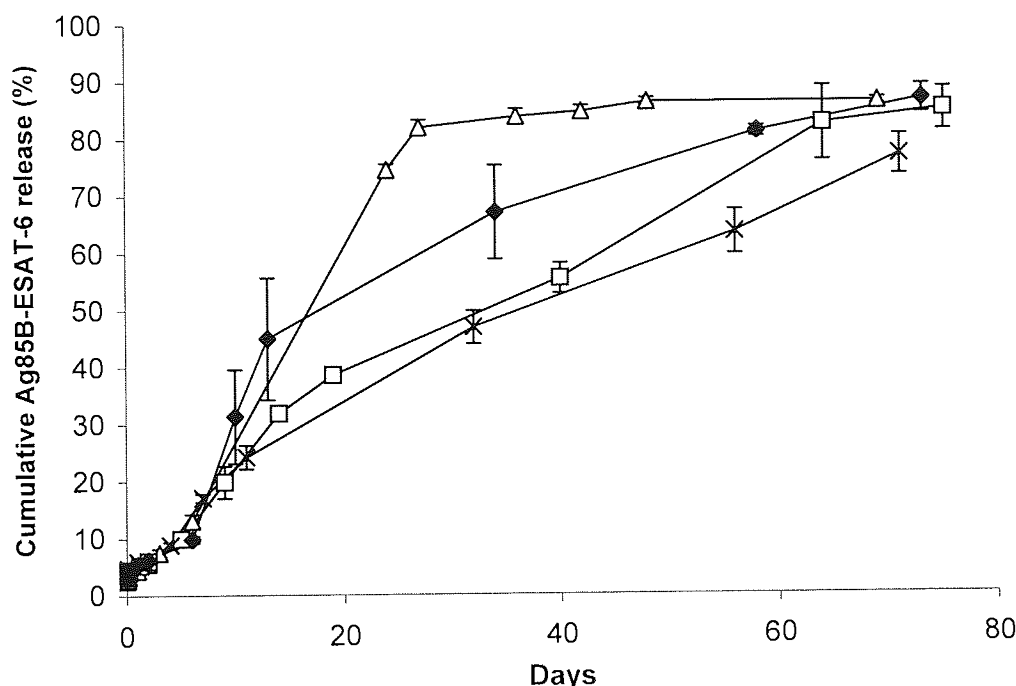


Fig. **4.8. Cumulative antigen release (%, w/w) vs time.** PLGA (△), PLGA + DDA (□), PLGA + TDB (♦) and PLGA + DDA + TDB (×) microspheres prepared by the w/o/w double emulsion solvent evaporation method (section 2.7.1.) were incubated in simulated physiological conditions (PBS, pH 7.4 at 37°C). Ag85B-ESAT-6 release was determined on the basis of radioactivity of ^{125}I-labelled Ag85B-ESAT-6 recovered in the suspended pellets after ultracentrifugation. Results represent percentage release of initially loaded antigen expressed as mean ± s.d., n= 3. Recovery of initial radioactivity was consistently within acceptable limits of 85-115%.

4.5. Immunological characterisation of microsphere based vaccines

The effect of each microsphere-formulated vaccine was investigated in mice. Mice were immunised three times with the dose of Ag85B-ESAT-6 fusion protein encapsulated in PLGA microspheres containing no adjuvant, DDA or TDB alone, and a combination of both DDA and TDB. Mice immunised with Ag85B-ESAT-6 adsorbed to DDA and

DDA-TDB liposomes were included for comparison. The ability of the various preparations to induce antibody responses after immunisation was investigated by measuring the antigen-specific titres of the IgG1 and IgG2b isotypes by ELISA (section 2.14.1.). As shown in Figure 4.9., the IgG1 and IgG2b titres determined were at the same level for PLGA, PLGA-TDB and PLGA, but lower for PLGA-DDA-TDB, whereas the highest antibody titres were observed for the DDA based formulations.

The apparent reduction in antibody response to the microsphere formulations as compared to the DDA based liposomes is likely to be due, in part, to the antigen association and release profiles of the systems. The DDA based liposomes (see Chapter 3) associate the antigen by surface adsorption, whereas the microspheres encapsulate the antigen. This difference in association will undoubtedly affect the way in which the antigen is presented to the cells of the immune system.

Further, the liposomal formulations exhibit an initial burst release of antigen (Fig. 3.6, Chapter 3), whereas the microspheres appear to sustain a steady release of antigen over time, with no initial burst. The immediate availability of the antigen from the liposomal formulations is evidently an advantage in stimulating a humoural immune response, since this provides enhanced accessibility to the antigen for the cells of the immune system, thereby leading to improved antigen processing from the early time points. Indeed, the duration of antigen stimulation and the cytokine environment to which the cells are exposed can polarise the subsequent response (Storni et al., 2005).

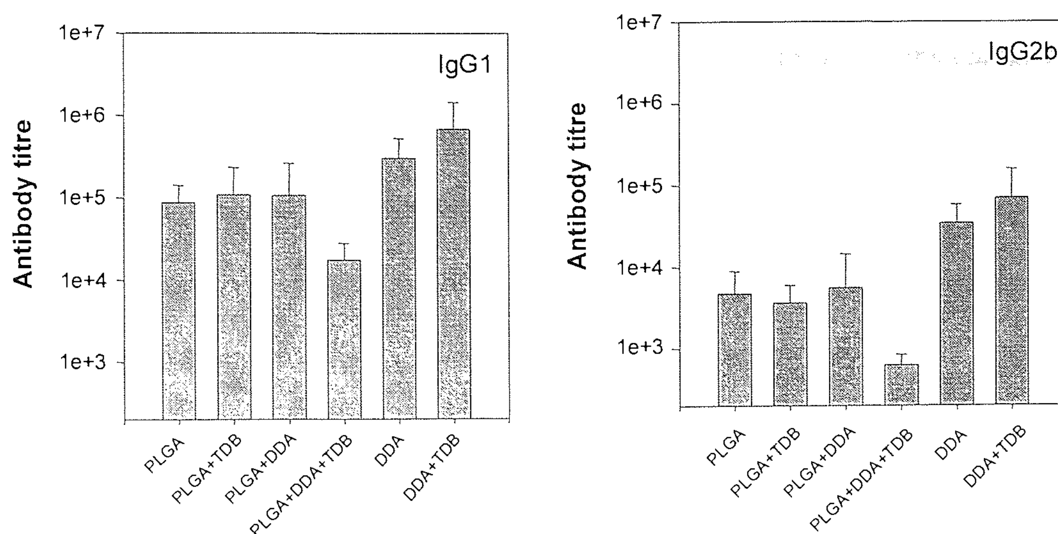


Fig. 4.9. Antibody responses against Ag85B-ESAT-6 generated by PLGA and DDA based vesicle preparations. C57Bl/6j mice (n=3-4) were immunised three times with 2 μg of Ag85B-ESAT-6, encapsulated in PLGA microspheres or adsorbed to DDA and DDA-TDB liposomes. Serum was drawn 7 weeks after the first immunisation for determination of IgG1 and IgG2b Ag85B-ESAT-6 specific antibody titres. All results have been compared to naïve mice, but calculation of midpoint titres was not applicable for the naïve group due to the low OD values measured. In vivo studies conducted by SSI.

In terms of IFN-γ production, the DDA-based liposomes again induced the strongest responses, with the PLGA formulation containing DDA alone inducing the most elevated response of the microsphere preparations studied (Fig. 4.10.), despite the mean particle size seemingly being in excess of the desired range. This may be explained by the observation that although the mean particle size of the formulation was over 20 μm (Table 4.2.), as a consequence of the multimodal size distribution (Fig. 4.1b.), approximately 50% of the volume of sample was in actual fact within the sub-10 μm range. In addition, it may be that the relatively large mean particle size may be a consequence of aggregates, as seen by the SEM images (Fig. 4.3.).

Subsequently, it is possible that the formulation containing DDA alone may simultaneously promote humoural and cell-mediated immunity by acting as a depot for

the release of antigens, and being available for uptake by macrophages. Indeed, the *in vitro* release profiles (Fig. 4.8.) reveal that the microsphere formulations containing DDA and DDA+TDB exhibit similar profiles, further implicating the effect of size or the presence of aggregates, and consequently the distribution of such systems *in vivo*, on the level of immune response achieved. Indeed, the presence of aggregates may enhance the immune response through recruitment of macrophages to the site of injection, thus leading to more effective antigen processing.

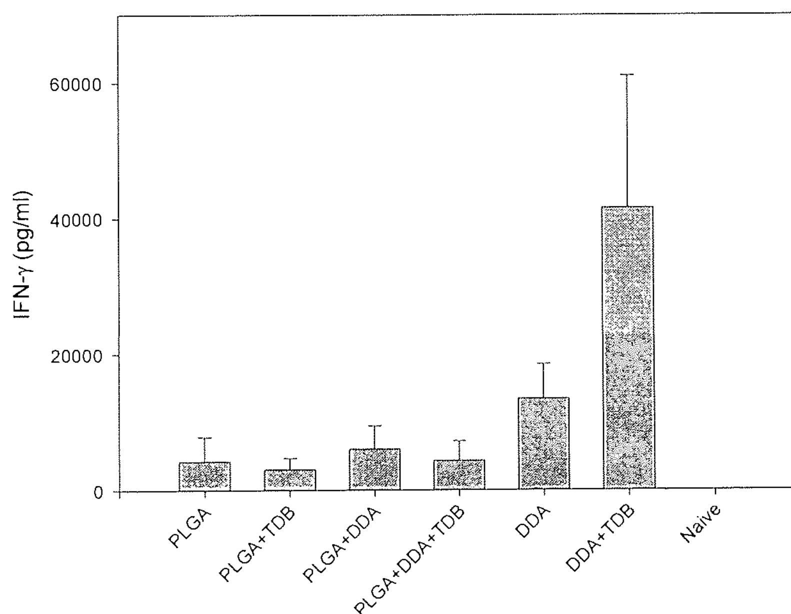


Fig. 4.10. Immune responses against the *Mycobacterium tuberculosis* Ag85B-ESAT-6 fusion protein generated by PLGA and DDA based formulations. Release of IFN-γ from spleen lymphocytes isolated from C57Bl/6j mice (n=4) immunised three times with 2 μg of Ag85B-ESAT-6 encapsulated in PLGA microspheres or adsorbed to DDA and DDA-TDB liposomes. Splenocytes were isolated 7 weeks after the first immunisation and re-stimulated in vitro with the Ag85B-ESAT-6 (5μg/ml). Studies conducted by SSI.

The enhanced response elicited by the liposome systems is, as before, a consequence of the way in which the antigen is presented to the immune system, and also the relatively small size as compared to the microsphere systems, which has been shown to affect

uptake of particles by APCs (Peyre et al., 2004). In addition, the relatively high cationic charge associated with the DDA-TDB liposomal formulation will be advantageous in terms of interacting with the cells of the immune system (Carmona-Ribeiro et al., 1997; Denis-Mize et al., 2003; Chung et al., 2007; Smith Korsholm et al., 2007).

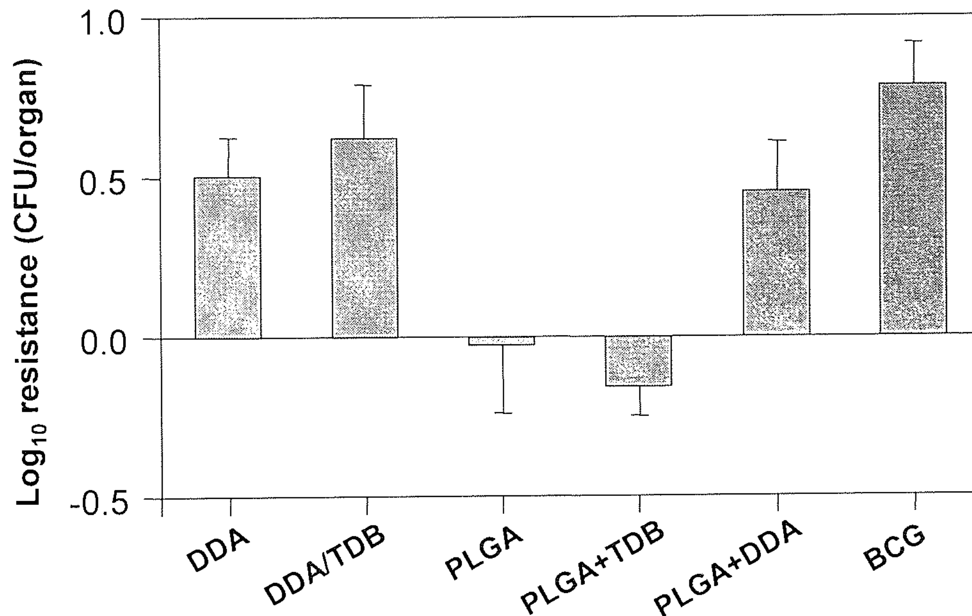


Figure 4.11. Protective efficacy of the microsphere formulations, expressed as the $\log_{10}$ reduction in bacterial load in the lung. N.B. Organs were taken 9 weeks instead of 6 weeks after challenge. Studies conducted by SSI.

Finally, the protective efficacy of the formulations was tested in comparison to that obtained with BCG following an infection challenge (Fig. 4.11.) The microsphere formulation PLGA+DDA+TDB was not tested due to the lack of positive results from the antibody and cytokine studies. In correlation with the results seen for antibody production and IFN-γ proliferation, the microsphere formulation incorporating DDA was the only microsphere formulation, of those tested, to induce significant levels of protection, whereas the microsphere formulations of polymer alone and PLGA+TDB showed no

protective efficacy. As before, the presence of aggregates may be responsible for recruitment of cells of the immune system, thereby leading to an enhanced immune response.

Further, the greater ability of the liposomal formulations to effectively initiate the desired immune response is likely to be due to several factors, including the size and surface charge of the liposomes, as well as the location and release profiles of each system.

Moreover, despite positive results for the microsphere formulation incorporating DDA and the liposomal preparations, the protection offered by BCG was greater than any of the other formulations studied.

4.6. Conclusion

This study has shown that biodegradable PLGA (75:25) microspheres, prepared by a modified double emulsion-solvent evaporation method, offer good potential for the delivery of the novel subunit TB vaccine, Ag85B–ESAT-6.

The formulation parameters investigated have provided an understanding of their effect on particle size and size distribution, and selection of the appropriate parameters can lead to production of microspheres well within the desired sub-10 μm range, with high entrapment efficiencies. Whilst the addition of the cationic lipid DDA to the formulations seems to have a potentially detrimental effect in terms of size and entrapment efficiency, the consequent ability to induce the desired immune response demonstrates the

importance of balancing such observations with other potentially desirable facets of the delivery system. The addition of immunomodulator TDB, either alone or in conjunction with DDA, is shown here to have a stabilising effect on the size and size distribution of the particles, underlining the potential of this substance to both act as an adjuvant and augment stability of particulate delivery systems. Encouragingly, the addition of these immunomodulators does not appear to have a detrimental effect on storage stability, and the *in vitro* release profiles generated show a more sustained release than in the absence of these molecules. Indeed, the slower release of antigen from the formulations containing DDA could suggest long-term efficacy, with an antibody response of a longer duration as compared to other systems, as suggested previously (Katz et al., 1996; Rosenkrands et al., 2005).

In terms of vaccine efficacy, despite protection levels not achieving those of BCG, these results advocate further investigation and optimisation of the microsphere formulation, since the use of particulate delivery systems for the administration of sub-unit vaccines clearly has the desired effect of enhancing the relatively low immunogenicity of the antigen, whilst also being able to offer the several associated advantages over attenuated vaccines. Therefore, these delivery systems offer excellent potential for the combination of immunomodulating agents in the design of vaccine delivery systems.

Chapter 5

Immunological and physico-chemical investigations into the optimisation of parameters involved in the formulation of microsphere-based TB vaccines

5.1. Introduction

Following the results from chapter 4, this chapter outlines the further optimisation of the microsphere formulation. Continuing with PLGA (75:25) in combination with DDA as before, the physico-chemical characteristics, and consequently the immunological efficacy of this formulation were studied for: (1) the effect of varying the emulsion stabiliser used in the external aqueous phase of the w/o/w double emulsion solvent evaporation method; (2) the effect of varying the preparation method, and thus the method of antigen association; and (3) the effect of using an alternative adjuvant, muramyl dipeptide (MDP), either alone or in combination with DDA.

5.1.1. Emulsifiers in the preparation of microspheres

Initially, a range of emulsion stabilisers were employed in the external aqueous phase of the w/o/w double emulsion solvent evaporation method (section 2.7.1.) to test their influence on the formulation process. 10% (w/v) PVA was used as a comparative control, in terms of the microsphere formulations, given the promising results from the previous chapter (chapter 4).

As alternatives, 0.5% (w/v) cetyl trimethyl ammonium bromide (CTAB) and 0.75% (w/v) chitosan (low molecular weight) were investigated for their ability to prepare microspheres and, consequently, any immunological effect. The concentrations of each were chosen due to previous reports of their use in microsphere formulation (Singh et al., 2000; Briones et al., 2001; Ravi Kumar et al., 2004; Wischke et al., 2006) and some initial optimisation (results not shown).

CTAB, a component of the antiseptic cetrimide, is a cationic surfactant similar in structure to DDA in that it possesses a quaternary ammonium head-group (Fig. 5.1.), although the single hydrophobic chain differs from the double hydrophobic chain present in DDA. Moreover, CTAB has previously been used to effectively prepare cationic microspheres for DNA vaccine delivery (Briones et al., 2001; Denis-Mize et al., 2003; Singh et al., 2003; O'Hagan et al., 2004), therefore making it an ideal candidate to compare with PVA.

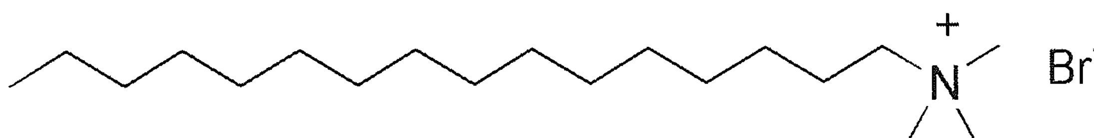


Fig. 5.1. Structure of cetyl trimethyl ammonium bromide (CTAB).

Chitosan (Fig. 5.2.) is a deacetylated derivative of chitin, the second most abundant polysaccharide found in nature after cellulose (Patel et al., 2005). Derived from crustacean shell waste, once the edible parts have been removed for the food industry, chitin is available to the extent of 10 gigatons a year. Furthermore, the biocompatibility, biodegradability and low toxicity of chitosan have lent its use to a wide variety of applications in a diversity of industries, including agriculture, cosmetics, water treatment, food/nutrition, materials science (Dodane & Vilivalam, 1998; Harish Prashanth & Tharanathan, 2007), and of course the medical sciences (Khor & Lim, 2003).

In addition to chitosan's anticoagulant and wound healing properties (which have made it useful for wound dressings and medical sutures) (Ueno et al., 1999), its orthopaedic

(Yokoyama et al., 2002) and tissue engineering applicability (Ma et al., 2001), and its antibacterial and antifungal properties (Vishu Kumar et al., 2007), chitosan is particularly useful for drug delivery. Chitosan has been shown to stimulate macrophage function (Nishimura et al., 1987; Peluso et al., 1994) and cytokine production (Mori et al., 1997), possess mucoadhesive properties (Illum et al., 1994; Alpar et al., 2005) and facilitate adjuvant activity (Moschos et al., 2004). Moreover, chitosan has previously been employed in the formulation of particulate delivery vehicles (Vila et al., 2002; Ravi Kumar et al., 2004; Luzardo-Alvarez et al., 2005), initiating enhanced Th1 immune responses (Strong et al., 2002), and therefore appears to be a viable alternative to PVA in the formulation of PLGA based microspheres.

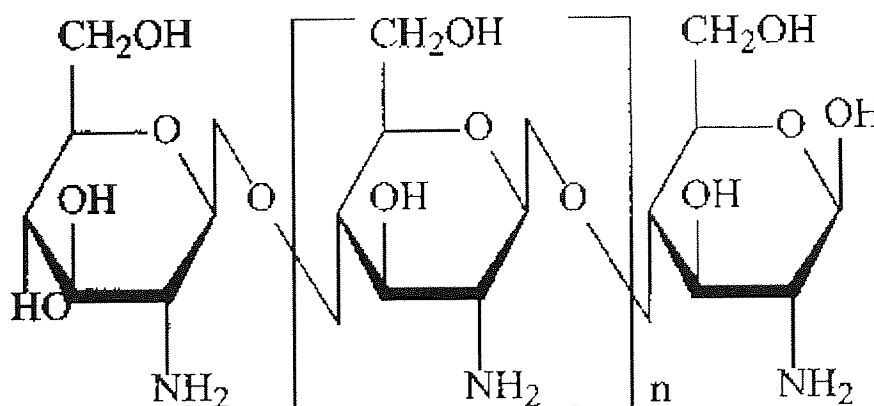


Fig. 5.2. Structure of chitosan.

5.1.2. Method of Preparation

The second part of the work outlined in this chapter concerns the investigation into the effect of altering the preparation method of the formulations. Microspheres prepared by the double emulsion solvent evaporation method (w/o/w), as previously (section 2.7.1.),

were compared to those prepared via the single oil-in-water emulsion solvent evaporation method (o/w) (see section 2.7.2.). These investigations would allow for the comparison between methods of antigen association, since the w/o/w method yields microspheres with entrapped antigen, whereas the o/w technique allows for adsorption of the antigen to the surface of pre-formed microspheres.

The adsorption of antigen to pre-formed microspheres adds the advantage of avoiding exposure of the antigen to solvents or shear stresses, as in the w/o/w process, whilst also allowing a more direct comparison between the DDA/TDB liposome system (see Chapter 3) and the polymer based microsphere formulations. Chitosan was chosen as the emulsion stabiliser for the o/w process due to the relatively high associated cationic charge, which would allow for effective adsorption of antigen, and also inherent Th1 biased adjuvanticity outlined above, allowing for stimulation of macrophages and cytokine production.

5.1.3. Effect of adjuvant: alternative and combination

Finally, muramyl dipeptide (MDP) (Fig. 5.3.) was investigated as an alternative or additional adjuvant to DDA in the microsphere formulation. MDP, the major component responsible for the active adjuvanticity of Freund's complete adjuvant (FCA) (Ellouz et al., 1974), has previously been shown to enhance production of cytokine and co-stimulatory molecules involved in cell mediated adaptive immunity (Inohara et al., 2003; Inohara & Nunez, 2003; Moschos et al., 2006). The use of an alternative adjuvant would clarify the importance of DDA and also determine potential adjuvant synergy.

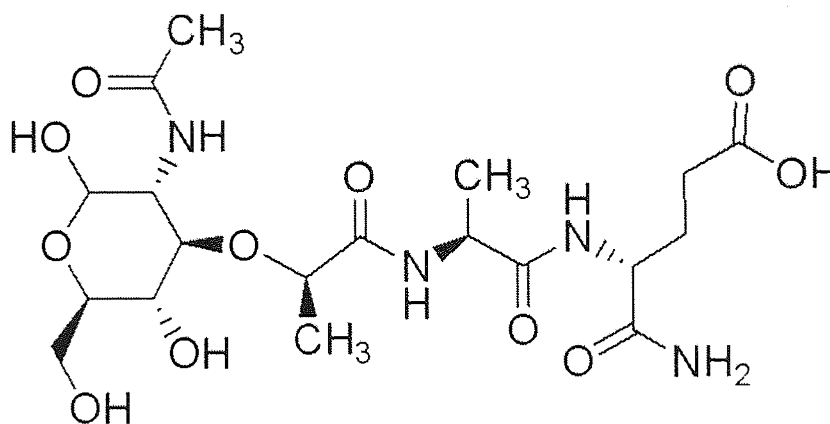


Fig. 5.3. Structure of muramyl dipeptide (MDP)

5.1.4. Immunological studies

The immunological analysis carried out in order to determine the efficacy of the microsphere formulations in achieving the desired immune response, encompassed quantification of Ag85B-ESAT-6 specific IgG antibody production, spleen cell proliferation and cytokine secretion (section 2.14.).

Being the most useful and most abundant of the serum immunoglobulins (Playfair & Bancroft, 2004), IgG is the most obvious choice for detection from blood samples. Indeed, it can activate both the complement system and mediate opsonisation, thus leading to enhanced phagocytosis (Goldsby et al., 2003). Further, the analyses will encompass the measuring of both the IgG1 and IgG2a subsets. Production of IgG1 generally follows activation of Th2 type cells, whereas IgG2a is produced following secretion of Th1 type cytokines such as IFN-γ (Zhu et al., 2007). Therefore, these investigations will give an indication of both the level and type of humoural response initiated by the formulations tested.

Cell proliferation analysis will provide an indication of both B cell and T cell activation in response to the antigen, giving further insight into the type and level of immune response achieved by each formulation.

Finally, the cytokine ELISAs will again assay markers of both Th1 (IFN-γ and IL-2) and Th2 (IL-5 and IL-6) responses, providing further elucidation of the method of action of each formulation. IFN-γ is considered the key cytokine marker for anti-mycobacterial immunity (Agger & Andersen, 2001), being the major macrophage activator element, indicative of CD4+ recruitment, whilst also playing a role in B cell differentiation (Playfair & Bancroft, 2004), therefore vital for cell mediated immunity (Mosmann & Sad, 1996).

IL-2 is a growth factor secreted by T-cells, thus indicative of T-cell activation, which in turn can lead to B-cell proliferation and differentiation, and is therefore another essential signal in directing cell mediated immunity (Playfair & Bancroft, 2004), whilst also playing a role in the humoural response.

In terms of the Th2 cytokine markers, IL-5 promotes B-cell proliferation and differentiation, and also leads to eosinophil production and activation, whereas IL-6 is key in differentiation to plasma cells (Playfair & Bancroft, 2004), an important facet in memory responses and innate immunity, and therefore useful in vaccines.

5.2. Emulsion stabiliser

5.2.1. Physico-chemical characteristics

The physico-chemical attributes of the particles produced by the double emulsion solvent evaporation method (w/o/w), using various emulsion stabilisers in the external aqueous phase, are summarised in Table 5.1. From these results, it becomes clear that the type of emulsifying agent employed in the w/o/w technique has a significant influence on the resulting particles' characteristics.

Emulsion stabiliser	Volume mean diameter (μm)	Span	Zeta potential (mV)	H1 entrapment efficiency (%)
PVA	1.4 ± 0.1	1.60 ± 0.06	14.3 ± 1.8	57.4 ± 0.8
Chitosan	3.0 ± 0.1	1.68 ± 0.01	39.1 ± 1.6	24.2 ± 4.2
CTAB	4.0 ± 1.1	4.33 ± 1.95	-27.5 ± 0.8	2.7 ± 0.5

Table 5.1. The effect of varying the emulsion stabiliser employed in the w/o/w double emulsion solvent evaporation process on the physico-chemical characteristics of PLGA+DDA microspheres produced. Size was measured using a Sympatec Helos (Sympatec, Germany). Zeta potential was measured using a Brookhaven Zetaplus (Brookhaven, NY). For entrapment efficiency, ^{125}I-labelled Ag85B-ESAT-6 was added to the internal aqueous phase, and non-entrapped antigen removed by ultracentrifugation. Results denote mean ± S.D. from at least 3 independently synthesised batches.

Contrary to results elsewhere (Esposito et al., 1999; Mollenkopf et al., 2004), the CTAB stabilised microspheres exhibited a negative surface charge, suggesting that the CTAB molecules are susceptible to loss on washing of the microspheres following solvent evaporation, as suggested by (Wischke et al., 2006). Indeed, it is evident from the antigen entrapment efficiency that CTAB is the least effective of the emulsifying agents studied in terms of stabilising the particles, since the antigen is evidently allowed to migrate to the external aqueous phase rather than remaining entrapped as an internal phase. In addition, this apparent loss of stabilising moieties did result in some aggregation of the

particles following lyophilisation, as suggested by the relatively large particle size and span of size distribution (Table 5.1.).

The PVA stabilised microspheres exhibited relatively small, uniform particles, and it has previously been shown that PVA forms a stable layer around the microspheres which is not fully removed on washing (Boury et al., 1995). The relatively high antigen entrapment efficiency of the PVA stabilised systems further supports this theory, whereas the positive charge associated with the particles is an indication of the presence of the immunomodulatory DDA.

The Chitosan stabilised particles exhibited a fairly uniform size, as evidenced by the span of the size distribution (Table 5.1.), although the mean diameter was greater than that of the PVA stabilised particles, which may be due to slight aggregation as a consequence of reduced wettability following lyophilisation (Wischke et al., 2006). Further, the reduction in antigen entrapment confirms a reduction in emulsion stabilising efficiency. However, ESEM analysis (see chapter 7) of the formulations showed that the chitosan stabilised particles were more robust as compared to PVA stabilised systems when subjected to reduced pressure and electron bombardment, possibly due to cross-linking or gelling of the chitosan or interaction with the microsphere surface. Nevertheless, the relatively high surface charge associated with these particles may help to enhance cellular uptake by greater interaction with the negative surface of the APC (Carmona-Ribeiro et al., 1997; Denis-Mize et al., 2003; Chung et al., 2007; Smith Korsholm et al., 2007) as compared to PVA stabilised microspheres.

5.2.2. Antibody production

Antibody ELISAs were performed at regular intervals (see section 2.14.1.) to determine the antigen specific antibody production initiated by each formulation (Fig 5.4a-c.). As can be seen from the results, the type of emulsifier used has a notable effect on the generation of antibodies, with a clear trend PVA > Chitosan > CTAB emerging. Indeed, the CTAB stabilised formulation appears to offer very little immunological effect, possibly due to low antigen loading and aggregation of the particles, which in turn can lead to ineffective release of antigen. Additionally, the negative charge associated with the microspheres suggests a lack of surface modification, whilst also indicating the absence of the immunostimulatory DDA, which may potentially impair the interaction with APCs (Tabata & Ikada, 1988b; Smith Korsholm et al., 2007). Indeed, although anionic particulate carriers have been effectively employed in targeting APCs (Allison & Gregoriadis, 1974; Heath et al., 1985; Copland et al., 2000; Ahsan et al., 2002; Myschik et al., 2006), other factors, such as size and protein loading, are likely to have an impact on effective presentation.

As for the PVA and Chitosan stabilised particles, there are instances of enhanced antibody production as compared to naïve control, although this is exclusively for the Th2 type antibodies (IgG1) (Fig. 5.4b.), with very little effect observed for the Th1 indicator IgG2a (Fig. 5.4c.). Indeed, the PVA stabilised formulation shows significantly enhanced IgG1 titres at the latter time points as compared to the CTAB stabilised group (Fig. 5.4b.).

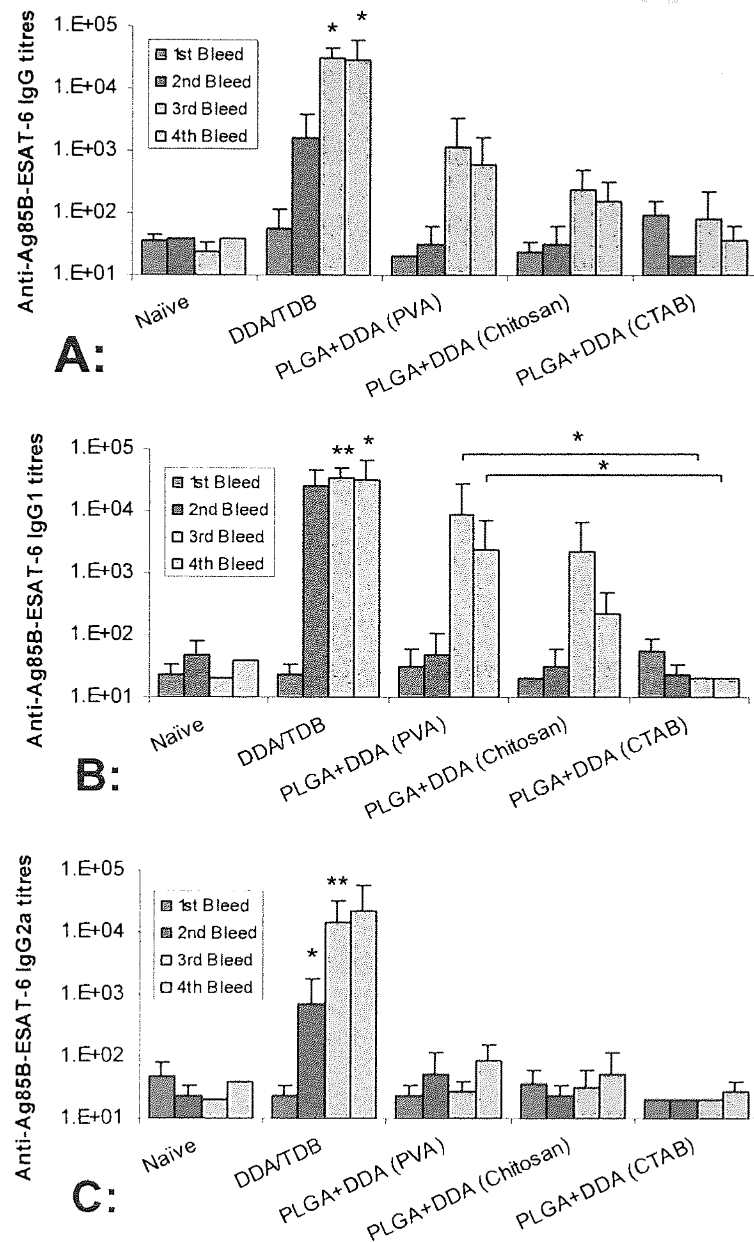


Fig. 5.4. Ag85B-ESAT-6 specific antibody titres. Microsphere formulations were prepared by the w/o/w method, as described in section 2.7.1., with various emulsion stabilisers. As a positive control, the liposomal formulation DDA/TDB, prepared as described in section 2.2.1., was administered due to its' ability to initiate strong protective immune responses against Mtb (see Chapter 3). Groups of five female BALB/c mice, approximately six weeks old, received doses of vaccine formulations containing 2μg of Ag85B-ESAT-6 in a 50μl volume. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks. Serum samples were taken at 12 days after the first administration and at two week intervals thereafter. Sera samples obtained at different time intervals after immunisation were analysed for the presence of anti-Ag85B-ESAT-6 IgG (**A**), IgG1 (**B**) and IgG2a (**C**) antibodies by enzyme-linked immunosorbent assay (ELISA).

* denotes significantly increased antibody titres (n=5, $p<0.05$) compared to naïve control

** denotes significantly increased antibody titres (n=5, $p<0.01$) compared to naïve control

The slightly lower levels of antibody production achieved with the chitosan stabilised formulation, when compared to the PVA stabilised group, may be as a consequence of the relative rigidity of the particles affecting the effective presentation of the antigen to the cells of the immune system, which is also likely to be affected by the slight aggregation seen with this formulation. In addition, factors such as size and surface will also influence immune response (Tabata & Ikada, 1988a; Peyre et al., 2004; Chung et al., 2007). Unfortunately, for all formulations, the antibody titres produced are less than those of the positive control (DDA/TDB liposomes).

5.2.3. Cell proliferation

Following on from the antibody ELISA, each formulation was then investigated for its ability to initiate antigen specific spleen cell proliferation (Fig. 5.5., section 2.14.2.1.). As seen for the antibody production, the effect of emulsifier again had a significant impact, with the results corroborating the immune-potentiating trend PVA > Chitosan > CTAB (Fig. 5.5.). Indeed, the PVA stabilised formulation showed levels of proliferation significantly higher than the naïve control and comparable to that of the positive control (DDA/TDB). The Chitosan stabilised formulation also showed significantly enhanced levels as compared to naïve control, with the CTAB stabilised group again failing to initiate any significant immunological effect compared to the control.

As was seen for the antibody results (Figs. 5.4a-c), the negative charge, low antigen loading and aggregation of the CTAB stabilised formulation will all play a part in the ability of the microspheres to effectively present the antigen and target the APCs.

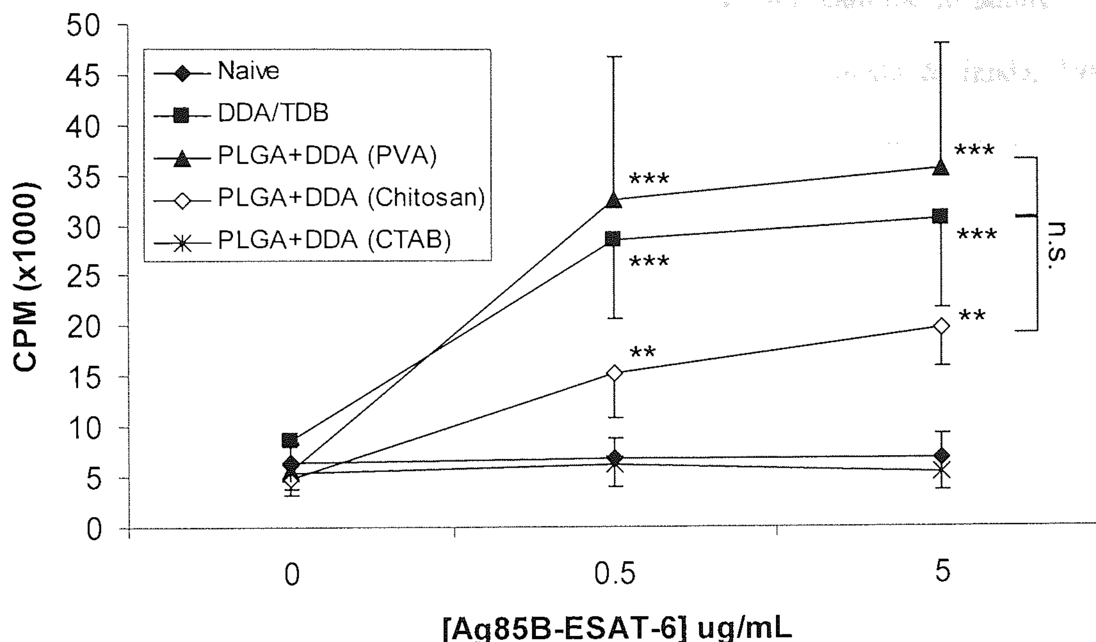


Fig. 5.5. Spleen cell proliferation in response to stimulation/re-stimulation with Ag85B-ESAT-6 antigen. Cell proliferation was measured by incorporation of ^3H into cultured splenocytes. Microsphere formulations were prepared by the double emulsion solvent evaporation method, as described in section 2.7.1., with various emulsion stabilisers. As a positive control, the liposomal formulation DDA/TDB, prepared as described in section 2.2.1., was administered.
** denotes significantly increased proliferation in comparison to naïve controls ($n=5$, $p<0.01$)
*** denotes significantly increased proliferation in comparison to naïve controls ($n=5$ $p<0.001$)
n.s. denotes no statistical difference between groups ($n=5$, $p>0.05$)

Conversely, the relatively high positive surface charge associated with the Chitosan stabilised microspheres will be advantageous in terms of interacting with the cells of the immune system (Thiele et al., 2003; Huang et al., 2004; Huang et al., 2005; Chung et al., 2007), thus leading to an enhancement in immune response.

Indeed, it has previously been shown that interaction with the surface of APCs, through electrostatic binding, increases the likelihood of uptake of both drug and particulate carrier (Smith Korsholm et al., 2007). Further, interaction with the cell surface is considered the rate limiting step in such a process (Ahsan et al., 2002). Finally, the PVA

stabilised formulation has the added advantage of being both cationic in nature, whilst exhibiting a mean diameter pertaining to enhanced uptake (Tabata & Ikada, 1988a), which may explain the enhanced immune response seen with this formulation. In addition, the relative rigidity of the Chitosan stabilised formulation is again likely to have affected the results.

Encouragingly, both the PVA and Chitosan stabilised microspheres elicited antigen specific spleen cell proliferation which was not significantly different from the positive control, DDA/TDB liposomes.

5.2.4. Cytokine production

The final immunological investigation of this study was determining cytokine production induced by each formulation (section 2.14.2.2.). The cytokines assayed encompassed indicators for both Th1 and Th2 immune responses. For Th1, IFN-γ (considered the key cytokine marker for tuberculosis immunity) and IL-2 were measured (Figs. 5.6a. and 5.6b. respectively) and for Th2, IL-5 and IL-6 were measured (Figs. 5.6c. and 5.6d. respectively).

As with both antibody production and cell proliferation, the type of emulsifier employed has an influence on the efficacy of the vaccine delivery systems, with the trend PVA > Chitosan > CTAB once again clearly evident. Encouragingly, the PVA stabilised microspheres initiated significantly higher levels of all cytokines assayed as compared to naïve control ($p<0.01$), with IL-5 levels greater than those produced by the positive

control (DDA/TDB) (Fig. 5.6c.). In addition, the Chitosan stabilised formulation showed significantly increased levels of IFN-γ (Fig. 5.6a) and IL-6 (Fig. 5.6d) when compared to naïve control ($p<0.01$). Once again, the CTAB stabilised formulation showed no positive results for any cytokine assayed.

Once more, the physico-chemical characteristics of the microsphere formulations (Table 5.1.) are likely to be the most influential factors in terms of efficacy in initiating an effective immune response. It has already been noted that the lack of surface modification and aggregation of the CTAB stabilised particles will profoundly affect their interaction with APCs (Tabata & Ikada, 1988b; Chung et al., 2007), and the cytokine results again confirm this hypothesis. Moreover, the positively charged systems, i.e. the PVA and Chitosan stabilised microspheres as well as the DDA/TDB liposomes, will have a greater tendency to interact with the negative surfaces of the cells of the immune system (e.g. macrophages) (Singh et al., 1992; Denis-Mize et al., 2003; Huang et al., 2004; Chung et al., 2007), allowing more effective presentation and processing of the antigen, as well as a greater likelihood of uptake by such cells.

Indeed, it has been reported that an important mechanism of the ability of DDA (present in all formulations) to act as an adjuvant in the delivery of antigen is the immediate electrostatic interaction with the cell surface, followed by induction of active uptake (Smith Korsholm et al., 2007). This observation again implicates the loss of DDA molecules from the CTAB stabilised formulation, thus leading to inefficient antigen uptake and lack of immunological effect.

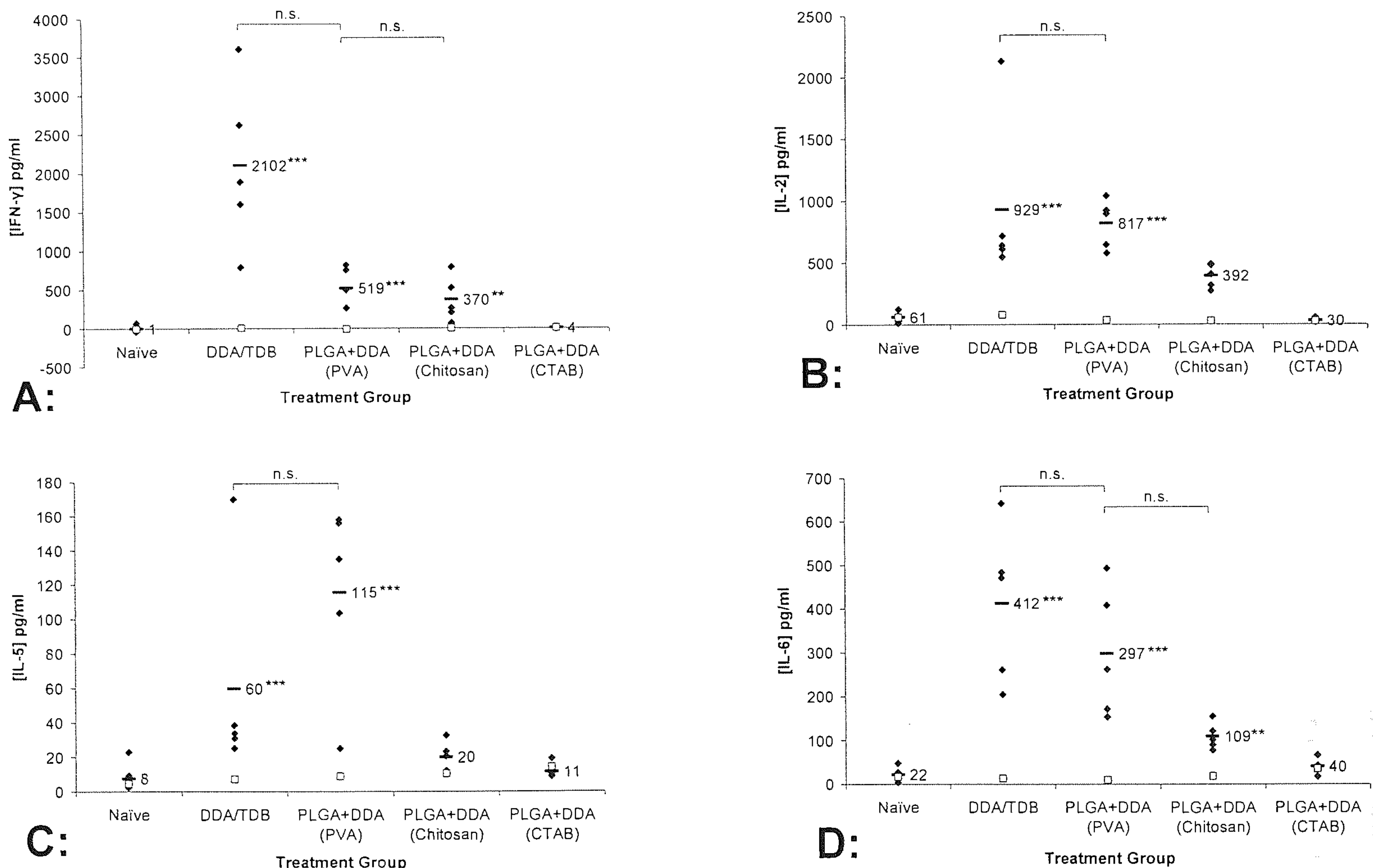


Fig. 5.6. Ag85B-ESAT-6 specific cytokine production. Cytokines were detected using DuoSet® capture ELISA kits (mouse IFN-γ (**A**), IL-2 (**B**), IL-5 (**C**), IL-6 (**D**)) purchased from R&D systems, Abingdon, UK, according to the manufacturers instructions. Filled diamonds represent the mean of three measurements for each individual spleen. Horizontal lines represent average group values (n = 5) and are shown numerically on the chart. Unfilled squares represent average of unstimulated group values (n=5). ** denotes significantly increased levels in comparison to naïve controls (n=5, $p<0.01$) *** denotes significantly increased levels in comparison to naïve controls (n=5, $p<0.001$) n.s. denotes no statistical difference between groups (n=5, $p>0.05$).

In addition, the positive cell proliferation and cytokine secretion results seen for the microsphere formulations stabilised by both PVA and chitosan, in conjunction with the relatively low levels of antibody production, suggest a greater emphasis on cell mediated immunity, a desired facet for anti-mycobacterial immunity.

5.3. Preparation method

5.3.1. Physico-chemical characteristics

PLGA+DDA microspheres, stabilised by chitosan, were then investigated in terms of formulation type. The single oil-in-water method (o/w) produced cationic particles of a similar diameter to those prepared by the double emulsion method (Table 5.2.), although there is a slight increase in measured size. The surface charge is also similar to that of the microspheres produced by the double emulsion method (w/o/w), however, the slight decrease, whilst not significant, may be due to the adsorbed layer of antigen masking the positive charge (Table 5.2.). Indeed, this masking of the positive charge may explain the increase in mean diameter, through a reduction in electrostatic repulsion between the particles. Once again, following freeze-drying there does appear to be some aggregation, suggesting the reduction in wettability, as for the w/o/w method.

Nevertheless, adsorption of the antigen to the surface of the microspheres does prove to be a more efficient method of association, with an increase of approximately three-fold when compared to the double emulsion method (Table 5.2.). This result may be expected, since adsorption of the antigen to pre-formed particles adds the advantage of avoiding potential loss of antigen through migration from the internal aqueous phase

during formation of the secondary emulsion, and also eliminates potential loss on washing. Additionally, this alternative method of antigen association allows for comparison between the way in which the antigen is presented by the particles, i.e. either entrapped within the polymer matrix and released over a period of time, or exposed on the surface and potentially more visible and accessible to the cells of the immune system.

Furthermore, adsorption of the antigen to cationic microspheres allows a more direct comparison between polymer based systems and the liposomal DDA/TDB formulation (see Chapter 3). Indeed, the release of antigen from the microspheres formulated via the single emulsion method bears closer resemblance to the DDA/TDB formulation than the alternative double emulsion method (Fig. 5.7.), with a notable burst release, particularly over the first 24 hours, followed by prolonged, sustained release, intimating that the majority of the initial antigen load remains adsorbed to the microspheres, potentially facilitating enhanced delivery within APCs.

Preparation	Volume mean diameter (μm)	Zeta potential (mV)	H1 entrapment efficiency (%)
w/o/w	3.0 ± 0.1	39.1 ± 1.6	24.2 ± 4.2
o/w	4.7 ± 1.1	34.2 ± 2.3	77.4 ± 6.5

Table 5.2. The effect of preparation method on the physico-chemical characteristics of PLGA+DDA microspheres produced. Microspheres composed of PLGA and DDA were prepared by either the double emulsion solvent evaporation (w/o/w) or the single emulsion solvent evaporation (o/w) method (section 2.7.), with chitosan as the emulsion stabiliser (0.75%, w/v). Size was measured using a Sympatec Helos (Sympatec, Germany). Zeta potential was measured using a Brookhaven Zetaplus (Brookhaven, NY). For entrapment efficiency, ^{125}I-labelled Ag85B-ESAT-6 was added to the internal aqueous phase, and non-entrapped antigen removed by ultracentrifugation. Results denote mean ± S.D. from at least 3 independently synthesised batches.

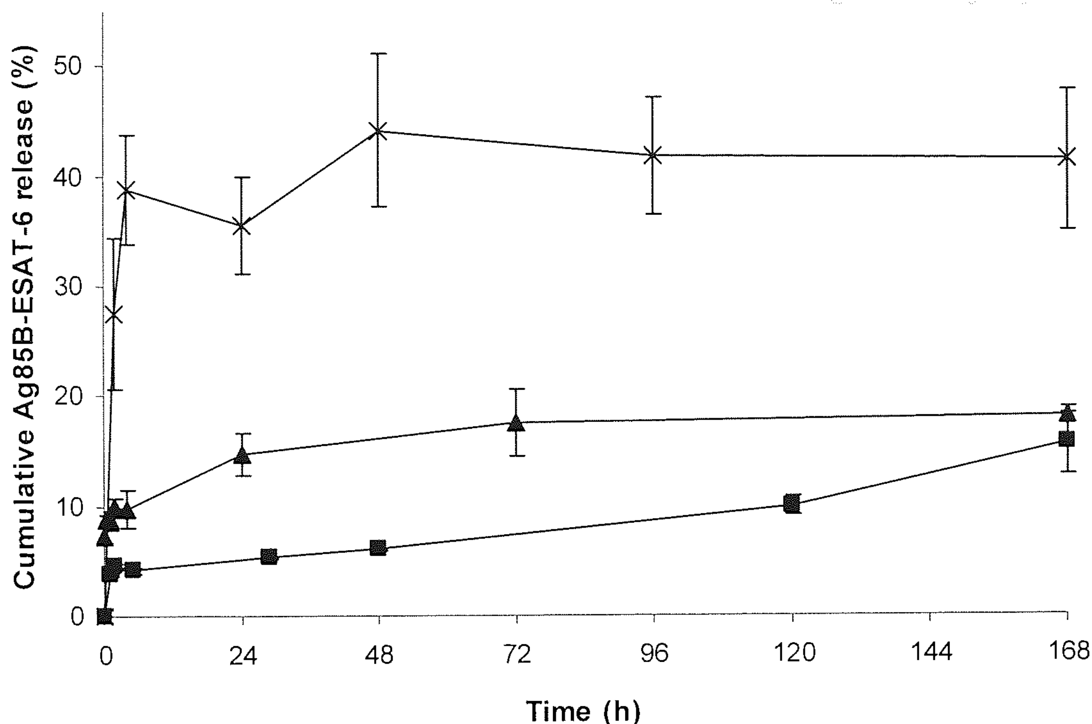


Fig. 5.7. Cumulative antigen release (%, w/w) vs time. PLGA + DDA (w/o/w) (■), PLGA + DDA (o/w) (▲) and DDA/TDB liposomes (×) were incubated in simulated physiological conditions (Tris-HCl, pH 7.4 at 37°C). Both microsphere formulations were stabilised by 0.75% chitosan. Ag85B-ESAT-6 release was determined on the basis of radioactivity of ^{125}I-labelled Ag85B-ESAT-6 recovered in the suspended pellets after ultracentrifugation. Results represent percentage release of initially loaded antigen expressed as mean ± s.d., n= 3.

5.3.2. Antibody production

In terms of formulation type, the location of the antigen clearly has an influence on the type and level of antibody response achieved, with the o/w formulation (antigen adsorbed) showing increased levels of all antibodies investigated as compared to the w/o/w (antigen entrapped) formulation (Figs. 5.8a-c). In addition, the o/w formulation shows a mixed antibody response, with both Th1 and Th2 type antibodies showing increased levels as compared to the naïve control.

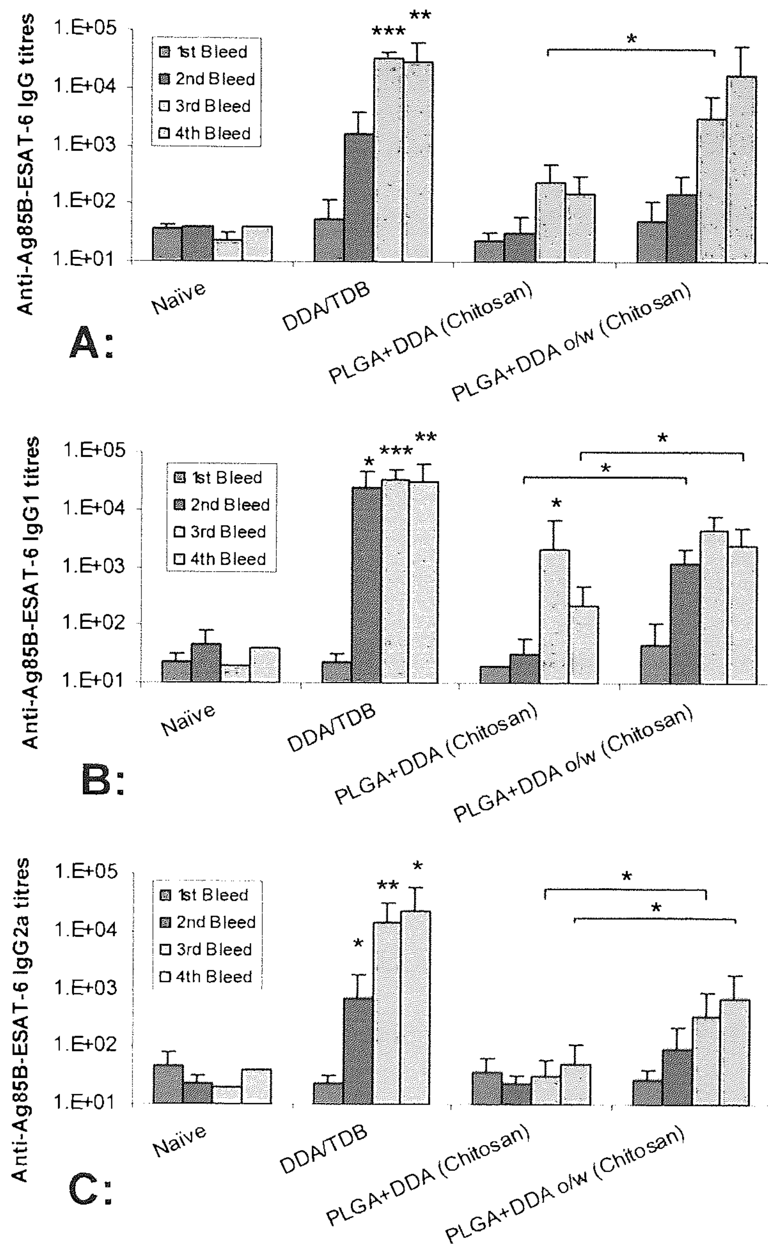


Fig. 5.8. Ag85B-ESAT-6 specific antibody titres. Microsphere formulations were prepared by either the single (o/w) or the double (w/o/w) emulsion solvent evaporation method, as described in section 2.7. As a positive control, the liposomal formulation DDA/TDB, prepared as described in section 2.2.1., was administered due to its' ability to initiate strong protective immune responses against Mtb (see chapter 3). Groups of five female BALB/c mice, approximately six weeks old, received doses of vaccine formulations containing 2μg of Ag85B-ESAT-6 in a 50μl volume. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks. Serum samples were taken at 12 days after the first administration and at two week intervals thereafter. Sera samples obtained at different time intervals after immunisation were analysed for the presence of anti-Ag85B-ESAT-6 IgG (**A**), IgG1 (**B**) and IgG2a (**C**) antibodies by enzyme-linked immunosorbent assay (ELISA).
* denotes significantly increased antibody titres ($n=5$, $p<0.05$) compared to naïve control
** denotes significantly increased antibody titres ($n=5$, $p<0.01$) compared to naïve control
*** denotes significantly increased antibody titres ($n=5$, $p<0.001$) compared to naïve control

This apparent difference in immune response between the two preparation techniques may be attributable to several factors, including size and zeta potential, although the most probable cause is the way in which the antigen is released and presented to the cells of the immune system. As revealed by the *in vitro* release profiles of the systems (Fig. 5.7.), the formulation with adsorbed antigen (o/w) shows an initial burst of antigen, and it is this immediate accessibility to the cells of the immune system and persistence of antigen that may explain the enhanced antigen specific antibody responses. Indeed, the efficiency of the DDA/TDB liposomes in eliciting an antibody response may also be attributable to the initial burst of antigen.

Once again, the positive control (DDA/TDB) did show the highest levels of antibody production, although the o/w microsphere formulation appeared to be eliciting increasing antibody production at each time point, particularly for the IgG2a (Fig. 5.8c), and it would have been interesting to have continued the study further to investigate if this continued to be the case. The earlier onset of an antibody response seen with the DDA/TDB group could again be ascribed to the greater burst release of antigen shown by the liposomes as compared to the microsphere formulations (Fig. 5.7.).

5.3.3. Cell proliferation

When comparing the formulation type for antigen specific spleen cell proliferation (Fig. 5.9.), the results show very little positive immunological effect for the microspheres prepared by the o/w process, with the w/o/w process showing significantly higher levels of proliferation ($p<0.05$).

This result, although disappointing considering the promising antibody production, gives an insight into the type of immune response generated by the various systems. It has already been noted that the burst release of antigen is likely to be the cause of the high antibody responses, whereas the low levels of cell proliferation initiated by the o/w microsphere preparation intimate that such systems are not ideal for cell mediated immunity. A possible explanation for this could be the difference in size of the three systems, further implicating the influence of physico-chemical characteristics on immunological efficacy as outlined above (section 5.2.).

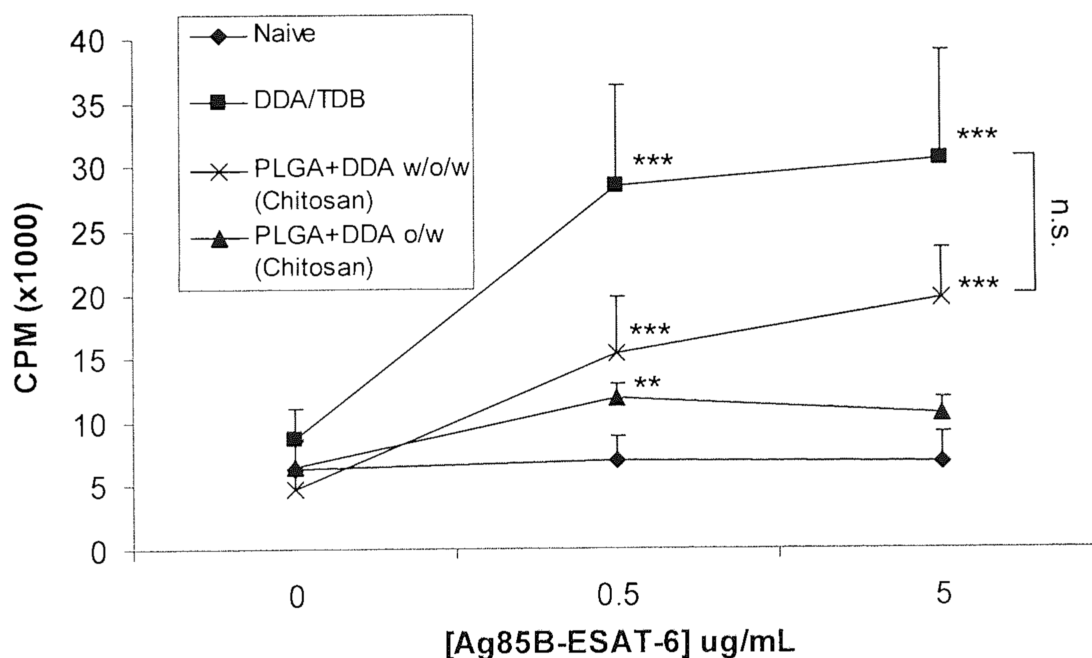


Fig. 5.9. Spleen cell proliferation in response to stimulation/re-stimulation with Ag85B-ESAT-6 antigen. Cell proliferation was measured by incorporation of ^{3}H into cultured splenocytes. Microsphere formulations were prepared by either the single (o/w) or the double (w/o/w) emulsion solvent evaporation method, as described in section 2.7. As a positive control, the liposomal formulation DDA/TDB, prepared as described in section 2.2.1., was administered.
** denotes significantly increased proliferation in comparison to naïve controls (n=5, $p<0.01$)
*** denotes significantly increased proliferation in comparison to naïve controls (n=5 $p<0.001$)
n.s. denotes no statistical difference between groups (n=5, $p>0.05$)

The particulate nature of the systems will lead to recognition and recruitment of cells of the immune system and the consequent immunological cascade (Storni et al., 2005; Bramwell & Perrie, 2005a), and seemingly the burst release allows for efficient processing and recognition of the antigen. However, uptake of the particles is dependent on size (Peyre et al., 2004), and results here suggest that this phenomenon may play a part in the immune response initiated by the various formulations. Although the o/w formulation does initiate positive antibody response due to the enhanced accessibility of antigen (Figs 5.8a-c.), the relatively large size associated with these particles (Table 5.2.) may be prohibitive to their uptake by APCs. Indeed, the smaller w/o/w particles, although unable to initiate the same antibody responses, do appear to yield good cell proliferation (Fig. 5.9.), suggesting a bias towards a cell mediated response. However, given that the difference in size is not great, several other factors, including duration of antigen presentation and presence of aggregates, are likely to influence the type of immune response initiated, and further analysis, with confocal microscopy for example, would help to clarify this point. Further, the DDA/TDB liposomes have the advantage of combining both the burst release of antigen with a yet smaller diameter, allowing for effective presentation of antigen and uptake by APCs.

5.3.4. Cytokine production

In terms of cytokine production (Figs. 5.10a-d.), the o/w formulation showed little effect immunologically, with no significant difference to the control group, whilst the w/o/w formulation initiated significantly higher levels of production for each cytokine assayed as compared to naïve control ($p<0.05$).

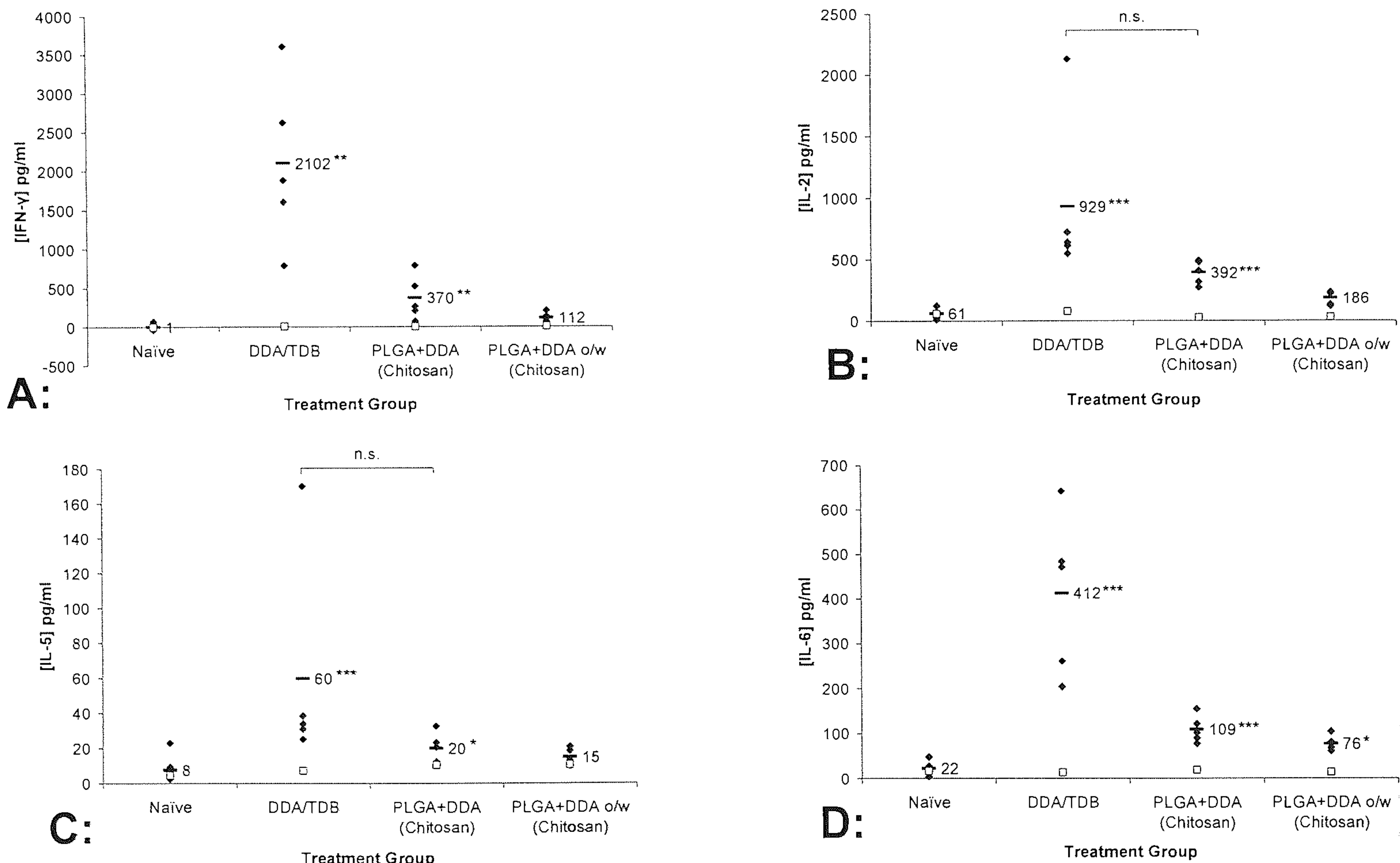


Fig. 5.10. Ag85B-ESAT-6 specific cytokine production. Cytokines were detected using DuoSet® capture ELISA kits (mouse IFN-γ (**A**), IL-2 (**B**), IL-5 (**C**), IL-6 (**D**)) purchased from R&D systems, Abingdon, UK, according to the manufacturers instructions. Filled diamonds represent the mean of three measurements for each individual spleen. Horizontal lines represent average group values (n = 5) and are shown numerically on the chart. Unfilled squares represent average of unstimulated group values (n=5). * denotes significantly increased levels in comparison to naïve controls (n=5, p<0.05) ** denotes significantly increased levels in comparison to naïve controls (n=5, p<0.01) *** denotes significantly increased levels in comparison to naïve controls (n=5, p<0.001) n.s. denotes no statistical difference between groups (n=5, p>0.05)

The o/w formulation did show increased levels compared to the naïve control (treatment group 1) for all cytokines assayed, although the only instance of significance was for IL-6 (Fig. 5.10d.). Furthermore, the w/o/w formulation showed significantly enhanced production of all cytokines studied compared to the o/w formulation ($p<0.05$), supporting the theory that the w/o/w formulation favours cell mediated immunity.

5.4. Alternative and combination of adjuvants

5.4.1. Physico-chemical characteristics

Microspheres were formulated by the double emulsion solvent evaporation process (Section 2.7.1.), with chitosan as the emulsion stabiliser present in the external aqueous phase, incorporating either MDP or DDA alone or in combination. In terms of the effect of MDP on the physico-chemical characteristics of the microspheres produced (Table 5.3.), there is a significant increase in volume mean diameter ($p<0.001$) and decrease in zeta potential ($p<0.01$) as compared to the formulation containing DDA alone.

Adjuvant	**Volume mean diameter (μm)**	**Zeta potential (mV)**
DDA	3.0 ± 0.1	39.1 ± 1.6
DDA + MDP	8.5 ± 0.3	30.8 ± 1.6
MDP	7.3 ± 0.1	28.3 ± 1.1

Table 5.3. The effect of adjuvant on the physico-chemical characteristics of microspheres produced. Microspheres composed of PLGA were prepared by the double emulsion solvent evaporation (w/o/w) method (section 2.7.1.), with various adjuvants (DDA and/or MDP), with chitosan as the emulsion stabiliser (0.75%, w/v). Size was measured using a Sympatec Helos (Sympatec, Germany). Zeta potential was measured using a Brookhaven Zetaplus (Brookhaven, NY). Results denote mean ± S.D. from at least 3 independently synthesised batches.

However, since the MDP is adsorbed to the pre-formed particles due to its hydrophilic nature, it is conceivable that the apparent increase in mean diameter is as a consequence of this adsorbed layer of peptide causing the microspheres to be present as agglomerates within a peptide reservoir, thereby skewing the sizing data towards a larger mean diameter. Furthermore, the formulation containing MDP alone still exhibited a cationic charge, despite the absence of DDA, confirming that the chitosan used to stabilise the particles confers this property on the microspheres.

5.4.2. Antibody production

The results for the antibody ELISA (Figs. 5.11a–c.) reveal that, from the microsphere formulations, those containing PLGA and MDP offer the strongest responses, although this is once again skewed towards a Th2 type response, with no formulation eliciting enhanced IgG2a production (Fig. 5.11c.). The trend MDP > DDA > DDA+MDP is evident, which suggests a potential interaction between the two adjuvants, DDA and MDP, which may thus impair their immunogenicity.

Indeed, the formulation containing MDP alone shows significantly ($P<0.05$) enhanced IgG1 production as compared to the DDA+MDP formulation (Fig. 5.11b.). This interaction is likely to be occurring through ion-dipole bonding between the cationic headgroup of the DDA and the carboxylic acid groups on the MDP molecule, thus impairing the immunological activity of both adjuvant. However, further investigations into the effect of pH on the physico-chemical characteristics of the formulations would be required to further elucidate this interaction.

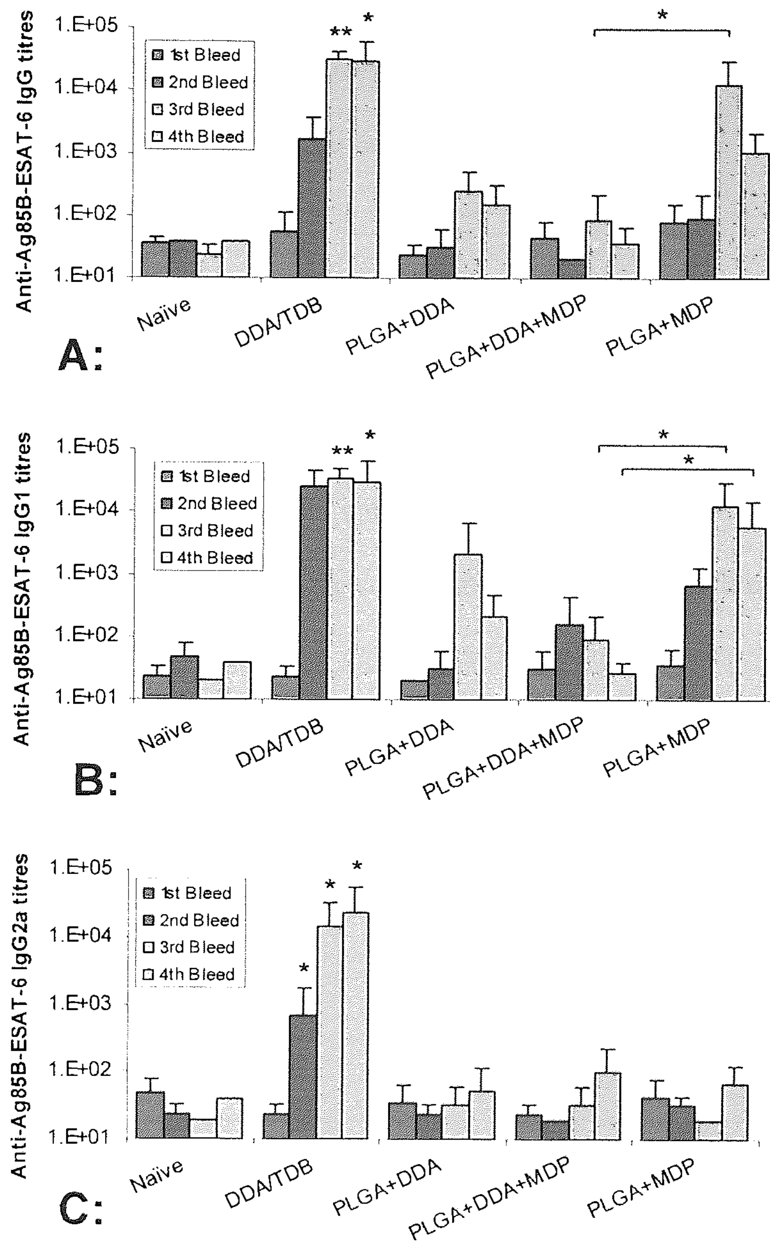


Fig. 5.11. Ag85B-ESAT-6 specific antibody titres. Microsphere formulations were prepared by the double emulsion solvent evaporation method, as described in section 2.7.1., incorporating DDA and/or MDP. As a positive control, the liposomal formulation DDA/TDB, prepared as described in section 2.2.1., was administered due to its' ability to initiate strong protective immune responses against Mtb (see chapter 3). Groups of five female BALB/c mice, approximately six weeks old, received doses of vaccine formulations containing 2μg of Ag85B-ESAT-6 in a 50μl volume. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks. Serum samples were taken at 12 days after the first administration and at two week intervals thereafter. Sera samples obtained at different time intervals after immunisation were analysed for the presence of anti-Ag85B-ESAT-6 IgG (**A**), IgG1 (**B**) and IgG2a (**C**) antibodies by enzyme-linked immunosorbent assay (ELISA).
* denotes significantly increased antibody titres ($n=5$, $p<0.05$) as compared to naïve control
** denotes significantly increased antibody titres ($n=5$, $p<0.01$) as compared to naïve control

Moreover, it has already been noted that the adjuvant activity of DDA relies upon electrostatic interaction with the surface of APCs (Smith Korsholm et al., 2007), whilst MDP is also thought to actively target macrophages (Wahl et al., 1979), lending further weight to the hypothesis that the two adjuvants are interacting in a way that is detrimental to their adjuvanticity.

5.4.3. Cell proliferation

Encouragingly, both the adjuvants alone and in combination promoted proliferative responses significantly greater than the naïve group (Fig. 5.12.), with the formulations containing DDA or MDP alone showing levels not significantly different to that of the positive control (DDA/TDB).

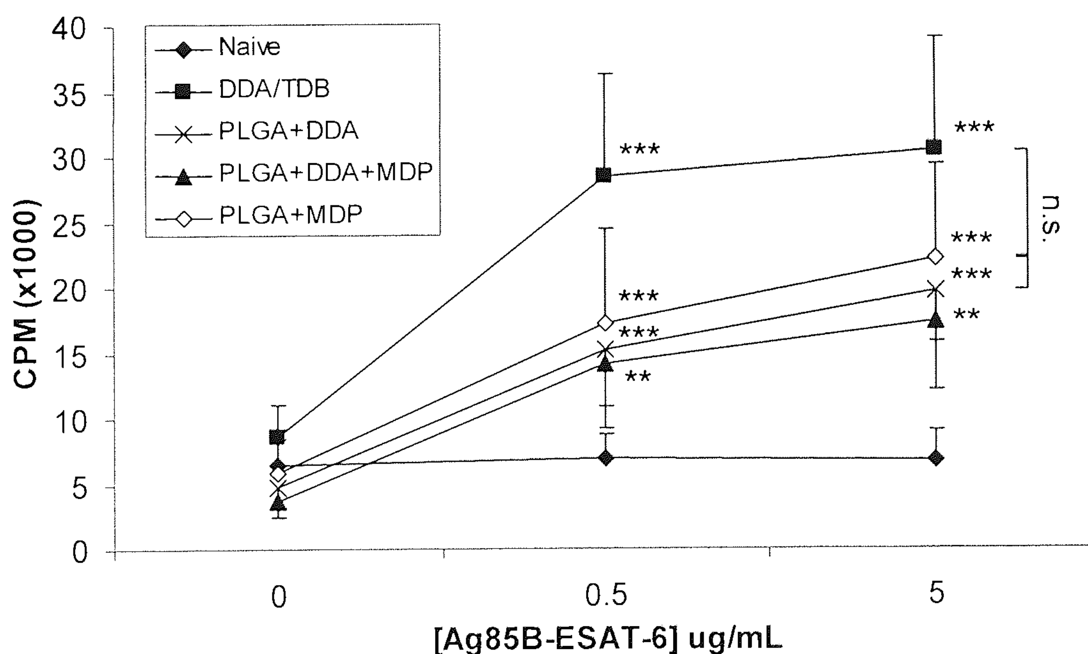


Fig. 5.12. Spleen cell proliferation in response to stimulation/re-stimulation with Ag85B-ESAT-6 antigen. Cell proliferation was measured by incorporation of ^{3}H into cultured splenocytes. Microsphere formulations were prepared by the double emulsion solvent evaporation method, as described in section 2.7.1., incorporating DDA and/or MDP. As a positive control, the liposomal formulation DDA/TDB, prepared as described in section 2.2.1., was administered. ** denotes significantly increased proliferation in comparison to naïve controls (n=5, p<0.01). *** denotes significantly increased proliferation in comparison to naïve controls (n=5 p<0.001). n.s. denotes no statistical difference between groups (n=5, p>0.05)

Although there was no significance between the microsphere groups, the formulation containing both DDA and MDP in combination elicited the lowest levels of cell proliferation, significantly lower ($p<0.05$) than that of DDA/TDB. This result corroborates the trend seen with the antibody results (Figs. 5.11a-c.), again suggesting possible detrimental interaction (in terms of immunological efficacy) between the two adjuvants.

5.4.4. Cytokine production

Production of cytokines was again investigated (Figs. 5.13a-d.), and in this regard, the formulation incorporating DDA alone gave the most consistently positive results, with, this formulation initiating significantly enhanced levels ($P<0.01$) of all cytokines except IL-5 compared to the naïve group, with no significance from the positive control DDA/TDB group for all studied but IL-6. Additionally, of the microsphere formulations tested in this study, those formulated with DDA alone showed significantly enhanced production of both the Th1 cytokine markers (IFN-γ and IL-2, Figs. 5.13a. and 5.13b., respectively) when compared to MDP alone or the combination of both DDA and MDP ($p<0.05$).

Despite the encouraging results from the antibody and cell proliferation assays, the microsphere formulation containing MDP alone showed no advantage over DDA alone or a combination of both in terms of any of the cytokines studied, although there were instances of significantly enhanced production compared to the naïve control (IFN-γ and IL-6, Figs. 5.13a. and 5.13d., respectively).

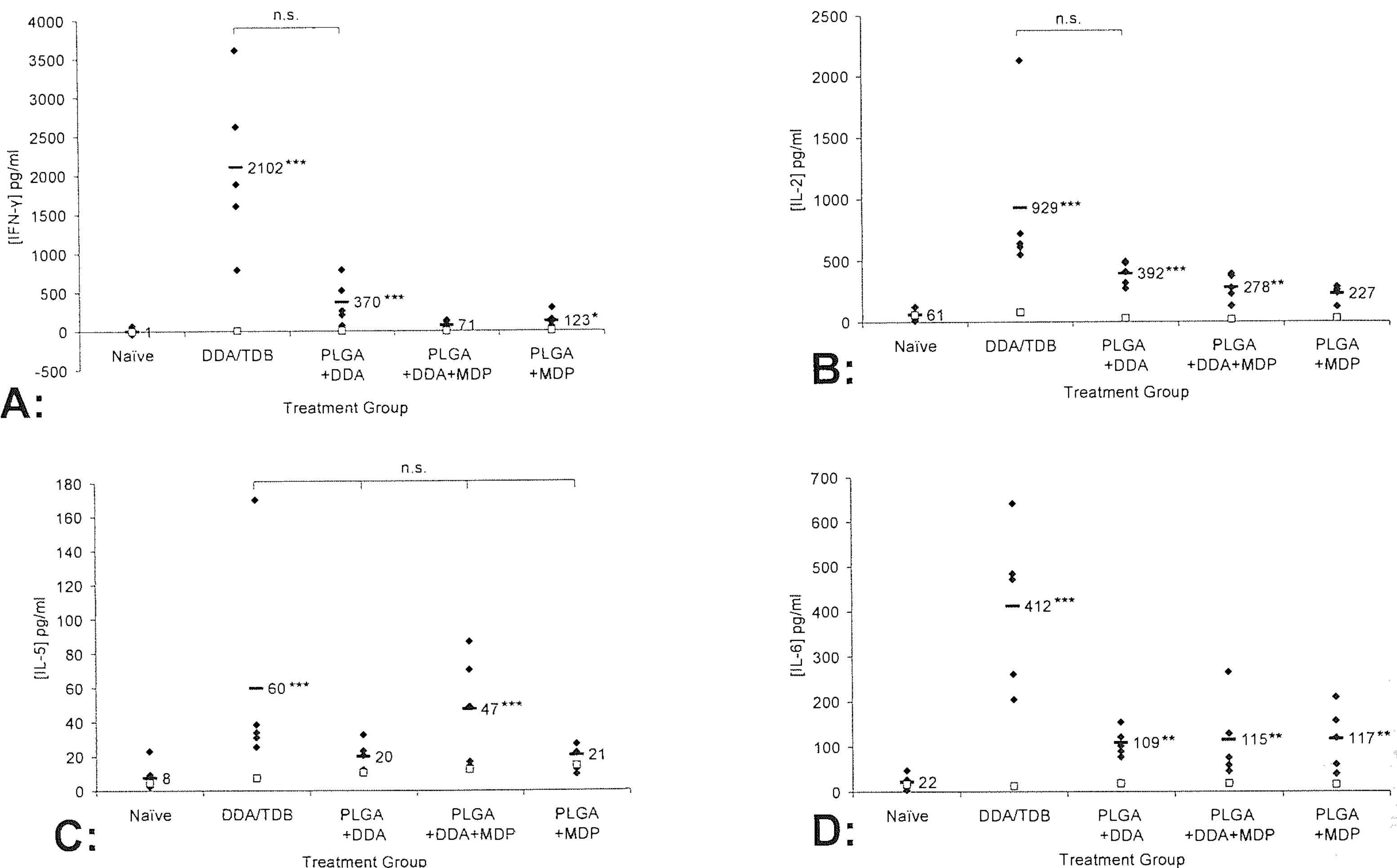


Fig. 5.13. Ag85B-ESAT-6 specific cytokine production. Cytokines were detected using DuoSet® capture ELISA kits (mouse IFN-γ (**A**), IL-2 (**B**), IL-5 (**C**), IL-6 (**D**)) purchased from R&D systems, Abingdon, UK, according to the manufacturers instructions. Filled diamonds represent the mean of three measurements for each individual spleen. Horizontal lines represent average group values (n = 5) and are shown numerically on the chart. Unfilled squares represent average of unstimulated group values (n=5). * denotes significantly increased levels in comparison to naïve controls (n=5, p<0.05) ** denotes significantly increased levels in comparison to naïve controls (n=5, p<0.01) *** denotes significantly increased levels in comparison to naïve controls (n=5, p<0.001) n.s. denotes no statistical difference between groups (n=5, p>0.05)

Furthermore, although the microsphere formulation incorporating both DDA and MDP appeared to inhibit IFN-γ production (Fig. 5.13a.), and to a lesser extent IL-2 (Fig. 5.13b.), there seems to be some benefit of combining the two adjuvants for the Th2 marker cytokines, and IL-5 in particular (Fig. 5.13c.). However, for this particular antigen, this is of no specific advantage, since TB is generally considered to require a Th1 biased response (Flynn et al., 1993; Ellner et al., 2000).

These results do suggest that DDA is an effective adjuvant for cell mediated immunity, although the combination of both DDA and MDP impairs this ability to an extent, possibly due to an interaction between the two adjuvants. Evidently the addition of MDP acts as a barrier to the interaction of DDA with the immune system, possibly by simply forming a steric barrier potentially through ion-dipole bonding, thus diminishing the accessibility of the DDA. Moreover, MDP is also thought to actively target macrophages (Wahl et al., 1979), so the interaction between the two adjuvants may also have a negative effect on the adjuvanticity of MDP, although further investigation would be required to confirm this.

5.5. Conclusions

There remains debate as to the correlates of protection against Mtb, since it is considered that antibody production may not be important for an effective immune response (McMurray, 2003), as this relies on Th1 cytokine responses, particularly IFN-γ (Flynn et al., 1993; Ellner et al., 2000). However, recent studies suggest that other parameters may need to be assessed (Agger & Andersen, 2001), advocating the need for a broad analysis

of immunogenicity for the overall evaluation of the effects of formulation changes on vaccine efficacy. With this in mind, the results seen for the PVA stabilised microspheres indicate good potential as a TB vaccine candidate, despite the diminished antibody responses as compared to the DDA/TDB liposomes (positive control), particularly for the Th1 dominant IgG2a. Indeed, the elevated levels of IL-5 (indicative of B-cell proliferation) elicited by the microsphere formulation, as well as antigen specific spleen cell proliferation at levels comparable to the positive control, signifies a role for such a formulation in cell mediated and memory immunity, a vital aspect for vaccine efficacy. In addition, although the DDA/TDB liposomes initiated greater IFN-γ secretion, the microsphere formulation produced levels significantly higher than naïve controls for this and all cytokines assayed, suggesting a good mix of Th1 and Th2 type response.

In terms of the effect of varying the nature of the emulsion stabiliser in the w/o/w process, a clear trend of PVA > Chitosan > CTAB emerged for both humoural and cellular immune responses. It has previously been reported that surface charge (Thiele et al., 2003) and size (Peyre et al., 2004) are likely to affect immune response, which may explain the inability of the CTAB stabilised formulation to offer any immunological benefit. In addition, the negative charge associated with these particles indicates loss of stabiliser on washing of the microspheres (Wischke et al., 2006), leading to unmodified surfaces, low antigen loading and presumably absence of the cationic immunomodulator DDA. Both the PVA and Chitosan stabilised microsphere formulations achieved positive results, but with an emphasis on Th2 type antibody production and cell mediated immunity. The difference in immune response initiated by the PVA and the chitosan

stabilised particles is likely due to factors such as the size of the microspheres produced, but may also be attributable to the relative rigidity of the chitosan stabilised particles, possibly as a consequence of direct interaction of the basic groups on the chitosan molecule with the acidic groups of the PLGA. In addition, although it has been reported that PLGA can enhance MHC II antigen presentation due to the acidic microenvironment following degradation of the polymer (Storni et al., 2005), it has also been postulated that chitosan, being basic in nature, can promote the MHC I presentation pathway, thus inhibiting the MHC II pathway (Strong et al., 2002).

In terms of preparation method, adsorption of the antigen to the surface of cationic microspheres prepared by the o/w process elicited enhanced antibody production as compared to their w/o/w counterparts, suggesting a difference in the way the antigen is presented to the immune system. However, it would appear that such formulations may be better suited for vaccines against diseases requiring antibody production rather than a cell-mediated immune response, such as malaria.

With regards the use of MDP as an alternative or in combination with DDA, there appears to be little synergistic effect between the two adjuvants. Indeed, the combination of both MDP and DDA seems to have a detrimental effect to the immunological efficacy of the microsphere formulation, possibly through ion-dipole interactions between the two adjuvants, and it would be interesting to investigate this interaction further. Nevertheless, the use of MDP as an alternative to DDA does offer some benefit in terms of antigen specific antibody production, although the same can not be said for either cell

proliferation of cytokine production, confirming the superior adjuvanticity of DDA for this particular application.

Therefore, in summary, the results described in this chapter suggest that, of the microsphere formulations investigated, the optimum parameters appear to be: preparation by the w/o/w method; the use of PVA as emulsion stabiliser; and DDA as the adjuvant of choice. The defining factors behind these results are likely to include the size, charge and method of presentation of antigen to the cells of the immune system.

Chapter 6

Formulating microsphere-based vaccines with enhanced shelf-life

6.1. Introduction

Freeze-drying, also referred to as lyophilisation, is used extensively in biological applications, particularly in the manufacture of vaccines, including BCG (Gheorghiu et al., 1996), proteins (Manning et al., 1989), blood products, antibiotics and enzymes (Aulton, 2002). The process involves freezing a liquid product, and then removing the water by sublimation under reduced pressure. Based on an understanding of the phase diagram for water (Fig. 6.1.), a liquid-to-solid-to-vapour transition occurs at low temperatures and reduced pressures, resulting in a dry product with low molecular mobility, thereby suppressing potential instabilities arising from physical or chemical interactions (Mohammed et al., 2006).

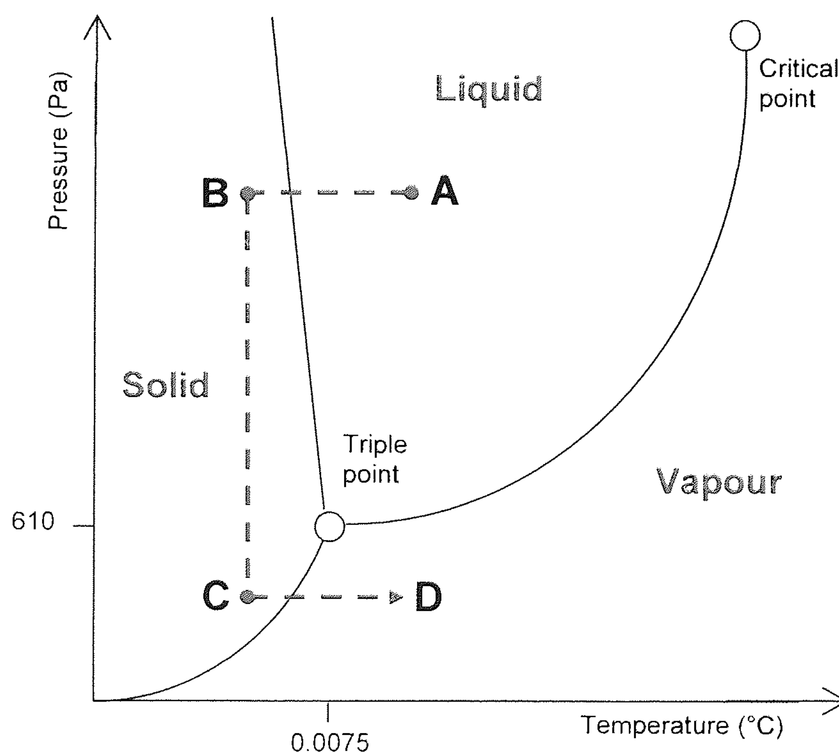


Fig. 6.1. The phase diagram for water (not to scale). The dashed arrow represents the freeze-drying process: A-B Freezing stage; B-C Vacuum application; C-D Sublimation and drying stage.

There are three stages involved in the freeze drying process: (1) initial freezing of the formulation; (2) primary drying; and (3) secondary drying. For the initial freezing stage, the formulation is usually frozen to around -10 to -30°C, which is well below the normal freezing point of water. However, addition of cryoprotectants can necessitate lower freezing temperatures (Aulton, 2002). Primary drying involves the removal of water by sublimation, to around 0.5% moisture content (Mohammed et al., 2007), during which the latent heat of sublimation must be provided. Further, the vapour formed must be continuously removed, normally by the use of a refrigerated condenser, in order to avoid a pressure rise which would stop sublimation. In order to remove any surface bound or residual water not removed by the primary drying stage, secondary drying is achieved by raising the temperature to as high as 50 or 60°C, leading to a free-flowing powder (Aulton, 2002).

Besides offering enhanced long-term stability, the production of a stable, dry powder product can eliminate the need for a cold chain, which accounts for millions of wasted doses of vaccines each year (Christensen et al., 2007; Schondorf et al., 2007), and is of particular value in developing regions where TB is a problem (i.e. sub-Saharan Africa and South East Asia), whilst also facilitating transport as compared to bulky products such as solutions. Indeed, it has been estimated that precluding the need for a cold chain for vaccine distribution through development of thermo-stable formulations could save around $200 million annually (Lloyd, 2000), due to the costs incurred implementing necessary infrastructure, reliable transport, functioning freezers and refrigerators and effective monitoring in order to maintain the cold chain (Christensen et al., 2007).

Moreover, freeze-drying has been shown to be a suitable method for improving the long-term stability of a wide variety of particulate formulations, including microspheres (Chacon et al., 1999; Duncan et al., 2005), nanoparticles (Lemoine et al., 1996; de Chasteigner et al., 1998; Bozdag et al., 2005), liposomes (Crowe & Crowe, 1988; Sun et al., 1996; Mohammed et al., 2006) and non-viral gene delivery vectors (Li et al., 2000; Seville et al., 2002).

However, it has been postulated by several authors that cryoprotectants are necessary to retain initial formulation characteristics (Crowe & Crowe, 1988; Roy et al., 1997; Anchordoquy et al., 2001; Mohammed et al., 2007), since freeze-drying can promote aggregation or fusion of particles, a phenomenon exploited elsewhere for the production of dehydration-rehydration liposomes (Kirby & Gregoriadis, 1984) and ISCOMs (Copland et al., 2000; Demana et al., 2005).

Having been found to play an important role in organisms capable of withstanding dehydration (Koster & Leopold, 1988; Crowe et al., 1996; Lloyd, 2000), the most commonly used cryoprotectants are sugars, and more specifically the disaccharides sucrose and trehalose (Fig. 6.2.), which have received much attention for their role as cryoprotectants in the freeze-drying of particulate formulations (Miyajima et al., 1986; Crowe et al., 1994a; Sun et al., 1996; Chacon et al., 1999; Saez et al., 2000; Mohammed et al., 2006). Various theories concerning the method of action have been proposed: the water replacement hypothesis has been postulated (Crowe & Crowe, 1988), whereby hydrogen bonding forms stable boundaries between particles; alternatively, others (Koster

& Leopold, 1988; Levine & Slade, 1988) have intimated that a glassy layer, formed via vitrification of the sugars, forms a viscous matrix whereby interaction and aggregation of particles is reduced; further, Allison and colleagues (2000) propose that particle isolation, caused by the concentration of solutes in the unfrozen fraction of the solution, is the most likely requirement for effective cryoprotection.

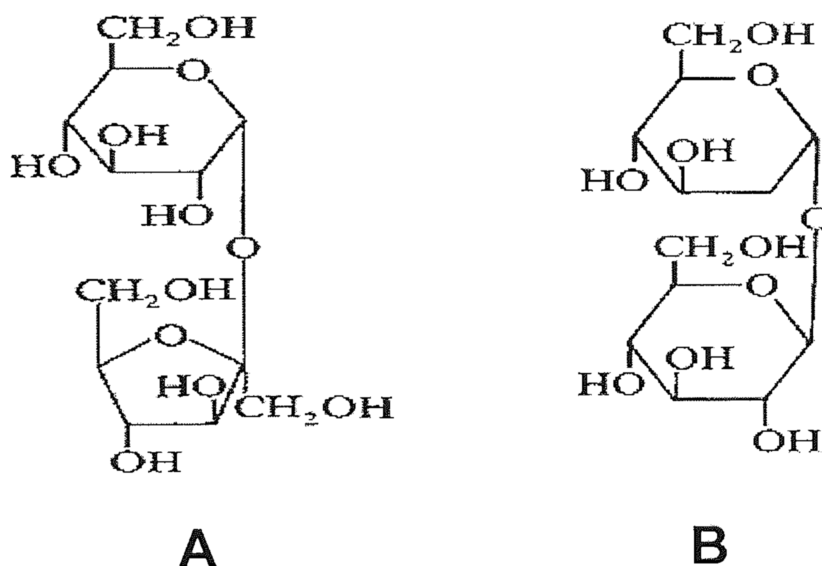


Figure 6.2. Chemical structure of (A) sucrose and (B) trehalose.

Although scant publications exist on the use of amino acids for the cryoprotection of particulate delivery systems (Anchordoguy et al., 1988; Crowe et al., 1994a; Schwarz & Mehnert, 1997), recent results reveal a potential role for such compounds in cryoprotection (Mohammed et al., 2006; Mohammed et al., 2007) as an alternative to the traditional sugar-based cryoprotectants. Indeed, amino acids have previously been employed as cryoprotectants for applications in cryobiology (Kundu et al., 2001; Bakaltcheva & Reid, 2003) and to enhance the aerosolisation of spray-dried powders containing non-viral gene delivery vectors (Li et al., 2003; Li et al., 2005).

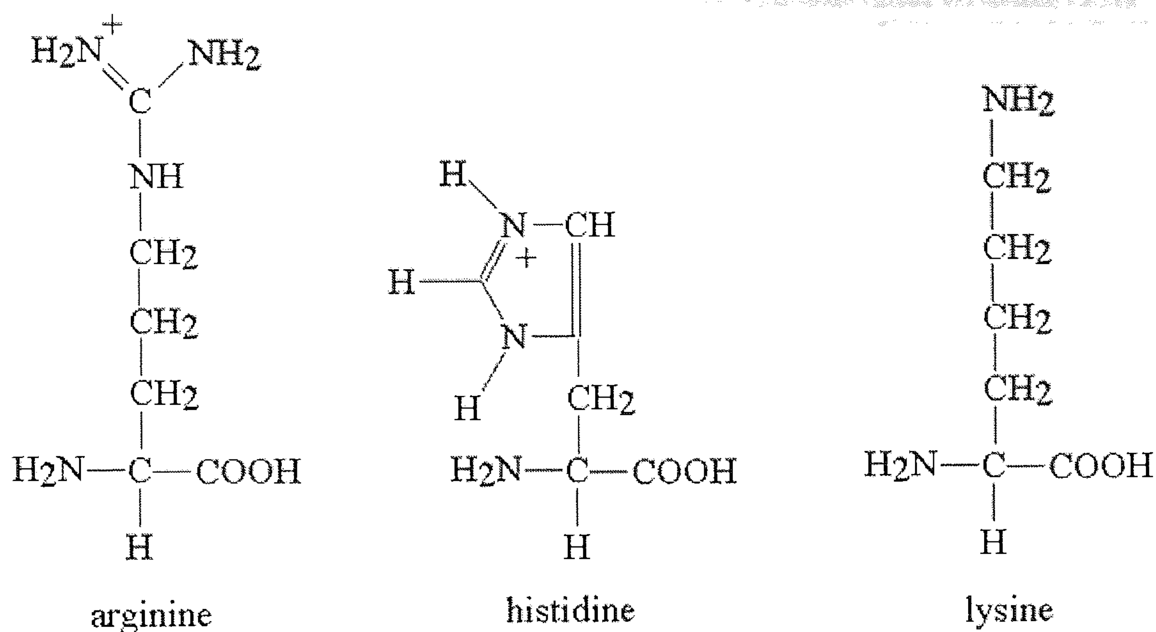


Fig. 6.3a. Chemical structure of amino acids bearing basic side chains

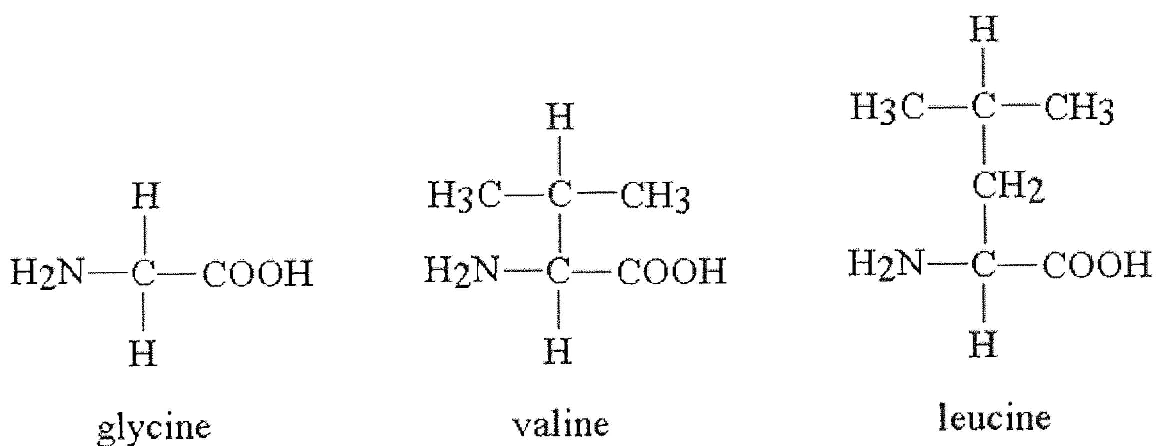


Fig.6.3b. Chemical structure of amino acids bearing hydrophobic side chains.

In order to further elucidate the mechanism of action of cryoprotectants, the work in this chapter describes the freeze-drying of polymer based microspheres with a range of cryoprotectants, including traditional sugar-based cryoprotectants (sucrose and trehalose) (Fig. 6.2.) and amino acids bearing either basic (lysine, arginine and histidine) (Fig. 6.3a.) or hydrophobic side chains (glycine, valine and leucine) (Fig. 6.3b.).

6.2. Freeze-drying of microspheres: cryoprotectant efficacy

6.2.1. Sugar-based cryoprotectants

Initial studies concentrated on the use of the disaccharide sugars, sucrose and trehalose. The results (Fig. 6.4.) reveal that cryoprotectants are necessary to maintain microsphere characteristics following lyophilisation. Indeed, freeze-drying of PLGA microspheres prepared by the w/o/w process (section 2.7.1.), both in the presence and absence of DDA, without the use of cryoprotectants (no cryo, Fig. 6.4.) leads to significant aggregation and, hence, significantly increased volume mean diameters ($p<0.01$) as compared to before freeze-drying (Fig. 6.4.).

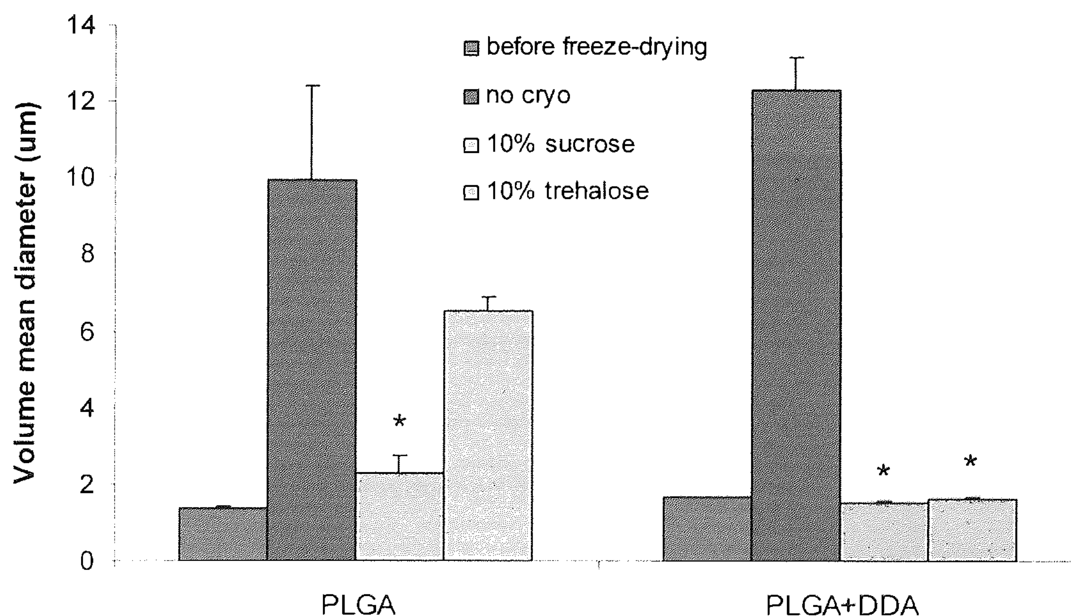


Fig. 6.4. Volume mean diameter of microspheres before and after freeze-drying in the presence of various cryoprotectants. Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in either double distilled water (no cryo), 10% (w/v) sucrose or 10% (w/v) trehalose. Following rehydration, sizes were measured using a Sympatec Helos (Sympatec, Germany). Results denote mean ± SD from at least 3 independently synthesised batches.
* denotes no significant increase in mean diameter in comparison to before freeze drying (bfd) ($p>0.05$)

In terms of the efficiency of the disaccharides to act as cryoprotectants, sucrose, at isotonic concentration (10% w/v), was consistently effective in maintaining particle size, both in the presence and absence of DDA, with microparticles retaining a size of around 2 microns post lyophilisation (Fig. 6.4.). Trehalose, on the other hand, is only effective at preventing significant aggregation when DDA is present in the formulation. However, there does appear to be some inhibition of aggregation as compared to no cryoprotectants when DDA is not present (Fig. 6.4.), which suggests that vitrification may be occurring.

These results suggest that the main function of the cryoprotective ability of sucrose appears to be vitrification and particle isolation (Allison et al., 2000; Konan et al., 2002), whereas trehalose requires additional interaction with the particles, as suggested previously (Crowe et al., 1994a; Ozaki & Hayashi, 1998). The cationic nature of the DDA headgroup will potentially allow for ion-dipole or electrostatic interactions to be formed with the sugar, thus leading to water replacement, and hence cryoprotection. Indeed, zeta potential analysis of the formulations reveals that, although there is no evidence of an altering of the surface charge when DDA is not present in the formulation (results not shown), freeze-drying of the PLGA+DDA formulation in the presence of trehalose leads to a significant reduction in zeta potential, from 15.7 ± 0.9 mV to -15.1 ± 0.2 mV, whereas employing sucrose as cryoprotectant maintains zeta potential (16.3 ± 6.8 mV). The apparent difference between the two sugars, in terms of altering the zeta potential of the formulation incorporating DDA, may be explained by the relative stability of the sugar glasses. It has previously been reported that trehalose retains a high T_g upon rehydration (~115°C) and thus remains in the glassy state (Aldous et al., 1995; Crowe et

al., 1996), whereas the lower T_g of sucrose (~65°C) enables the sugar glass to become more readily mobile (Crowe et al., 1996). Therefore, the trehalose may be masking the charge of the microsphere surface by maintaining the glassy state around the particles, whereas the sucrose is more readily hydrated, which again implicates vitrification as a necessary function of cryoprotection in the case of the disaccharides. However, the explanation of this result is unclear, and further investigations would be required in order to determine the type of interaction occurring and the origin of the negative charge. Furthermore, although sucrose prevents significant aggregation in the PLGA formulation (i.e. without DDA), there is still an increase in mean diameter following lyophilisation, from 1.39 ± 0.02 μm to 2.30 ± 0.43 μm (Fig. 6.4.), as well as an increase in the distribution of sizes, with an increase in span from 1.54 to 2.50 (results not shown). In contrast, the formulation containing DDA shows no significant change in mean diameter (Fig. 6.4.) or span (results not shown) when freeze-dried in the presence of either cryoprotectant, suggesting interaction of both the disaccharides with the cationic DDA headgroup, leading to more efficient cryoprotection. This further supports the hypothesis that vitrification alone, although necessary, may not be sufficient for stabilisation of particulate formulations (Crowe et al., 1994a).

6.2.2. Amino acid based cryoprotectants

6.2.2.1. Basic side chains

The use of amino acids was then investigated as an alternative to traditional sugar-based cryoprotectants employed in the freeze-drying of PLGA+DDA microspheres. Initial investigations focused on lysine, due to recent results suggesting efficient cryoprotection

of liposomal formulations (Mohammed et al., 2007). In terms of maintaining microsphere particle size following lyophilisation, lysine does offer a degree of cryoprotection, with concentrations ≥ 1.5% (w/v) offering significantly enhanced cryoprotection as compared to no cryoprotectant (Fig. 6.5a., $p<0.01$). Nevertheless, compared to before freeze-drying, there is a significant increase in mean diameter ($p<0.05$) by a factor of at least 1.8 (3% (w/v) lysine), whilst the span of the size distribution is also significantly increased ($p<0.05$, Fig. 6.5a.).

Furthermore, zeta potential measurements (Fig. 6.5b.) reveal that the addition of lysine as a cryoprotecant profoundly affects the surface charge of the particles, suggesting that the mode of action of cryoprotection in this case is more likely to be through interaction with the microsphere surface, and thus the water replacement hypothesis (Crowe & Crowe, 1988). Indeed, it has been reported that amino acids can stabilise freeze-dried protein through formation of ion-dipole bonds (Tian et al., 2006). However, in contrast to the disaccharides, the interactions are likely to be with the carboxylic acid group of the polymer, rather than the DDA headgroup.

Further investigations into the effect of pH on the cryoprotective ability of lysine would, however, be required, in order to elucidate if this interaction remains apparent over a range of pH where the basic side chain is either protonated or not protonated, thus determining if ion-dipole or hydrogen bonding are indeed a function of cryoprotection in this case.

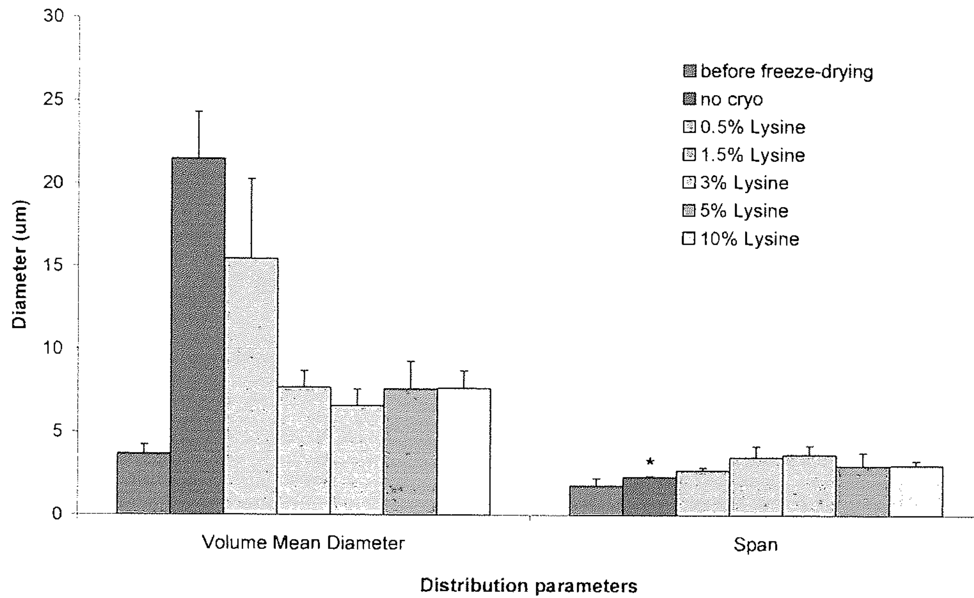


Fig. 6.5a. Volume mean diameter and span of size distribution of microspheres before and after freeze-drying in the presence of lysine as cryoprotectant. Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in varying concentrations of lysine or double distilled water (no cryo). Following rehydration, sizes were measured using a Sympatec Helos (Sympatec, Germany). Results denote mean ± SD from at least 3 independently synthesised batches.
* denotes no significant increase in distribution parameter in comparison to before freeze drying (bfd) ($p>0.05$)

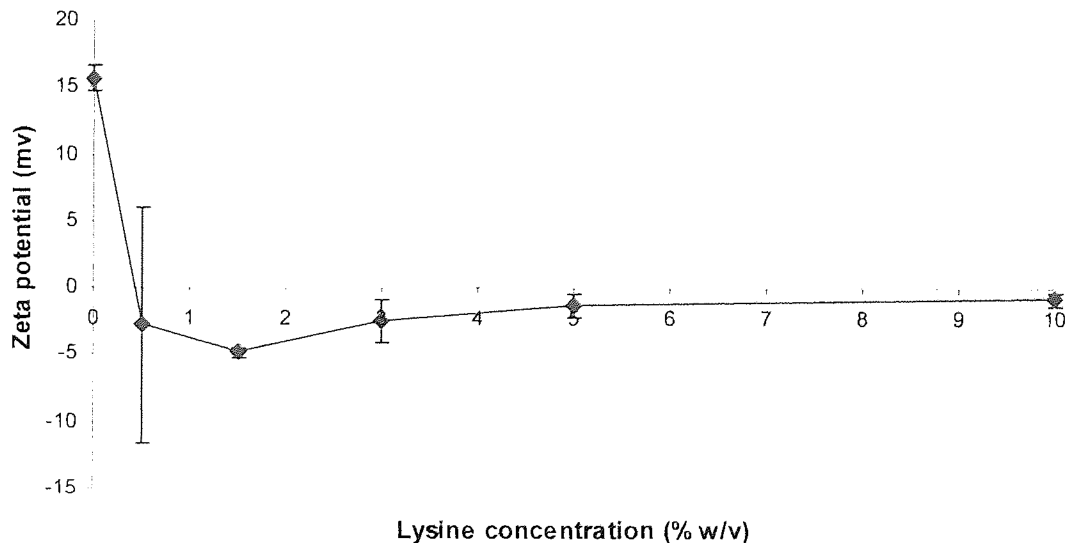


Fig. 6.5b. **Zeta potential of microspheres after freeze-drying in the presence of increasing lysine concentration.** Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in varying concentrations of lysine. Following rehydration, zeta potential was measured with a Brookhaven ZetaPlus (Brookhaven, NY). Results denote mean ± SD from at least 3 independently synthesised batches. NB: zeta potential before freeze-drying = 15.67 ± 0.9 mV.

Therefore, as a continuation of the studies investigating amino acids as cryoprotectants, arginine and histidine were then assayed for their ability to act as cryoprotectants due to their structural similarity (in terms of the presence of basic groups in the side chain) to lysine.

As shown in figure 6.6a., the efficiency of arginine to prevent aggregation of microspheres following freeze-drying shows concentration dependence. Arginine at both 1% (w/v) and 5% (w/v) showed significantly increased mean diameters and span of particle sizes as compared to before freeze-drying (Fig. 6.6a.), with only the highest concentration studied, 10% (w/v), proving effective. This evident trend for concentration dependence suggests that particle isolation is occurring, with possible interaction between the cryoprotectant and the microsphere surface.

Interestingly, the presence of arginine at a concentration of 1% (w/v) actually led to an increase in volume mean diameter and span as compared to no cryoprotectant (Fig. 6.6a), which suggests that the presence of arginine at low concentrations encourages aggregation. Although the explanation of this observation is unclear, this result could suggest that greater concentrations of arginine are required in order for electrostatic repulsion (arising from the positively charged side chain) to prevent fusion or further interactions between the particles and the basic side chain of the cryoprotectant, thus leading to aggregation of microspheres.

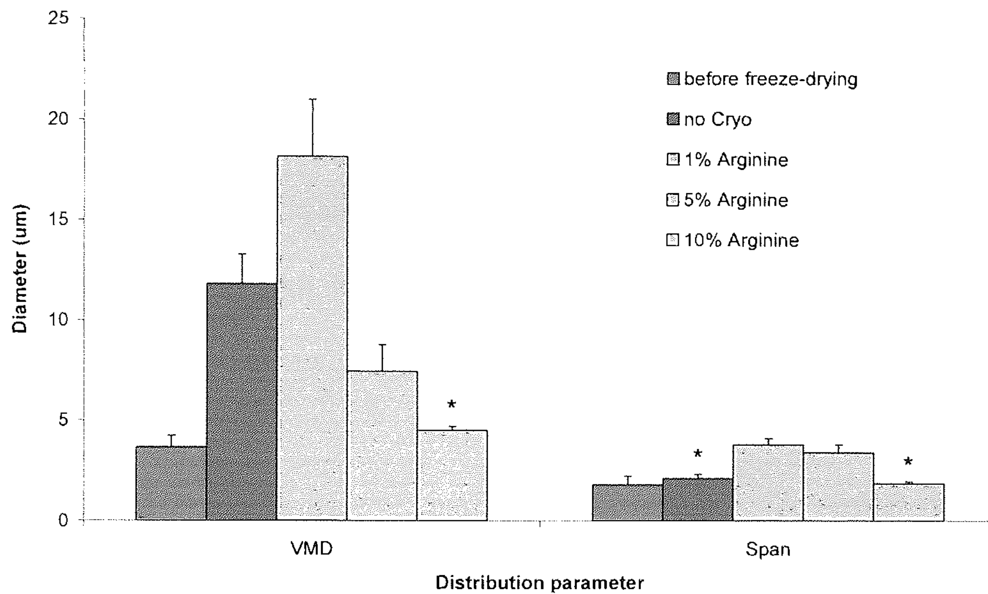


Fig. 6.6a. Volume mean diameter (VMD) and span of size distribution of microspheres before and after freeze-drying in the presence of arginine as cryoprotectant. Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in varying concentrations of arginine or double distilled water (no cryo). Following rehydration, sizes were measured using a Sympatec Helos (Sympatec, Germany). Results denote mean ± SD from at least 3 independently synthesised batches. * denotes no significant increase in distribution parameter in comparison to before freeze drying (bfd) ($p>0.05$)

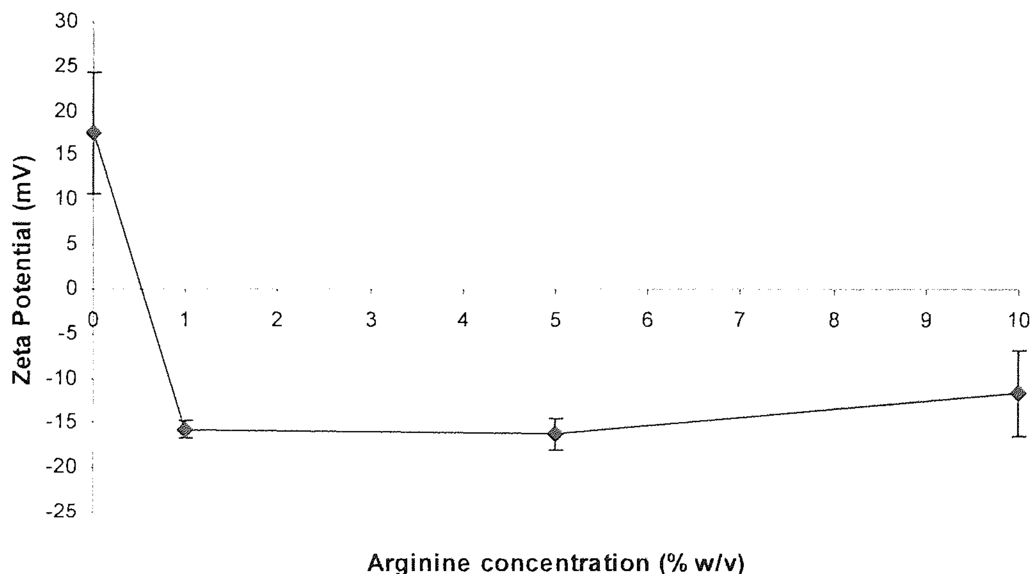


Fig. 6.6b. Zeta potential of microspheres before and after freeze-drying in the presence of increasing arginine concentration. Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in varying concentrations of arginine. Following rehydration, zeta potential was measured with a Brookhaven ZetaPlus (Brookhaven, NY). Results denote mean ± SD from at least 3 independently synthesised batches. NB: zeta potential before freeze-drying = 15.67 ± 0.9 mV.

Further, the reduced concentration of cryoprotectant may lead to inefficient particle isolation. Indeed, previous studies have suggested that the basic amino acids are capable of forming amorphous glasses during freeze-drying (Mattern et al., 1999), thus suggesting vitrification is again a function of cryoprotection as with the disaccharides. In addition, the significant increase in span (Fig. 6.6a, $p<0.01$) may also suggest inefficiency in particle dispersion by the particle sizing equipment, particularly for the larger aggregates, which can lead to variability in results.

Similarly to microspheres freeze-dried in the presence of lysine, the surface charge of the microspheres was greatly affected when freeze-dried in the presence of arginine (Fig. 6.6b.). This once again implicates the effect of interaction between the cryoprotectant and the microspheres, probably through ion-dipole or hydrogen bonding, as an important function of cryoprotection.

Due to the reduced solubility of histidine (4.19g per 100 mL) as compared to lysine (> 500g per 100 mL) and arginine (15g per 100 mL), lower concentrations were investigated in the following part of the study. Although none of the concentrations studied was able to prevent significant increases in mean diameter following lyophilisation ($p<0.05$), concentration dependence was again evident when histidine was employed as cryoprotectant (Fig. 6.7a.). The lowest concentration studied, 0.5% (w/v) showed no significance in mean diameter as compared to no cryoprotectant ($p>0.05$), whereas concentrations of 1% (w/v) and 2% (w/v) showed significantly reduced aggregation as compared to no cryoprotectant ($p<0.05$ and $p<0.01$, respectively) (Fig. 6.7a).

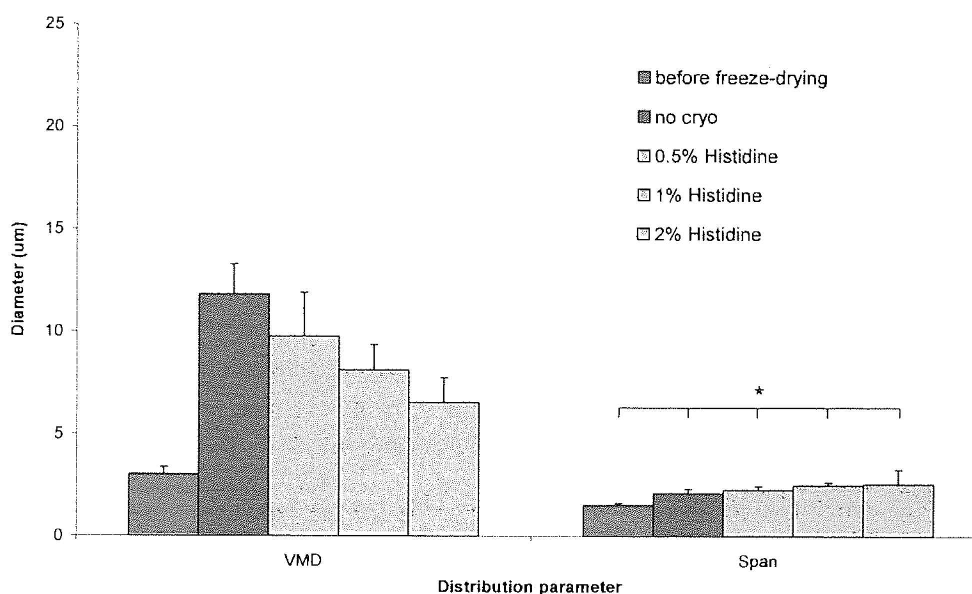


Fig. 6.7a. Volume mean diameter (VMD) and span of size distribution of microspheres before and after freeze-drying in the presence of histidine as cryoprotectant. Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in varying concentrations of histidine or double distilled water (no cryo). Following rehydration, sizes were measured using a Sympatec Helos (Sympatec, Germany). Results denote mean ± SD from at least 3 independently synthesised batches.* denotes no significant increase in distribution parameter in comparison to before freeze drying (bfd) ($p>0.05$)

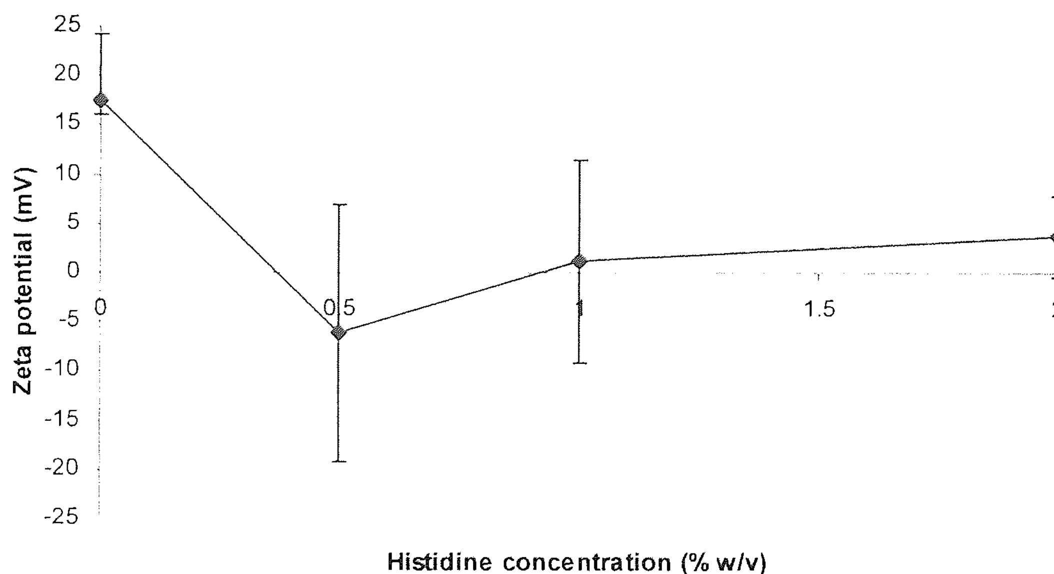


Fig. 6.7b. Zeta potential of microspheres after freeze-drying in the presence of increasing histidine concentration. Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in varying concentrations of histidine. Following rehydration, zeta potential was measured with a Brookhaven ZetaPlus (Brookhaven, NY). Results denote mean ± SD from at least 3 independently synthesised batches. NB: zeta potential before freeze-drying = 15.67 ± 0.9 mV.

However, as for the other positively charged amino acids investigated, zeta potential measurements again suggest interaction between the cryoprotectant and the microspheres (Fig. 6.7b.), once again corroborating the hypothesis that ion-dipole or hydrogen bonding may well be a determining factor in the cryoprotective ability of such moieties.

Furthermore, the extent of reduction in zeta potential correlates with the isoelectric points (pI) of the amino acids studied, in that arginine > lysine > histidine. Indeed, arginine (pI = 10.76) possesses three amine groups in its side chain, which is likely to facilitate ion-dipole or hydrogen bonding compared to lysine (pI = 9.74) or histidine (pI = 7.59), as evidenced by the zeta potential measurements (Fig. 6.6b). Indeed, it has previously been reported that the nature of the positively charged second amine group of the amino acid is important in cryoprotection (Anchordoquy et al., 1988).

6.2.2.2. Hydrophobic side chains

As a continuation of the investigations into the ability of amino acids to act as cryoprotectants, glycine, valine and leucine were also studied. Bearing hydrophobic side chains (He et al., 2006) with pI values around 6, these three amino acids offer an alternative to the more basic lysine, arginine and histidine studied above, whilst also providing an insight into the method of action of such cryoprotectants.

From the results (Fig. 6.8a.), there again appears to be concentration dependence for all three of the amino acids, most notably glycine. Indeed, only the higher concentrations (2% (w/v)) of glycine and valine were effective in preventing significant aggregation of

the PLGA+DDA microspheres following freeze-drying. However, leucine shows efficient cryoprotection in terms of maintaining mean diameter at all concentrations studied. Furthermore, leucine maintains the span of the size distribution, as does glycine at the higher concentration (2% (w/v)) (Fig. 6.8a. insert).

Interestingly, zeta potential measurements reveal no significant alteration of the surface charge associated with the microspheres for all three amino acids at all concentrations studied (Fig. 6.8b.). This result suggests that the mode of action of cryoprotection in this case is more likely to be through particle isolation rather than ion-dipole or hydrogen bonding, particularly since the hydrophobic amino acids investigated here possess inactive side chains that rarely take part in chemical reactions.

Since formation of ice during freezing causes solutes and particles to be concentrated in the unfrozen fraction (Anchordoquy et al., 2001), it is possible that the lower solubility of the hydrophobic amino acids may lead to more efficient particle isolation in the unfrozen fraction, and hence improved cryoprotection. Further, glycine has been employed as a bulking agent in the freeze-drying of proteins, since it crystallises on freeze-drying leading to enhanced physical stability, formation of stronger dried cakes and improved dissolution properties as compared to amorphous agents (Chang et al., 1996; Passot et al., 2005). These properties further support an alternative method of cryoprotection to the basic amino acids.

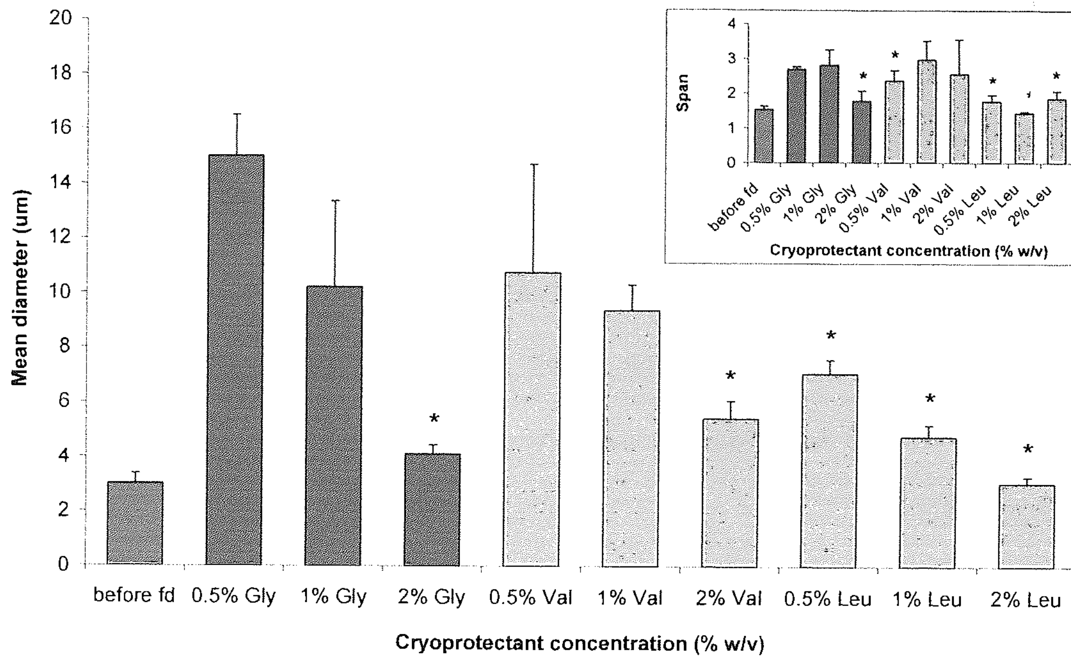


Fig. 6.8a. Volume mean diameter and span of size distribution (insert) of microspheres before and after freeze-drying in the presence of various cryoprotectants. Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in varying concentrations of glycine, valine or leucine. Following rehydration, sizes were measured using a Sympatec Helos (Sympatec, Germany). Results denote mean ± SD from at least 3 independently synthesised batches. * denotes no significant increase in distribution parameter in comparison to before freeze drying (bfd) (p>0.05).

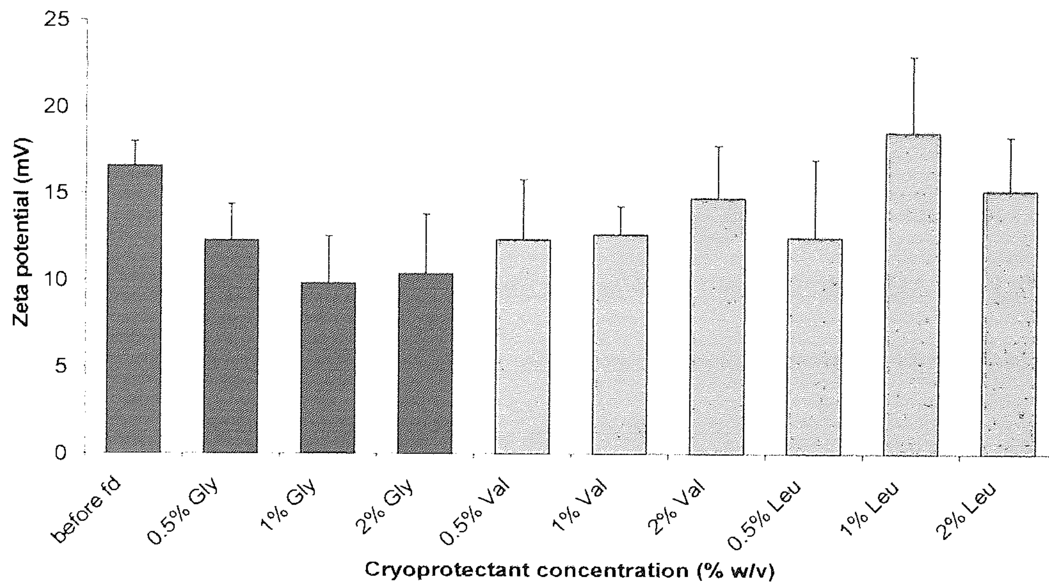


Fig. 6.8b. Zeta potential of microspheres before and after freeze-drying in the presence of increasing concentrations of various cryoprotectants. Microspheres were produced via the w/o/w/ double emulsion solvent evaporation method, as described in section 2.7.1., harvested and freeze-dried in varying concentrations of glycine, valine or leucine. Following rehydration, zeta potential was measured with a Brookhaven ZetaPlus (Brookhaven, NY). Results denote mean ± SD from at least 3 independently synthesised batches. NB: zeta potential without cryoprotectant = 17.45 ± 6.8 mV.

Therefore, it is likely that hydrophobic interactions and particle isolation are the main functions of cryoprotection for these particular amino acids. Indeed, there does appear to be a relationship between the length of the side chain of the amino acids and their cryoprotective ability, with leucine > valine > glycine.

In addition, the relative success of leucine as a cryoprotectant may also be due in part to its surfactant like properties (Gliniski et al., 2000), which have also been exploited for its use as a dispersibility enhancer for spray-dried powders (Rabbani & Seville, 2005).

6.3. Conclusions

From the results outlined above, it becomes clear that cryoprotectants are indeed necessary in order to maintain the physico-chemical characteristics of the microsphere formulations investigated.

Of the traditional sugar-based cryoprotectants studied, sucrose shows the most consistency in maintaining particle size and zeta potential, although it would appear that both the sugars investigated require interaction with the microsphere surface, possibly through hydrogen bonding or ion-dipole interaction with the cationic DDA headgroup, as well as vitrification in order to offer efficient cryoprotection. This observation corroborates results seen elsewhere (Crowe et al., 1994a).

Amino acids offer potential as an alternative to sugar-based cryoprotectants, although the mode of action appears to differ depending on the nature of the side chain present on the

amino acid backbone. The three amino acids studied with a basic side chain (lysine, arginine and histidine) are all capable of forming hydrogen bonds, and zeta potential measurements appear to reveal that there is indeed some interaction with the microsphere surface, substantiated by a decrease in surface charge. This would suggest that the water replacement hypothesis is the most likely method of cryoprotection for such amino acids. However, investigations into the effect of pH, on both the zeta potential and the cryoprotective ability of these amino acids, would be necessary in order to confirm whether ion-dipole or hydrogen bonds are indeed being formed between the basic groups present on the amino acid side chain and the microsphere surface. Further, results seen previously with cationic liposomal formulations (Mohammed et al., 2007) implicate the occurrence of hydrophobic interactions at higher concentrations of amino acid, which could also play a part in cryoprotection. Indeed, lysine does possess the longest side chain of the 20 amino set, along with arginine, which may explain the possibility of hydrophobic interactions. Additionally, the amorphous nature reported upon freeze-drying could implicate the role of vitrification in the cryoprotective function of the basic amino acids.

Furthermore, the three amino acids investigated bearing hydrophobic side chains (glycine, valine and leucine), showed good potential as alternatives to sugar-based cryoprotectants at much lower concentrations. Zeta potential measurements also indicate that, conversely to the basic amino acids, the hydrophobic amino acids do not appear to interact with the surface of the microspheres, implicating that particle isolation is the most likely method

of action of cryoprotection for such molecules, possibly as a consequence of their reduced solubility.

As detailed in Chapter 5, the physico-chemical characteristics of the microspheres greatly influence the immunological efficacy of such formulations, therefore implying that the hydrophobic amino acids, particularly leucine, offer a genuine alternative to sugar-based cryoprotectant. However, the basic amino acids studied, through interaction with the surface of the microspheres, could potentially affect the efficacy of the formulation *in vivo*, since previous results (Chapter 5) have highlighted the benefit of microspheres possessing a cationic nature, thus offering less applicability in this case. Nevertheless, this altering of the surface charge could potentially be exploited in other instances.

Chapter 7

Environmental Scanning Electron Microscopy (ESEM) as a morphological tool in the analysis of microspheres

7.1. Introduction

In contrast to conventional Scanning Electron Microscopy (SEM), environmental scanning electron microscopy (ESEM) allows for the imaging of wet samples in their natural state without the need for prior sample preparation (e.g. drying, fixing, freezing or metallic coating), making it a particularly useful method for morphological investigations in biological applications (Collins et al., 1993; Manero et al., 2003; McKinlay et al., 2004; Muscariello et al., 2005).

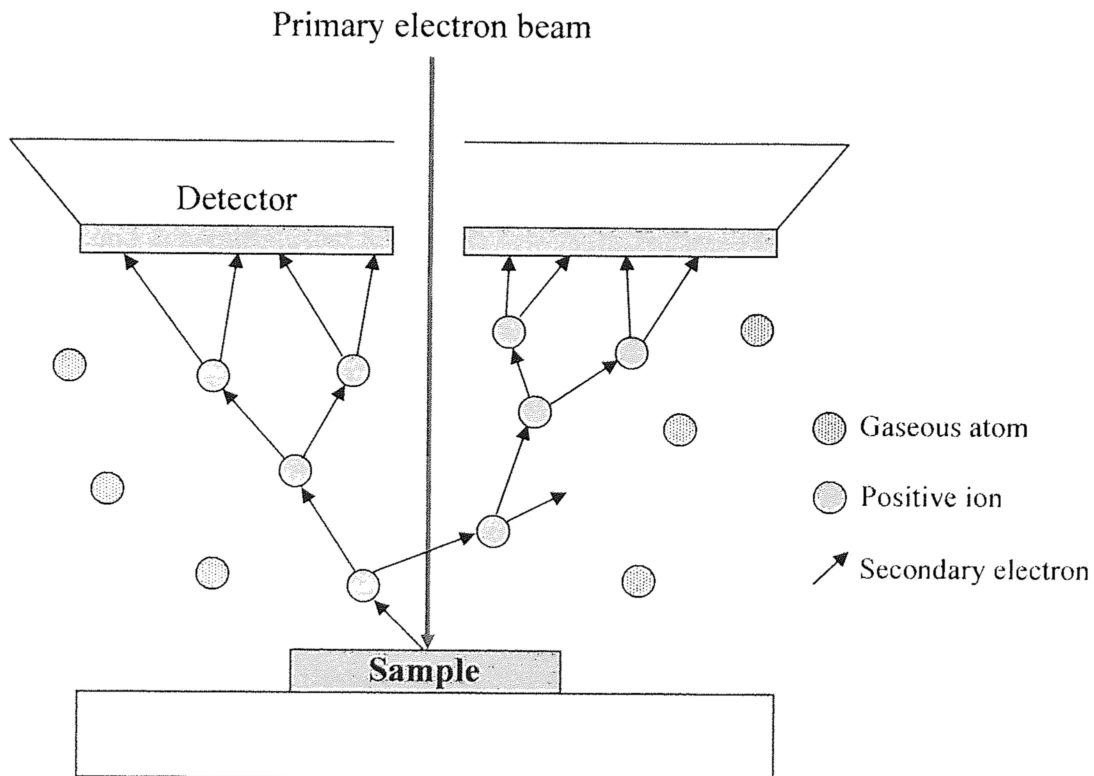


Fig. 7.1. Schematic representation of the gaseous amplification process. Primary electron beam generates secondary electrons (black arrows) from the sample, which in turn generate secondary electrons from vapour molecules present in sample chamber, leading to cascade amplification of the signal before reaching the detector.

Following initial observations in the late 70s (Robinson, 1975; Moncrieff et al., 1978; Moncrieff et al., 1979) and the subsequent development of the gaseous detection device (Danilatos, 1988; Danilatos, 1990), ESEM became commercially available towards the end of the 1980s. The main distinguishing feature of ESEM as compared to SEM is the presence of vapour (usually water) in the sample chamber, made possible by a system of differential pumping zones, which maintains the required high vacuum for the electron gun (10^{-6} torr) whilst allowing for partial vacuum in the sample chamber (10 torr) (Danilatos, 1993; Donald et al., 2000; Stokes, 2003).

The presence of water vapour molecules within the sample chamber plays a vital role in ESEM imaging (Fig. 7.1.). The primary electron beam generates secondary electrons from the sample surface, which then encounter vapour molecules, which in turn become ionised and generate further secondary electrons, which in turn encounter adjacent vapour molecules, and so on, leading to a "cascade" amplification of the signal before reaching the detector (Fig. 7.1.) (Stokes, 2003; Muscariello et al., 2005; Perrie et al., 2007b). Further, the positive ions resulting from the ionisation of the water vapour molecules are attracted to the negatively charged surface of the sample, compensating for the negative charge build up, hence precluding the need for conductive coating of the sample (Moncrieff et al., 1978; Stokes, 2003).

In addition, ESEM allows for the variation of parameters within the sample chamber, such as temperature, pressure and gas composition, permitting real-time studies into the effect of environmental changes on the sample (Perrie et al., 2007b).

Consequently, ESEM has previously been used to investigate several particulate formulations, including liposomes (Mohammed et al., 2004; Perrie et al., 2007b), niosomes (Vangala et al., 2006), microspheres (Elvira et al., 2004), thermo-responsive microspheres (D'Emanuele & Dinarvand, 1995), chitosan beads (Fu et al., 2006), polymeric surfactant micelles (Cao & Li, 2002), dendrimers (Sui et al., 2000) and colloidal latex dispersions (Donald et al., 2000).

Therefore, the aim of the work outlined in this chapter was to employ ESEM in the morphological characterisation of microsphere delivery systems for the novel sub-unit tuberculosis vaccine, Ag85B-ESAT-6, in order to elucidate the potential advantages of using such a technique over conventional SEM. Investigations involved studying the effect of various parameters involved in the formulation process on the morphology of microspheres produced, namely nature of emulsifying agent (PVA or Chitosan) and adjuvant (DDA or DDA+TDB), emulsion type (w/o/w or o/w) and effect of freeze-drying.

7.2. Morpological effect of microsphere formulation parameters

7.2.1. Emulsion stabiliser

Initial morphological investigations of Ag85B-ESAT-6 loaded PLGA+DDA microspheres studied the effect of the type of emulsifying agent employed in the double emulsion solvent evaporation process (w/o/w) (section 2.7.1.), with PVA (10%, w/v) (Figs. 7.2a-c) and Chitosan (0.75%, w/v) (Figs. 7.4a-d) being compared as in previous chapters.

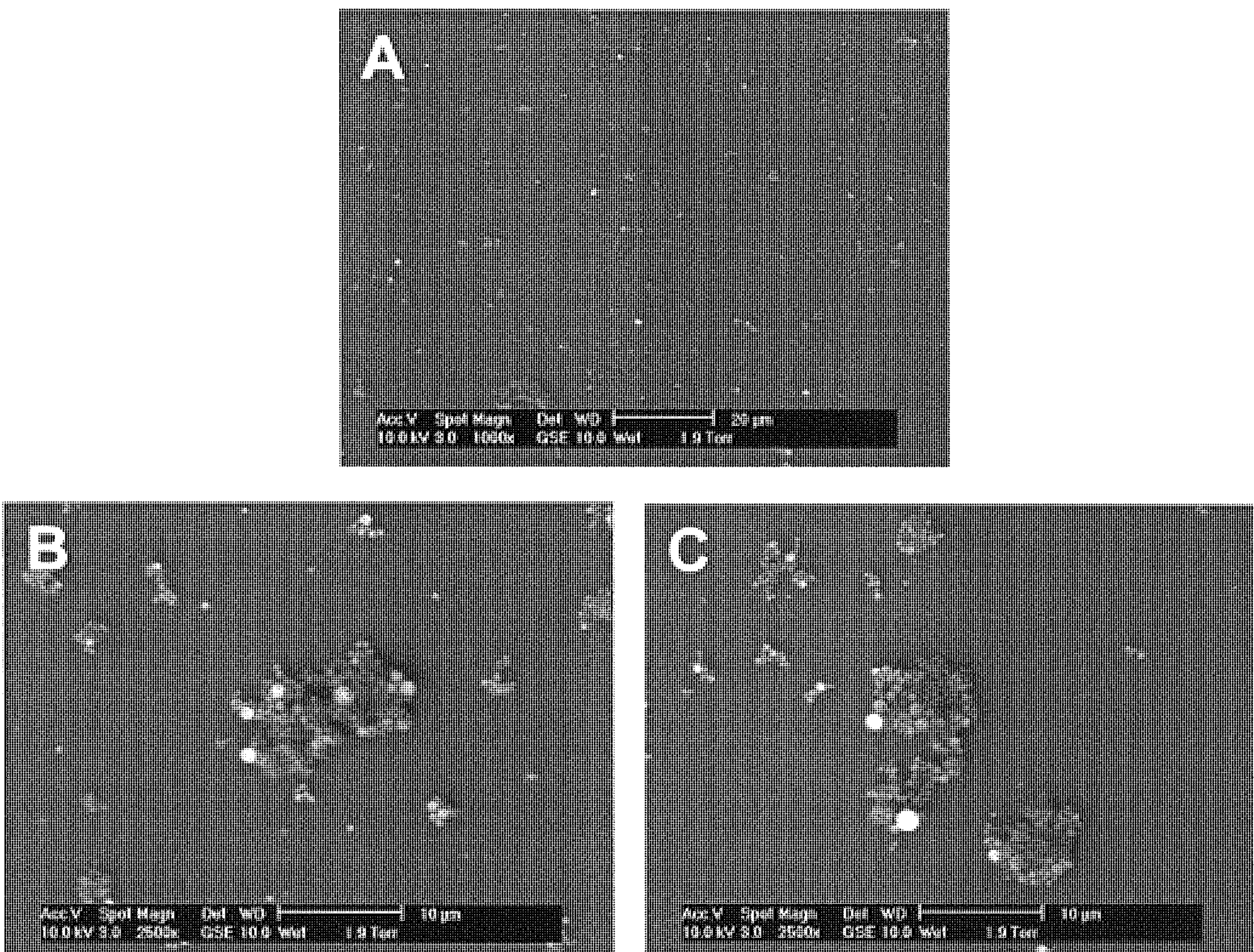


Fig. 7.2. ESEM micrographs of PLGA+DDA microspheres formulated via the w/o/w process, with PVA (10%, w/v) as emulsion stabiliser. Although relatively small individual particles were seen (**A**), clusters of microspheres were also evident (**B** and **C**).

PVA stabilised microspheres exhibited smooth, spherical particles, with diameters well within the desired sub-10 μm range (Figs. 7.2a-c) optimal for targeting antigen presenting cells (Audran et al., 2003). However, there was evidence of clusters of microspheres (Figs. 7.2b-c), with some reaching around 10 μm in diameter, whereas individual particles were generally less than 1 μm. The presence of such aggregates will not only influence the results of particle size analysis (Fig. 7.3.), but will undoubtedly impact on the function of the formulation *in vivo*, possibly forming depots as suggested previously (Chapters 5 and 6).

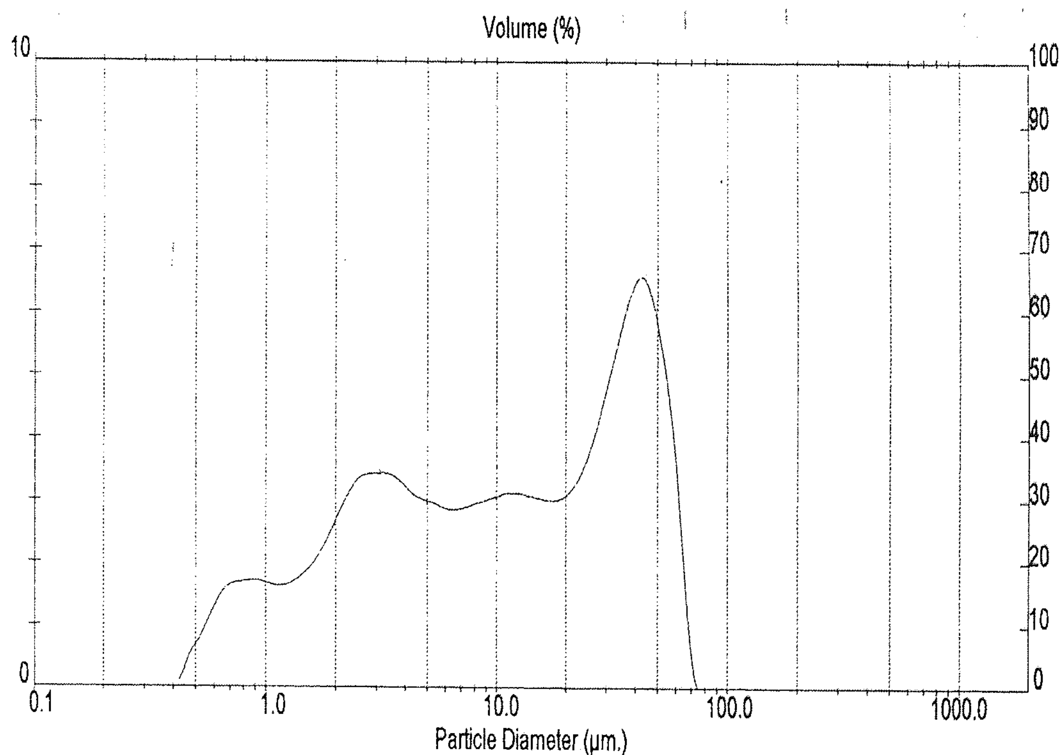


Fig. 7.3. Size distribution diagram of PLGA+DDA microspheres as derived from laser light scattering analysis. Microspheres consisting of PLGA and DDA were formulated via the w/o/w process (section 2.7.1.), with 10% (w/v) PVA as emulsion stabiliser. The multi-modal distribution derived by laser light scattering using a Mastersizer X (Malvern, UK), is likely to be as a consequence of aggregates present in the microsphere sample, since individual particles are generally around 1μm (as indicated by ESEM micrographs (see Fig. 7.2.)).

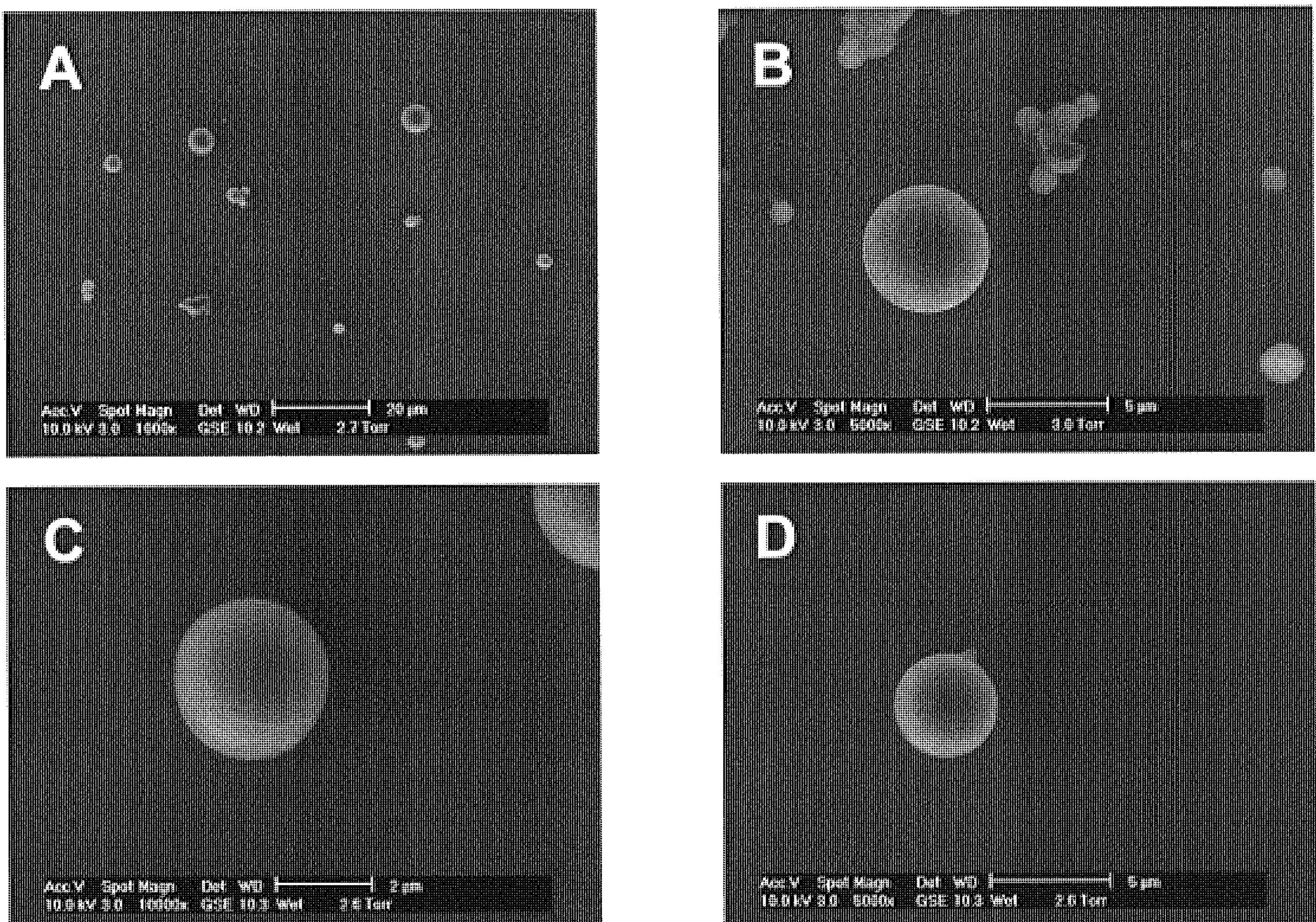


Fig. 7.4. ESEM micrographs of PLGA+DDA microspheres formulated via the w/o/w process, with Chitosan (0.75%, w/v) as emulsion stabiliser. More individualised particles were present as compared to the PVA stabilised formulation (Figs. 7.2a-c). These chitosan stabilised systems were also more robust when subjected to the electron beam, allowing for higher magnification (e.g. 10000x magnification (**C**)).

In contrast to the PVA stabilised microspheres, the use of chitosan as emulsion stabiliser produced more individualised particles, although there remained some evidence of clusters (Figs. 7.4a-d). Consequently, the average diameter of the particles imaged by ESEM correlated well to the volume mean diameters calculated by laser light diffraction (3.02 ± 0.1 μm).

Interestingly, the microspheres stabilised by Chitosan (Figs. 7.4a-d) were more robust when subjected to the electron beam, allowing high magnification without beam damage, whereas the PVA stabilised particles (Figs. 7.2a-c) were prone to fusion and beam damage, suggesting greater stability imparted by the Chitosan. This increased stability could possibly be attributed to crosslinking of the chitosan, or indeed simply a greater affinity between the chitosan and the microsphere surface as compared to PVA, leading to an enhanced boundary of protection surrounding the particles.

7.2.2. Method of preparation

Since the immunological effect of the method of preparation (i.e. w/o/w or o/w (section 2.7.1. and 2.7.2., respectively) was previously investigated (Chapter 5), ESEM analysis was undertaken to investigate any morphological differences between the microspheres produced, whilst continuing the comparison between the use of either PVA or chitosan as emulsion stabiliser. Initially, Ag85B-ESAT-6 loaded (via adsorption) PLGA+DDA microspheres formulated via the single emulsion method with PVA (10%, w/v) as emulsion stabiliser were investigated (Figs. 7.5a-b).

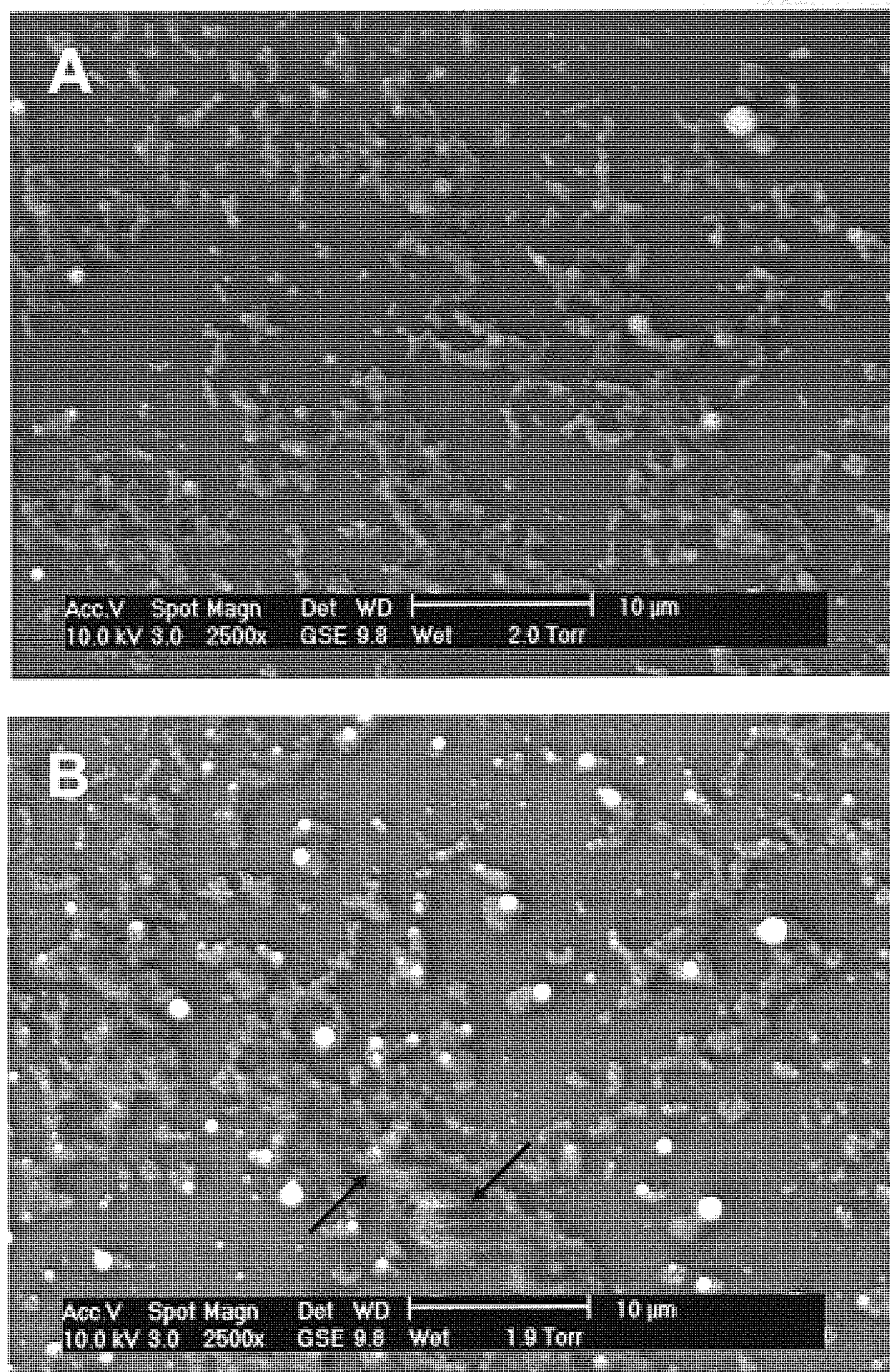


Fig. 7.5. ESEM micrographs of PLGA+DDA microspheres formulated via the o/w process, with PVA (10%, w/v) as emulsion stabiliser. Individual microspheres can be discerned within an adsorbed layer of "jelly", possibly of antigen. Arrows indicate areas of beam damage.

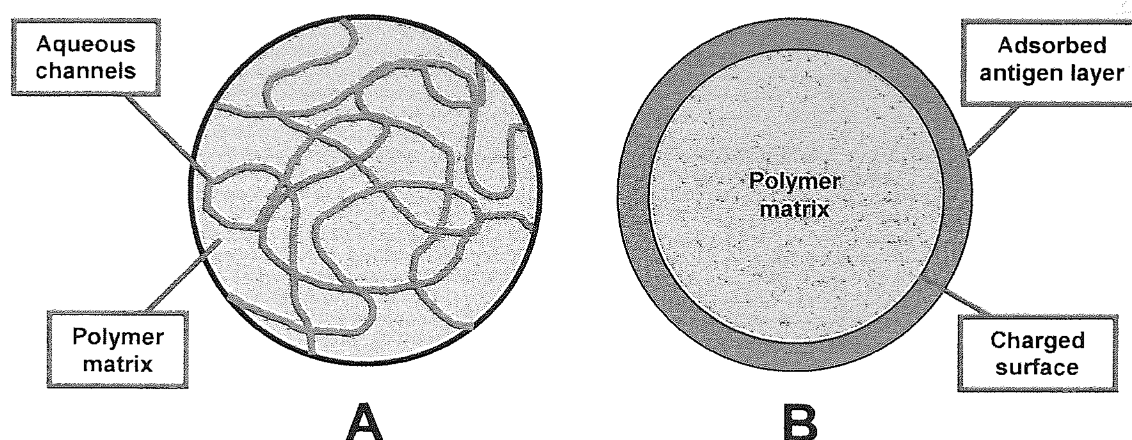


Fig. 7.6. Schematic representation of antigen loaded microspheres prepared by either the w/o/w (A) or o/w (B) process.

Although the diameters of the individual particles appear to be similar to the w/o/w method (Fig. 7.2a-c), the location of the antigen seems to be different depending on the method of preparation, as can be expected theoretically (Fig. 7.6.) Indeed, the ESEM images (Fig. 7.5a-b) showed microspheres present as agglomerates within a "jelly" of possibly the protein, although this would need to be confirmed by investigating antigen-free microspheres and will form the basis of future work. This observation was only made possible by the principles of ESEM imaging, allowing for the samples to be viewed in the hydrated and partially dehydrated state. As observed for the w/o/w formulation, the microspheres were prone to beam damage, as indicated on Fig. 7.5b.

When chitosan was employed as the emulsion stabiliser in the o/w process, there was again an obvious difference in morphology as compared to PVA stabilised particles (Figs. 7.7a-c). Although there were certain instances of clusters as for the PVA stabilised formulation (Fig. 7.7a), the majority of microspheres were individualised (Figs. 7.7b-c), similar to the w/o/w process.

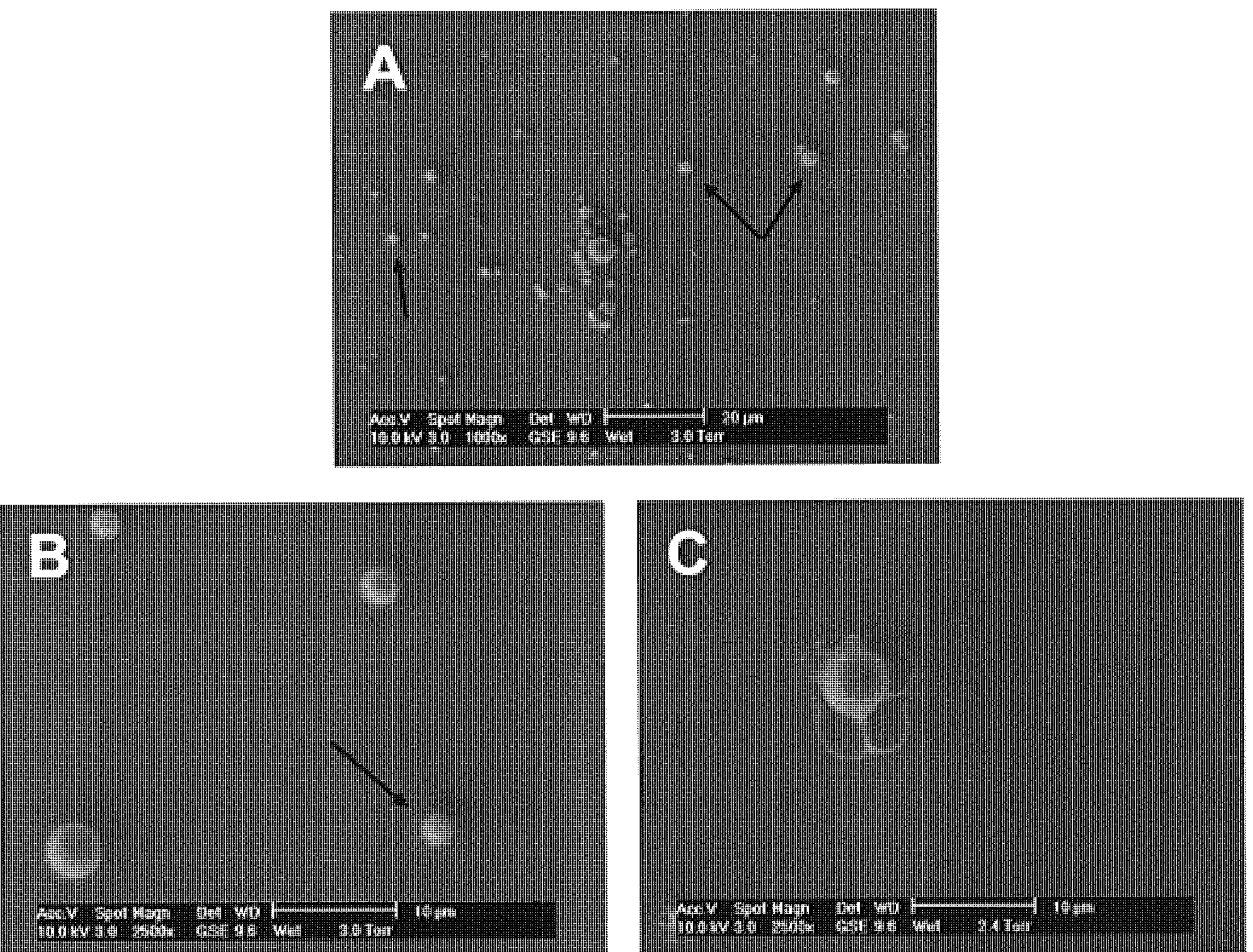


Fig. 7.7. ESEM micrographs of PLGA+DDA microspheres formulated via the o/w process, with Chitosan (0.75%, w/v) as emulsion stabiliser. Arrows indicate presence of an adsorbed layer, possibly of antigen, as a corona-like ring associated with the surface of the microspheres (**A** and **B**), which was seen to "bubble off" at reduced pressures within the sample chamber (**B** and **C**).

Additionally, the presence of a surface coating, possibly of antigen, was again distinguishable, although, in contrast to the PVA stabilised formulation, the chitosan stabilised microspheres appear to associate the antigen as a corona-like ring on the surface of the particles, which was then seen to "bubble off" as the pressure in the sample chamber was reduced (Figs. 7.7b-c). Further investigations of antigen-free microspheres would, however, be needed to confirm this. Again, this phenomenon was only made visible by the nature of the microscopic technique, since ESEM not only allows visualisation of the sample in the hydrated state, but also allows for the alteration of the environment within the sample chamber, in this case pressure.

7.2.3. Additional adjuvant

Further to the investigations into what may be considered external factors affecting microsphere morphology, the effect of additional adjuvant, TDB, within the microsphere formulation (w/o/w, 10% PVA (w/v)) was also studied (Fig. 7.8.).

Although not obviously different in terms of size and morphology, the microspheres containing TDB did appear to dry out as a sheet like arrangement as the sample was dehydrated within the sample chamber (Figs. 7.8.), with less evidence of cluster formation of particles as compared to the PLGA+DDA formulation (Figs. 7.2a-c). This observation may further implicate the role of TDB in stabilising the microspheres, as noted previously for both the microspheres (Chapter 4) and the liposomal formulation (Chapter 3 and (Davidsen et al., 2005)), possibly by inserting in between DDA molecules and thus reducing electrostatic interactions between the cationic headgroups through a

steric barrier. However, this sheet like arrangement may be an artefact of the way in which the sample is loaded or dehydrated within the sample chamber, although samples were standardised in terms of concentration. Nevertheless, there remained evidence of association between the particles, and further investigation would be required to confirm this hypothesis. Furthermore, studies have shown that microspheres incorporating TDB still require the addition of an emulsion stabiliser in order to form particles (results not shown), suggesting that the stability imparted by TDB is not as significant in microspheres as it is in the liposomal formulation.

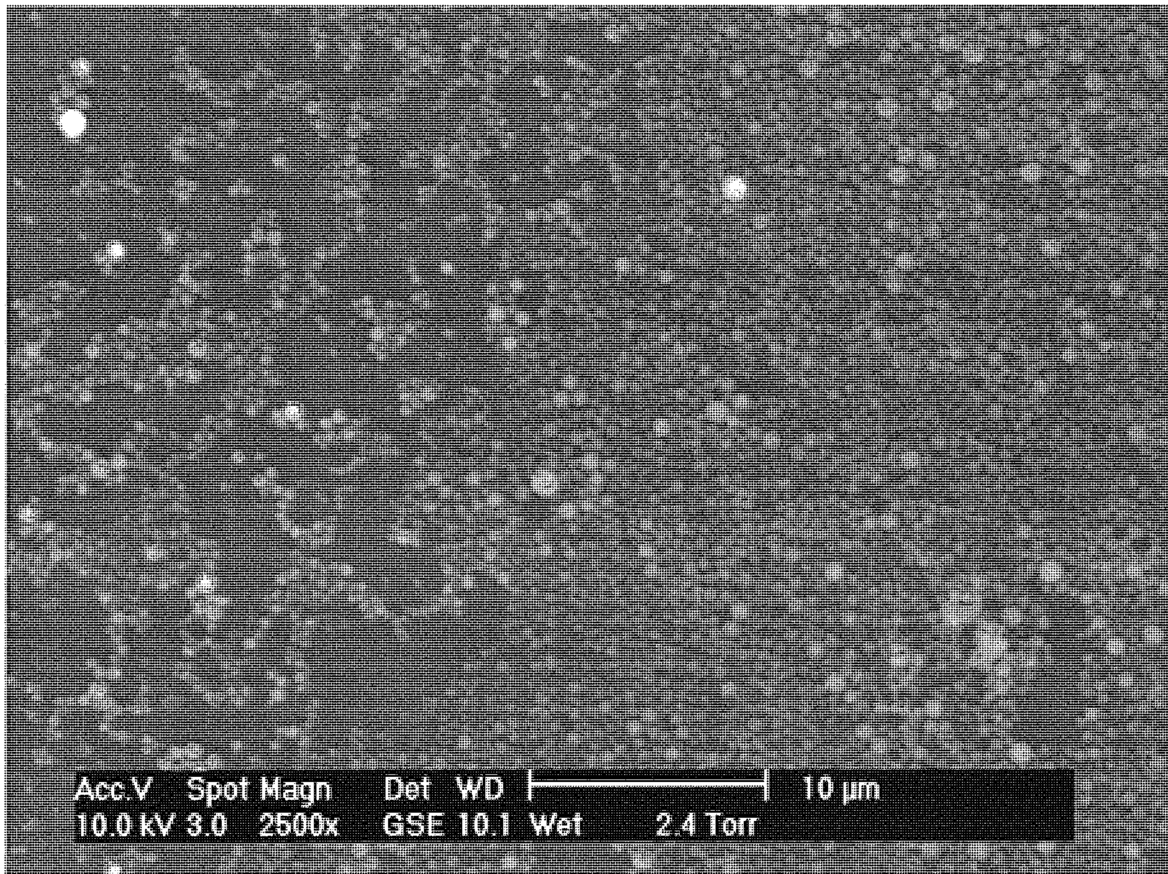


Fig. 7.8. ESEM micrograph of PLGA+DDA+TDB microspheres formulated via the w/o/w process, with PVA (10%, w/v) as emulsion stabiliser. In contrast to microspheres without the addition of TDB (Figs. 7.2a-c), the presence of TDB appears to reduce cluster formation, whilst exhibiting a sheet like arrangement upon dehydration.

7.2.4. Freeze-drying

Finally, ESEM was used to investigate the morphology of freeze-dried PLGA+DDA microspheres (w/o/w, 10% PVA (w/v)), with no cryoprotectants present during the lyophilisation process (Figs. 7.9a-b).

It is clear from the ESEM images that there is obvious aggregation of the microspheres following lyophilisation, which would explain the relatively large sizes obtained by laser light scattering measurements (Chapter 6). Indeed, the aggregates visible by ESEM tend to be around 10 μm, which is again in good agreement with previous particle sizing data (11.81 ± 1.5 μm). Interestingly, the aggregates formed are often rod-like structures, possibly due to the way in which the ice crystals form during the freezing stage of the freeze-drying process dictating the subsequent morphology, although this again may be an artefact of the imaging technique. However, the morphology of the individual particles and those present within the aggregates does not seem to be affected by the freeze-drying process, which lends weight to the applicability of such a process in enhancing shelf life whilst retaining structural integrity, although this would again need further investigation.

Once more, it is the nature of the ESEM microscope that enables the comparison between the samples pre- and post-lyophilisation, which would not have been possible in conventional SEM due to the drying of the sample in the necessary preparation of the samples prior to imaging, therefore only allowing the visualisation of the dried sample.

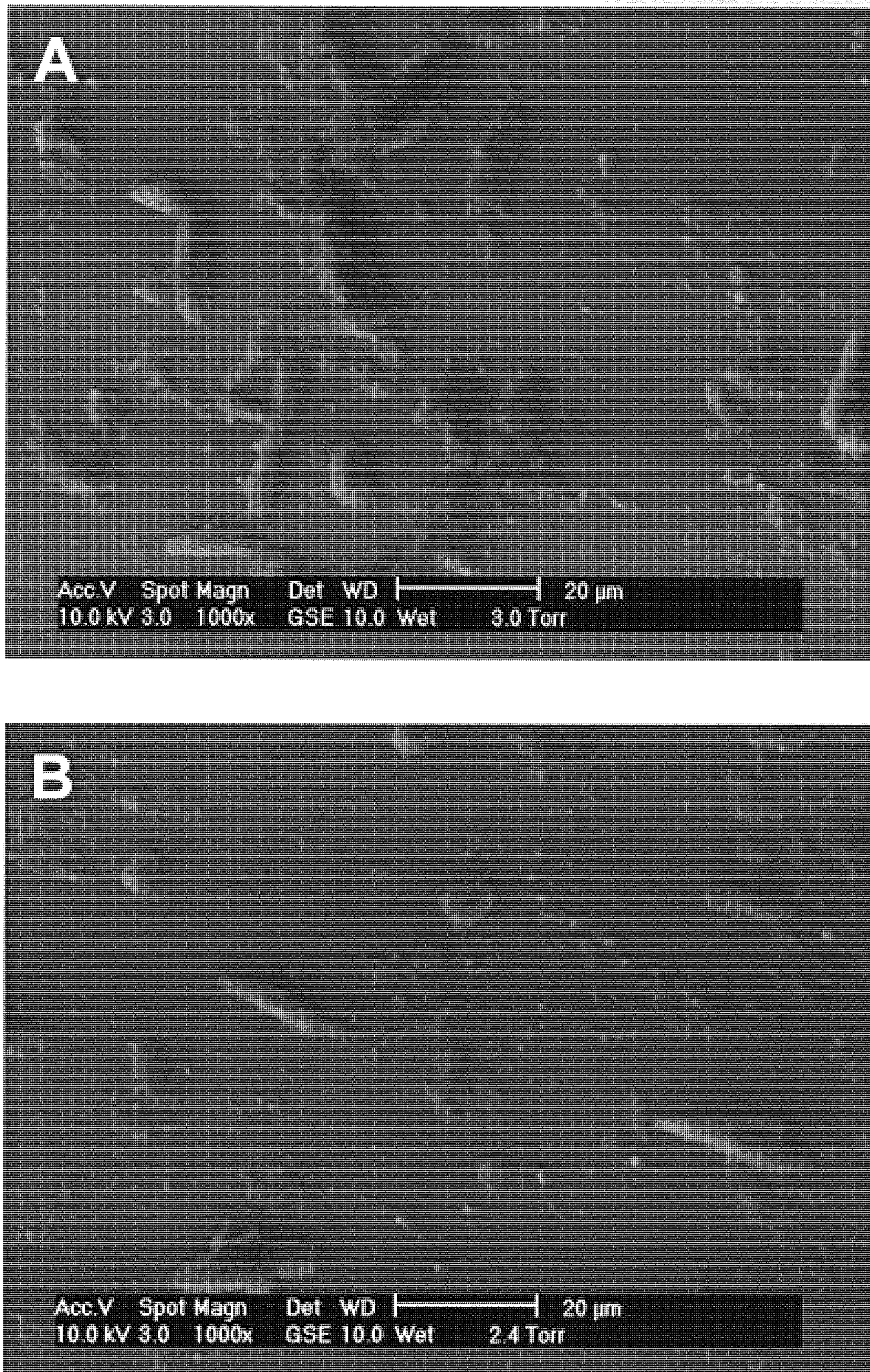


Fig. 7.9. ESEM micrographs of PLGA+DDA microspheres formulated via the w/o/w process, with PVA (10%, w/v) as stabiliser, following freeze-drying without cryoprotectant. There is clear evidence of aggregation of microspheres as a consequence of freeze-drying, with rod-like formations being seen.

7.3. Conclusion

Although not as sensitive to high vacuum as some biological samples, which require imaging in the hydrated state, and despite conventional SEM generally being considered adequate, ESEM has proved to be a valuable tool in the morphological analysis of polymeric microspheres.

In particular, the possible ability to visualise the location of the antigen – in the partially hydrated state – on the surface of the microspheres prepared by the single emulsion technique, and the subsequent "bubbling off" (Figs. 7.7a-c), would not have been possible using conventional, high vacuum, SEM. Indeed, little difference would have been evident in conventional SEM since the samples would have been dried prior to imaging.

In addition, the apparent difference in the robustness when comparing the emulsion stabilisers PVA and chitosan (Figs. 7.2a-c and 7.4a-d), which may be an indication of the stability of the particles, again may not have been possible under conventional SEM conditions. This further adds to the possible advantages of using ESEM, since this type of observation could be exploited as an alternative assay of formulation/stability relationships, as proposed for liposomes (Perrie et al., 2007b). Indeed, previous reports of controlled rehydration of liposome samples within the sample chamber led to visualisation of formation of vesicle structures through a "budding off" mechanism, whereas controlled dehydration revealed an enhancement in stability when drug was incorporated within the liposome bilayer (Mohammed et al., 2004; Perrie et al., 2007b).

Moreover, the altered morphology of the microspheres subjected to freeze-drying (Figs. 7.9a-b) would have been rendered less obvious using conventional SEM. Indeed, it would be interesting to use ESEM to investigate the effect of the addition of cryoprotectants of varying nature during the freeze-drying process (as in Chapter 6), and also the method of freezing during the freezing stage, to determine any morphological effects on the microspheres produced. Indeed, previous reports suggest that the temperature employed during the freezing stage can have a dramatic effect on the performance of cryoprotectants (Mohammed et al., 2007), although this will of course depend on the nature of the cryoprotectant and its effect on freezing point depression, as indicated elsewhere (Chapter 6).

Furthermore, it would be interesting to utilise the ability to alter the environment within the sample chamber in order to investigate the effect of temperature on the stability of microspheres of various compositions, or indeed the effect of varying the nature of the dispersion medium (e.g. pH, presence of salts) on the resultant morphology. Certainly, the ability of ESEM to analyse hydrated samples would allow for the study of morphology over time in order to evaluate the nature or extent of degradation of the formulation.

Chapter 8

General discussion and conclusions

8. General discussion and conclusions

Tuberculosis has been a persistent scourge of humankind that shows no sign of abating. Indeed, cases of TB are on the increase, and the emergence of HIV/AIDS has further exacerbated the global devastation caused by the disease. In addition, the appearance of drug resistant strains of TB aggravate the situation further, making treatment with antibiotics increasingly difficult, whereas the only currently licensed vaccine, BCG, has highly variable efficacy and fails to prevent the adult population against active TB (Fine, 1995; Colditz et al., 1995; Behr & Small, 1997).

Therefore, the development of an effective TB vaccine remains a global research priority, and one of the most promising vaccine categories is the use of sub-unit antigens. Aided by the complete genome sequencing of several pathogens – including *Mycobacterium tuberculosis*, the causative agent of TB (Cole et al., 1998) – sub-unit antigens are purified extracts of immunodominant cell wall components responsible for the immunogenicity of the related pathogen (Mora et al., 2003).

One such sub-unit vaccine antigen, the fusion protein Ag85B-ESAT-6, formed the basis of the work described in this thesis, and consists of the immunodominant antigen 85B (Ag85B) and the 6kDa early secretory antigenic target (ESAT-6), which have previously been identified as mycobacterial immunodominant epitopes (Olsen et al., 2001).

Nevertheless, although sub-unit vaccines offer several advantages in terms of safety, this is counterbalanced by the relative lack of immunostimulatory ability, necessitating the use

of adjuvants to confer vaccine efficacy. However, the vaccine adjuvants presently in use generally promote a weak humoural response, which is not desirable for diseases such as TB, where a cell mediated response is required (O'Hagan et al., 2001). Therefore, current research trends are towards adjuvants that initiate a more specific response, with emphasis on cell mediated immunity.

One particular class of adjuvants, which is a common feature of all the pharmaceutical vaccine adjuvant formulations currently being tested, including the work described in this thesis, is the use of particulate based formulations (Storni et al., 2005; Perrie et al., 2007a). Such systems can promote uptake, transport or presentation of the antigen to Antigen Presenting Cells, and include a wide variety of technologies, including liposomes, microspheres, ISCOMs, niosomes and emulsions to name but a few.

Alternatively, adjuvants can be derived from pathogens, and thus possess pathogen associated molecular patterns (PAMPs) required for stimulation of immunity. Again, several types of immunostimulatory adjuvant exist, including those dealt with in the context of this thesis, DDA, TDB and MDP.

Furthermore, it has been shown that the immunogenicity of a combination of subunit antigen plus immunostimulatory adjuvant can be even further enhanced if these are delivered by particulate carrier systems (Lendemans et al., 2005), which forms the basis of the rationale behind the work that has been described in this instance.

Initial investigations concentrated on the use of liposome formulations based on the immunostimulatory DDA, which self-assembles into vesicles in an aqueous environment (Brandt et al., 2000). Such systems have the advantage of being both particulate in nature and composed of adjuvant, although the lack of physical stability limits their use as vaccine adjuvants. Therefore, the initial focus of the work presented in this thesis was to resolve the stability issues associated with DDA-based liposome formulations, in order to produce a more clinically viable vaccine product.

Enhancement of the stability of DDA liposomes was achieved, first of all, through the optimisation of the nature of the aqueous medium in which the liposomes were prepared, avoiding the presence of salts that induce aggregation of the vesicles (Carmona-Ribeiro & Chaimovich, 1986). Further, through the development of an alternative method of preparation (i.e. the lipid hydration method (Section 2.2.)) and the addition of TDB to the formulation, stability issues relating to the liposomes were effectively resolved. Moreover, the addition of TDB not only stabilised the liposomes, but simultaneously improved the immunogenicity of the formulation considerably, initiating robust immune responses, supporting the theory that concomitant delivery of adjuvant and antigen within a particulate carrier can enhance the immunogenicity of the vaccine formulation. Indeed, the commendable ability of the DDA-TDB liposome formulation to stimulate both a cell mediated and antibody response makes this adjuvant advantageous for several disease targets, including infectious diseases such as TB, and studies are currently in progress in this regard (Vangala et al., 2007).

Following these investigations, further studies have led to the development of a freeze-dried, sterile product (Mohammed et al., 2006), lending further acceptability to such a formulation in the clinical setting, and the DDA-TDB formulation is now due to enter phase I clinical trials (Smith Korsholm et al., 2007).

As an alternative to the DDA-based liposome formulation, investigations then turned towards the development of a microsphere based formulation for the delivery of the Ag85B-ESAT-6 antigen, with PLGA as the polymer of choice due to its proven safety record and use in preparing controlled release delivery systems, which are licensed for use in humans in Europe and the USA (Furr & Hutchinson, 1985; Burns, Jr. et al., 1990). Initial studies centred on the optimisation of the parameters involved in the formulation process, with addition of the immunostimulatory DDA and TDB, either alone or in combination, also being investigated for physico-chemical and immunological effects.

As a consequence of these initial studies, the formulation comprising PLGA in combination with DDA was then carried forward as the lead microsphere formulation for further optimisation, due to its ability to induce enhanced protection against an aerosol challenge of TB as compared to the other microsphere formulations tested, further affirming the hypothesis that combined delivery of both adjuvant and sub-unit antigen within a particulate carrier can enhance immunogenicity. Surprisingly, the combination of both DDA and TDB within the microsphere formulation failed to enhance the immunogenicity further, despite the positive results for the liposomal systems. A possible explanation may be the relative stability of the formulations *in vivo*, since if the

formulation containing PLGA+DDA exhibits heterogeneously distributed sizes, with a tendency to form aggregates, this may in fact aid recognition and recruitment of macrophages, for example, to the site of injection. However, further investigations with the use of confocal microscopy or biodistribution studies, for example, would be required in order to elucidate any differences in the immunological mechanism of action of the formulations.

The further optimisation of the lead microsphere formulation (PLGA in combination with DDA) investigated the effect of the emulsion stabiliser employed in the formulation process (PVA, chitosan or CTAB), the type of formulation process itself (w/o/w or o/w), and also the addition of MDP as an alternative or additional adjuvant.

The nature of the emulsion stabiliser employed in the w/o/w process had a significant effect on the physico-chemical and subsequently the immunological characteristics of the microsphere formulation. Indeed, there was a clear correlation between the physical characteristics, particularly size and surface charge, and the relative ability to induce the desired immune response, with the CTAB stabilised microspheres – characterised by a negative surface charge and relatively large diameter – failing to show any immunological benefit as compared to naïve controls. In contrast, the formulations stabilised by both PVA and chitosan – characterised by a cationic surface charge – were able to enhance immune responses, with particular emphasis on cell mediated immunity through cell proliferation and cytokine secretion. However, of the two formulations showing positive immunogenicity, it was the PVA stabilised microspheres that continued

to show the most promise, with spleen cell proliferation and cytokine secretion comparable to that of the positive control DDA-TDB formulation. This apparent difference in ability to initiate the desired immune responses is again likely to be due to the physico-chemical characteristics of the particles produced, with the chitosan stabilised formulation showing increased mean diameter and also relative stability (as evidenced by ESEM) possibly through interaction of the chitosan with the microsphere surface, which may affect the relative accessibility of the antigen. In this regard, it would be beneficial to investigate the relative amounts of residual PVA and chitosan, and indeed CTAB, remaining in the formulation following washing, in order to clarify and quantify the presence (or absence) of an adsorbed layer of emulsion stabiliser on the surface of the microspheres that may affect formulation stability and interaction with cells of the immune system. Further, although it has been shown that PLGA can enhance MHC II presentation due to the acidic microenvironment on degradation (Storni et al., 2005), it has also been postulated that chitosan may enhance MHC I pathways, thus leading to restriction of MHC II antigen presentation (Strong et al., 2002), which may play a role in the relative immunological differences between the two formulations. However, further investigations into pH of the formulations upon degradation would help to clarify this.

The method of preparation of microspheres, either by the w/o/w or the o/w process (section 2.6.), allows for the comparison between formulations with either entrapped (w/o/w) or adsorbed (o/w) antigen. Results revealed that the method of antigen association does indeed have an effect on the type and level of immune response initiated, with the surface bound antigen formulation (o/w) showing a propensity towards humoural

immunity, with enhanced antibody production as compared to the entrapped antigen formulation (w/o/w), whilst generally failing to induce significant cytokine secretion as compared to naïve controls. Indeed, although factors such as particle size may have an impact on the type of immune responses initiated, it is likely that the method of antigen association, and thus presentation, will have a profound effect on the consequent processing of antigen by the cells of the immune system, since duration of antigen stimulation and persistence will affect the polarisation of the subsequent response (Storni et al., 2005). Once again, the use of techniques such as confocal microscopy would assist in elucidating the mechanism of action of the different formulation types, potentially revealing any variation in the interaction with the cells of the immune system.

In order to investigate the relative specificity of DDA as immunostimulatory adjuvant in this particular case, the use of MDP, either alone or in combination, was then studied. As for the combination of DDA and TDB within the microspheres, there was no advantage in terms of concomitant use of both adjuvants, suggesting a level of detrimental interaction, and it would be interesting to investigate this further. Conversely, the use of MDP alone in the microsphere formulation appeared to initiate a greater humoural response as compared to the microsphere formulation incorporating DDA, with significantly enhanced Ag85B-ESAT-6 antibody production. However, this enhanced antibody production was limited to the Th2 marker, IgG1, therefore indicating that MDP is less applicable in this particular instance as compared to DDA, which consistently promotes cell mediated immunity.

As stated previously, the development of a thermo-stable vaccine formulation with enhanced shelf-life has become a pre-requisite in the production of vaccines, particularly for the developing world (Christensen et al., 2007). Therefore, the lead microsphere formulation – PLGA in combination with DDA, prepared by the w/o/w process – was thus the subject of freeze-drying studies in order to produce a more stable product. Initial results suggested that the use of cryoprotectants was essential in stabilising the formulation during freeze-drying, leading to the investigation of several cryoprotectants, encompassing the traditional sugar-based (sucrose and trehalose) and the alternative amino acid-based (lysine, arginine, histidine, glycine, valine and leucine) cryoprotectants. Results revealed that choice of cryoprotectant can greatly influence resulting particle characteristics, including aggregation and surface charge, which, as highlighted by the results described above, are likely to have an influence on the resulting immunological efficacy.

Of the traditional sugar-based cryoprotectants, sucrose was shown to effectively prevent aggregation of the microspheres, whilst having little effect on the surface charge of the particles, suggesting that vitrification was the main function of this particular cryoprotectant. In terms of the use of amino acids as cryoprotectants, those possessing a basic side chain (lysine, arginine and histidine), although able to reduce aggregation as compared to no cryoprotectant, did appear to interact with the microsphere surface as evidenced by the significant reduction in zeta potential, which again is likely to affect immunological efficacy. However, further investigations would be required to determine

if this interaction persists over a range of pH whereby the basic group of the amino acid is both protonated and non-protonated, thereby clarifying the origin of this interaction.

Nevertheless, the use of amino acids with hydrophobic side chains (glycine, valine and leucine) offered protection against aggregation, whilst also maintaining the zeta potential of the microspheres, suggesting that particle isolation, rather than direct interaction, is the main function of such cryoprotectants. In addition, the amino acids bearing hydrophobic side chains were able to act as effectively as sucrose as cryoprotectants, although at much lower concentrations.

Therefore, with regards continuing this work, it would be necessary to further analyse formulations freeze-dried in the presence of both sugars and amino acids, at the respective effective concentrations, in terms of: residual water content, using thermo-gravimetric analysis (TGA), with the acceptable limit being 3% (w/v) (Mohammed et al., 2007); effect on transition temperature of the formulations, by differential scanning calorimetry (DSC), in order to determine the extent of interaction between microsphere and cryoprotectant; *in vitro* antigen release profiles to identify any potential effect on microsphere structural stability during freeze-drying; viscosity of the formulations following rehydration in order to assess ease of administration; and finally long-term stability at various conditions in order to confirm the suitability of a freeze-dried product from a practical perspective. Moreover, in order to further enhance the suitability of the microsphere formulation within a clinical setting, γ-sterilisation, as for the liposomal systems, would be a viable option in terms of producing a desirable vaccine product.

Also, SDS-PAGE analysis would be useful to investigate the stability of the antigen throughout the whole microsphere formulation process, from bench to clinic, in order to determine if any damage occurs (due to exposure to solvent, shear stresses, freeze-drying, sterilisation etc.) which may impair the resulting immunogenicity.

Finally, environmental scanning electron microscopy (ESEM) was investigated for its potential to morphologically study the microsphere formulations. In contrast to conventional SEM, ESEM allows visualisation of samples in their hydrated (at least partially) state, without the need for prior sample preparation (i.e. conductive coating). Indeed, in this particular instance, ESEM allowed for the visualisation of the relative rigidity of microspheres stabilised by chitosan as compared to those stabilised by PVA, allowing for relatively high magnification whilst avoiding fusion of particles or beam damage as a consequence of the electron bombardment. Furthermore, ESEM facilitated the observation of antigen adsorbed to the surface of microspheres prepared by the o/w process, either as a jelly or a corona-like ring around the particles, which was seen to "bubble off" as the pressure in the sample chamber was reduced.

However, it would be necessary to carry out further ESEM studies with antigen free microspheres in order to confirm that the layer was in fact antigen, and not simply an artefact of the imaging technique. Additionally, ESEM analysis of microspheres freeze-dried in the presence of various cryoprotectants (i.e. sugars and amino acids) would give a further indication as to the extent of the detrimental effect, if any, of the freeze-drying process on particle morphology.

The results detailed in this thesis go some way to achieving the primary objective of the project, that is the development of an effective delivery vehicle for the sub-unit vaccine antigen, Ag85B-ESAT-6. Indeed, it has been shown that the use of a particulate delivery system, whether liposomal or microsphere-based, can improve the efficacy of the poorly immunogenic sub-unit antigen. Moreover, the concomitant delivery of adjuvant and antigen in association with a particulate carrier can further improve the immunogenicity of the vaccine, whilst achieving a polarised immune response desirable for the particular disease target through appropriate selection of materials.

Encouragingly, following the initial investigations presented herein, the DDA/TDB liposome formulation has now been further optimised and taken forward into clinical trials, and appears to be a promising adjuvant system for several antigens and disease targets.

With regards the microsphere formulation, although not as effective as DDA/TDB liposomes, the positive immunological results suggest a role for such formulations as potential alternatives if any problems were to be encountered with the use of the DDA/TDB formulation. Furthermore, there remains several formulation variables that may be investigated in order to attempt to improve the immunological the efficacy of the microsphere formulation, including the use of alternative polymers (e.g. PCL, alternative ratios of PLGA), adjuvants and alternative preparation techniques (e.g. spray-drying, nano-precipitation), since the investigations carried out within the remit of this thesis concentrated on altering aspects of the formulation process, rather than the formulation

itself. However, selection of the appropriate variables will rely heavily on the particular application, and consideration of physico-chemical parameters, as highlighted throughout, is an essential aspect in the choice of variable investigated.

As for the use of particulate delivery systems and sub-unit vaccines in general, these will undoubtedly play a major role in future vaccine development, particularly with the trend towards improved vaccine safety as a consequence of the negative publicity and increased need within immunocompromised patient groups, as well as the ability to produce a product which can be cost-effective and practical in terms of manufacture and distribution.

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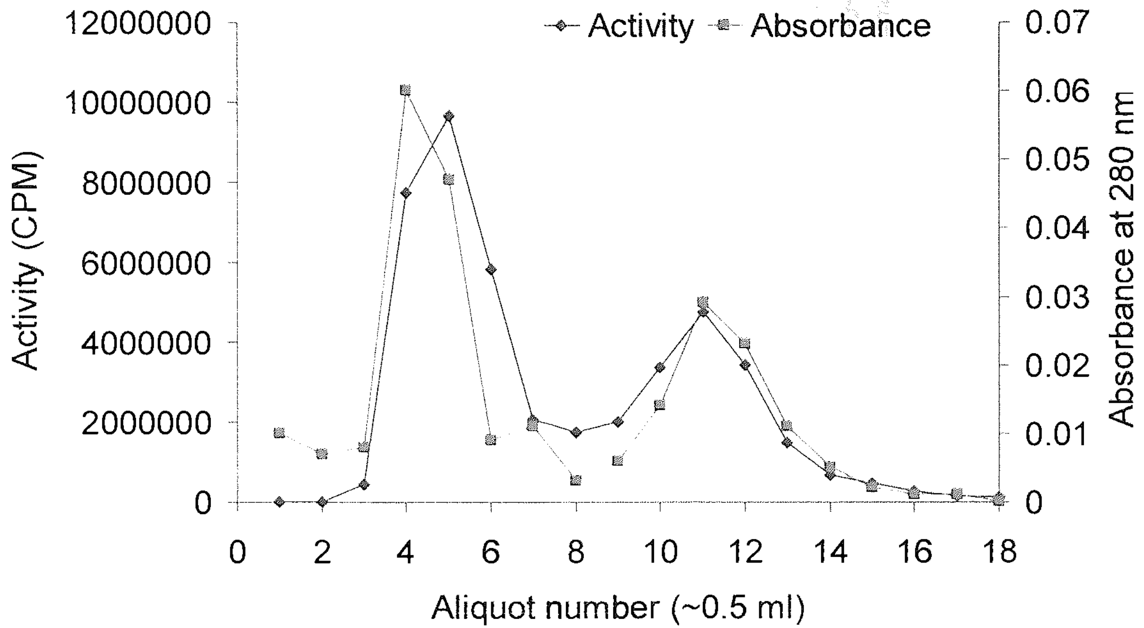

Fig. A.1a. Determination of γ-radiation activity and UV absorbance of Ag85B-ESAT-6 antigen following ^{125}I radiolabelling. Ag85B-ESAT-6 antigen was radiolabelled as described in section 2.5., and then passed through a Sephadex G-75 column. Aliquots (~0.5 mL) were collected and measured for γ–radiation and UV absorbance to confirm the presence of antigen.

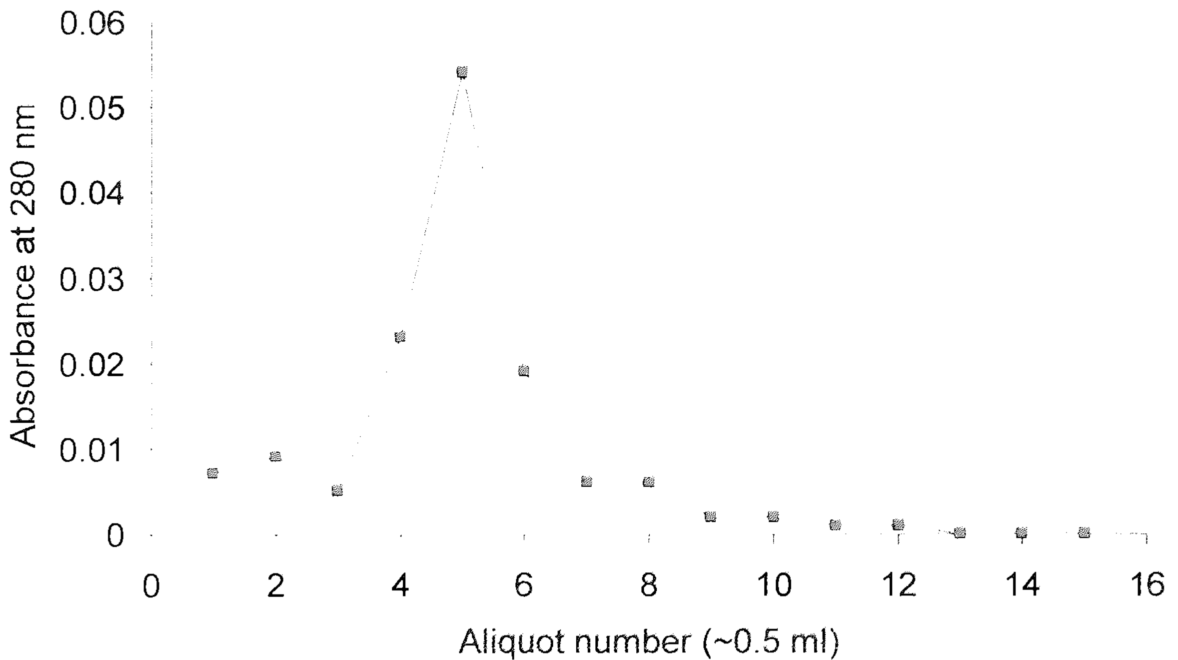

Fig. A.1b. Determination of UV absorbance of Ag85B-ESAT-6. Ag85B-ESAT-6 was passed through a Sephadex G-75 column. Aliquots (~0.5 mL) were collected and measured for UV absorbance (@ 280 nm) in order to identify the protein peak following ^{125}I radiolabelling (Fig. A.1a.).

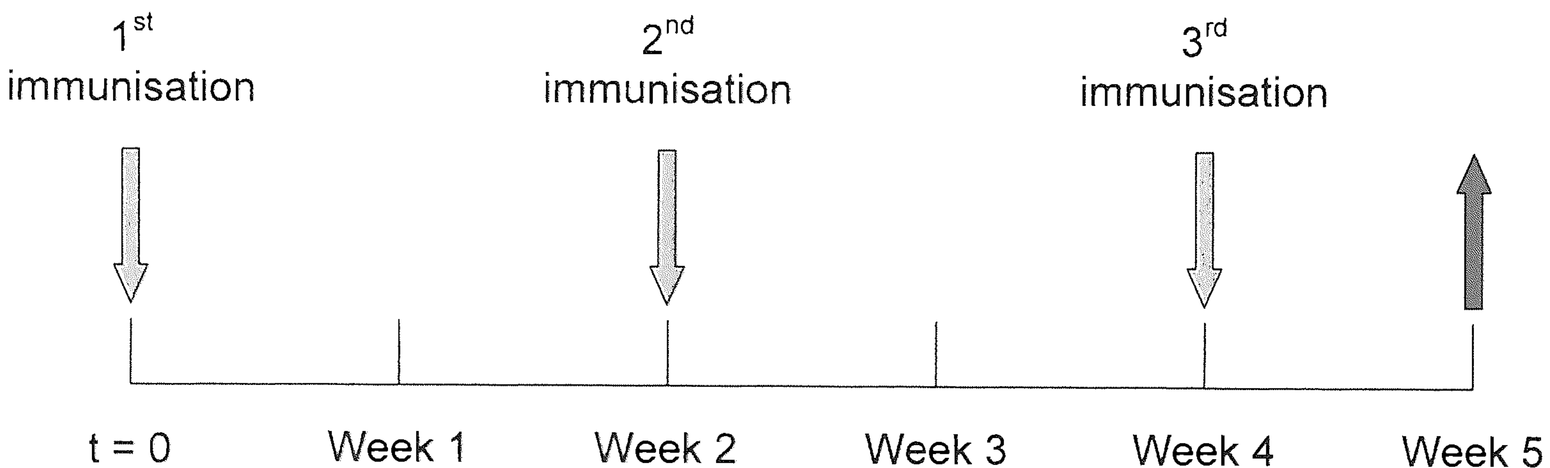

Fig. A.2. Immunisation schedule for DDA and DDA-TDB liposomes as performed by Statens Serum Institut. Female C57BL/6 mice, 8 to 12 weeks old, received three vaccine doses (containing 2 μg Ag85B-ESAT-6, 250 μg DDA and 50 μg TDB, where appropriate) at intervals of 2 weeks. One week after the third immunisation, the specific immune response of the blood cells was investigated by re-stimulation with Ag85B-ESAT-6 *in vitro* and subsequently measuring splenocyte cultures for secretion of the cytokines IFN-γ and IL-5 as indicators of a Th1 and Th2 response, respectively.