Particulate Carriers for Vaccines

Thesis submitted in accordance with the requirements of the School of Pharmacy, University of London for the degree of Doctor of Philosophy

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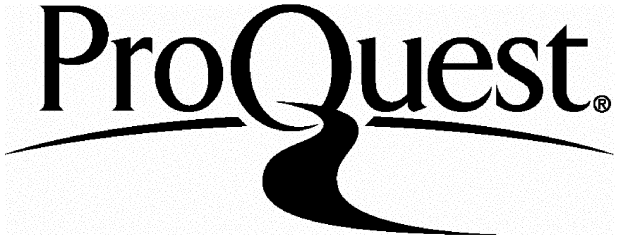

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Abstract

Soluble antigens and antigens synthesized by recombinant DNA techniques are weakly immunogenic and need protection against passage through the mucosal epithelium. Encapsulation of antigens into polymeric carriers is one of the techniques developed for delivering these agents. However, this technology needs further improvements to enhance the immune responses and stabilization of the antigen within the carriers. The incorporation of adjuvants to enhance immune responses is well known, and therefore incorporation of adjuvants with the polymeric carriers could also be beneficial. The focus of this study, therefore, was to co-encapsulate known and unknown adjuvants with polymeric particles.

Polymeric particulate carriers were prepared using the solvent evaporation or spray drying technique. The biodegradable polymers selected were polylactic acid and polycaprolactone. Diphtheria toxoid (DT) and hepatitis B surface antigen (HBsAg) were used as model antigens. The adjuvants used for co-encapsulation with antigens were penetration enhancers and mineral compounds. These adjuvants were either mixed with the polymer in the organic phase or in the internal phase during the preparation of the particles. The penetration enhancers used in the study were tocopherol acetate (TA), tocopherol nicotinate (TN), D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), cholic acid, lithocholic acid and melittin. Mineral compounds such as alum, zinc sulphate and zinc oxide were also co-encapsulated to evaluate their effect on the physicochemical properties and adjuvancy of the carriers. As surface charge plays an important role in the particle uptake, chitosan was used as an external phase stabilizer in some formulations to impart a positive charge on the particles. The penetration enhancers used here have not been used to co-encapsulate antigens in polymeric particles before, and there is not much data available for co-encapsulation of mineral compounds. The formulations were characterized with respect to size, surface charge, encapsulation efficiency, uptake in the cell lines and toxicity. These formulations were also evaluated in mice for antibody responses and cytokine analysis.

Incorporation of tocopherol derivatives into the particulate formulations significantly increased the encapsulation efficiency of the antigen. The *in vitro* toxicity studies showed that these formulations were non-toxic. *In vivo*, the HBsAg-loaded particles containing TPGS significantly improved the serum antibody levels when compared with free HBsAg. TA and TN also improved the serum antibody levels when administered intramuscularly and intranasally. However, the intramuscular responses were higher than the intranasally administered formulations. DT-loaded particles containing TPGS significantly improved the antibody levels.

Co-encapsulation of zinc sulphate and alum in nanoparticles improved the encapsulation efficiency of HBsAg. Sizes were in the range of 300 to 500nm for these formulations. The serum immune responses of particles containing zinc sulphate were equivalent to particles containing alum. However, the intranasal route elicited much lower antibody responses when compared with the intramuscular groups. Particles coated with chitosan significantly improved the antibody levels.

The co-encapsulation of bile salts (cholic acid or lithocholic acid) and melittin along with HBsAg did not have any effect on the encapsulation efficiency. Particles containing bile salts had a higher cellular uptake compared with particles without bile salts. Particles containing bile salts or melittin significantly improved the antibody levels when compared with free antigen and antigen plus cholera toxin B.

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List of Abbreviations

µg	microgram
µl	micro litre
APC	antigen presenting cell
BCG	bacilli Calmette- Guérin
BSA	bovine serum albumin
CpG	cytidine-phosphate-guanosine motifs
CTAB	cetyltrimethyl ammonium bromide
CT	cholera toxin
CTB	cholera toxin B
DC	dendritic cell
DCM	dichloromethane (methylene chloride)
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DT	Diphtheria Toxoid
EDTA	ethylenediaminetetraacetic acid
EPI	Expanded programme of immunization
FCS	foetal calf serum
HBsAg	hepatitis B surface antigen
HIV	human immunodeficiency virus
IL-4	Interferon 4
IL-6	interferon 6
IFN-γ	interferon gamma
i.m	Intramuscular
i.n	Intranasal
J774A.1	mouse monocyte/macrophage cell line
kcps	kilo counts *per* second
kDa	kilo Daltons
LD_{50}	lethal dose, 50%
M	Mole
mA	milliamps
ml	millilitre
mM	milli mole
m/m	mass *per* mass

mV	millivolt
m/v	mass *per* volume
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MVD	mean volume diameter
$NaHCO_3$	sodium hydrogen carbonate
Na_2HPO_4	sodium phosphate (dibasic)
Na_2SO_4	sodium sulphate
NaCl	sodium chloride
NaOH	sodium hydroxide
OVA	ova serum albumin
PBS	phosphate buffered saline
PCL	Poly caprolactone
PCS	photon correlation spectroscopy
PEG	polyethylene glycol
PEI	polyethylenimine
pg	picograms
PLGA	poly(dl-lactic acid-co-glycolide)
PLG	poly glycolide
psi	pounds *per* square inch
PVA	Poly (vinyl alcohol)
PVP	Poly (vinyl pyrrolidone)
rpm	revolutions *per* minute
s.c	subcutaneous
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TA	Tocopherol acetate
TN	Tocopherol nicotinate
TPGS	D α-tocopheryl polyethylene glycol 1000 succinate
TT	Tetanus Toxoid
UV	ultra violet
V	volts
v/v	volume *per* volume
w/o/w	water-in-oil-in-water
WHO	World Health Organization

Chapter 1

Introduction

1. Introduction

Vaccination is one of the major achievements of modern medicine. As a result of vaccination, diseases such as polio and measles have been controlled and small pox has been eradicated. It consists of stimulating an active immunologic protection by prior infection with a weaker or related version of lower inoculum of a pathogen. However, despite these successes there are still many microbial diseases such as malaria and AIDS, that cause tremendous suffering because there is no vaccine or the vaccines available are inadequate. The research in the field of vaccinology aims to develop the ideal vaccine which should be safe, economical and effective after a single-dose, stable as well as delivered non-invasively to increase compliance (as proposed by the Children's Vaccine Initiative at the Declaration of New York in September 1990).

1.1. History of vaccines

The term vaccination is derived from *vacca*, the Latin word for cow. The ancient Chinese protected against smallpox by the process of variolation, in which small quantities of scabs from a lesion of an infected person were intranasally inoculated. The process was promoted by Lady Mary Montagu, who had observed variolation in Turkey in the early 1700s.

In 1796 Edward Jenner used cowpox as a related immunogen against smallpox and by testing the procedure scientifically, established the scientific precedent for using a related but less dangerous pathogen to induce immune responses that are cross-reactive against the more virulent pathogen. During those days milkmaids who developed cowpox lesions were observed to be resistant to smallpox. This observation fascinated Jenner who collected the cowpox lesion on the hand of milkmaid Sarah Nelmes and inoculated a young boy, James Phipps. Following Phipps resistance to smallpox on subsequent exposure Jenner started experimenting with other children, with the same outcome. Initially, vaccination was performed with cowpox virus, but over time, vaccinia virus, the origin of which is unknown, became the preferred virus for vaccination (Fenner *et al*, 1998).

Nearly a century elapsed before Louis Pasteur disproved the theory of spontaneous generation and advanced the germ theory of infection. Pasteur was able to prove that

protection against a disease could be afforded by the infection of weakened germs which cause silent and relatively harmless infections. At the turn of the 19th century, two human virus vaccines (Jenner's small pox vaccine and Pasteur's rabies vaccine) as well as three human bacterial vaccines (typhoid, cholera and plague) existed (Liu, 1998).

The success of smallpox vaccine is phenomenal, it was the first and so far the only vaccine which has been able to eradicate smallpox from all over the world. Polio vaccination is also very successful in attaining the eradication targets so far. The World Health Organization (WHO) has initiated many projects for mass immunization of polio which has resulted in virtually eliminating the virus from the industrial nations and considerably reducing the worldwide prevalence of this potentially devastating pathogen. Similarly the success of diphtheria, *Haemophilus influenzae* type b, hepatitis, measles, rubella and tetanus is also remarkable. There are still no vaccines for diseases such as malaria or human immunodeficiency virus (HIV) and there is a need for better vaccines for diseases such as tuberculosis (TB) and hepatitis B. The requirement for multiple injections of the currently licensed hepatitis B, diphtheria and tetanus vaccines makes the task of health workers tedious because of the economics and logistics.

Following the 19th century, the emergence of new vaccines has been slower with much emphasis on improvements of existing vaccines and extending the number of vaccine-preventable diseases. The pharmaceutical industry is constantly looking for new ways to improve marketed therapeutics. One of the options is to develop new formulations of currently marketed therapeutics for a novel delivery route. In recent years, there has been a tremendous growth of biotechnology, which has resulted in the demand for improved methods for the delivery of protein pharmaceuticals. Traditionally the drug development of proteins has relied on parenteral injections, but in the future, the success of protein drug development will depend upon innovative methods for delivery (Cleland *et al*, 2001).

1.2. Traditional vaccines

Following the discoveries of the new vaccines, numerous attempts were made to develop safer and more efficacious vaccines. However, traditionally vaccines are classified into four categories, which are summarized as follows (Bloom, 1989; Weiner and Kennedy, 1999):

1. Live attenuated vaccines: the oldest method of vaccination introduced by Jenner. These are the most efficacious vaccines and give the best immune responses (both humoral and cell-mediated immune responses are induced). These vaccines are obtained by treating the viruses with physicochemical processes, while the bacteria are grown in poor culture. However, they may not be as safe due to the potential risk of reversion to virulence. Examples: Polio Sabin, Bacillus Calmette-Guerin (BCG), Measles, Mumps, Rubella.

2. Whole killed vaccines: introduced 100 years later by Pasteur. The pathogens are inactivated by heat or chemicals (formaldehyde, acetone), but they are still able to induce specific immune responses. The advantage of these preparations is their stability and capacity to induce antibody responses while the major drawback is their inability to produce cell-mediated immune responses. Examples: Pertussis whole cell, Hepatitis A.

3. Whole inactivated vaccines: the vaccine consists of a toxin, which is treated at high temperature in order to lose its toxicity. They induce only humoral responses. Examples: Diphtheria toxoid, Tetanus toxoid.

4. Purified subunit vaccines and recombinant subunit antigens: the vaccine is only made of a portion, which can stimulate the specific antibodies. They are considered safer than conventional vaccines. Due to their low immunogenicity they need to be administered with adjuvants to elicit immune responses. Examples: Acellular Pertussis, Hepatitis B.

Although all these vaccines have been successful, it is also observed that some diseases which are believed to be under control or eradicated can resurge if the immunization program is relaxed or circumstances otherwise change to favour the spread of infection or reduction in the resistance.

The currently used vaccines have several drawbacks: they are expensive, injected, mostly multivalent and unstable, resulting in high drop out rates from the schedule program of immunization in developing countries. The current strategies in vaccine delivery and targeting aim to increase immunogenicity (especially to sub-unit antigens), develop a mucosal delivered vaccine and create single-dose vaccines (Babiuk *et al*, 2000). Nowadays most of the research is focused on DNA-based vaccines (viral and non-viral). These vaccines induce immune responses against antigens synthesized *in vivo* after direct introduction of the DNA's encoding sequence. Since the protein antigen is produced *in vivo*, these vaccines should be able to induce both protective cellular and humoral immune responses (Davis, 1997).

1.3. Immunological aspects

The immune response occurs in two stages. The primary immune response occurs when the body is exposed to a new antigen, while the secondary immune response occurs to any further exposure. During the primary response along with the activated lymphocytes, the body also produces a group of long lived T and B lymphocytes called memory cells. Thus, in the case of a second exposure, the immune response is faster, more massive and longer and is often able to eliminate the antigen before the disease occurs. The goal of vaccination is to elicit a primary immune response thus generating an immunological memory in order to prevent the disease in case of infection (Davis, 1997).

1.3.1. Innate and Adaptive immunity

'Immunity' refers to the ability of the host to resist the attack of microbes that are harmful to the host. The immune system is classified into two subsystems, the innate immune system and adaptive immune systems. The innate immune system is nonspecific response whereas the adaptive immune system is very specific or in other words innate immunity can be called as 'natural immunity' or 'innate resistance' and adaptive immunity as 'acquired immunity'. The stimulation of the innate immune system has an important role in the evolution of the adaptive immune response (Hoebe *et al.*, 2004; Lavelle *et al.*, 2005).

The innate immunity provides the first line of defence when the host is attacked because adaptive immunity is slower as it is based on receptors that are generated by somatic

mechanisms. These somatic mechanisms generate various antigen receptors having random specificities which are clonally distributed on two types of lymphocytes: T and B cells, thus providing specificity and long-lasting immunological memory. The innate immunity also leads to a rapid burst of inflammatory cytokines and activation of antigen-presenting cells (APCs) such as macrophages and dendritic cells. This conditions the immune system for subsequent development of specific adaptive immune responses (Medzhitov and Janeway, 2000; Gourley *et al.*, 2004).

The innate immune receptors are germline encoded and their expression might be restricted to particular cell types but they are not clonally distributed. These innate recognition sensors are called pattern-recognition receptors (PPRs) and include the Toll-like receptor (TLR) family (Medzhitov *et al.*, 1997). The genes encoding the innate receptors show various degrees of polymorphism. The innate receptor ligands can also be polymorphic such as the natural killer (NK) receptor-ligand pairs (killer cell immunoglobulin-like receptor (KIR)–major histocompatibility complex (MHC) class I or NKG2D–NKG2D ligands). The distinctive features of innate versus adaptive immunity are summarized in the Table 1.1.

Table 1.1: The distinctive features of innate versus adaptive immunity (adapted from Vivier and Malissen, 2005).

	Innate immunity	**Adaptive immunity**
Receptors	Germline-encoded	Antigen receptors are products of site-specific somatic recombination
Distribution	Subset-specific but nonclonal	Antigen receptors are clonally-distributed
Repertoire	Limited	Immense
Memory	No	Yes

1.3.2. Pattern Recognition Receptors (PRRs)

The innate immune system uses a range of receptors called pathogen-associated molecular patterns (PAMPs) to distinguish the pathogens from the self-components. Recently pattern-recognition receptors (PRRs) involved in the innate immune responses to PAMPs were identified which has helped in understanding the mechanism behind the potentiation of immune responses (Iwasaki and Medzhitov, 2004). The PPRs consists of the following receptors (Figure 1.1):

- Non-phagocytic receptors (TLRs and nucleotide-binding oligomerization domain (NOD) proteins).

- Phagocytic receptors (scavenger receptors, mannose receptors and -glucan receptors).

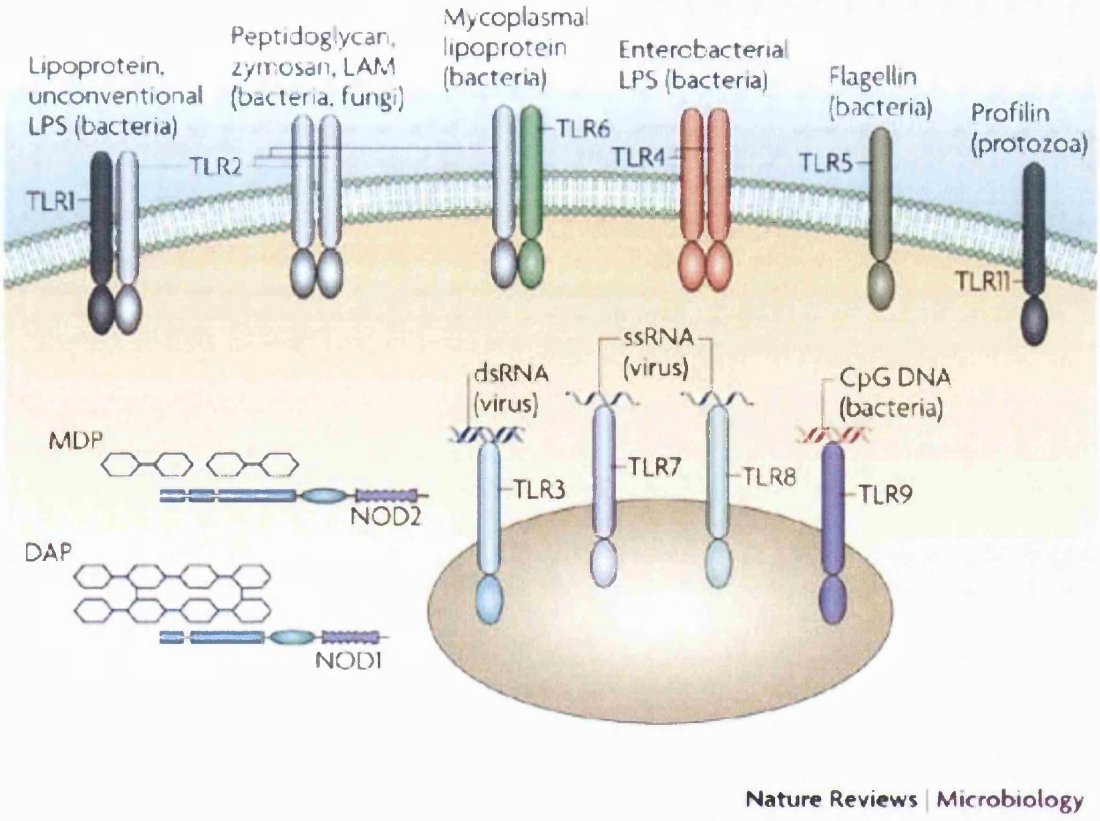

Figure 1.1: Pattern-recognition receptors: TLRs and NODs. The Figure focuses on the Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NODs). DAP, diaminopimelic acid; ds, double-stranded; MDP, muramyl dipeptide; LPS, lipopolysaccharide; LAM, lipoarabinomannan; ss, single-stranded (from Kaufmann, 2007, with permission from publishers).

The non-phagocytic receptors recognize PAMPs extracellularly (certain TLRs) or intracellularly (NOD family of proteins) leading to an elaborate signal transduction cascade whereas the phagocytic receptors directly recognize ligands on the surface of pathogenic microbes leading to their engulfment by macrophages. The NOD family of PRRs contains a leucine-rich, pattern-recognition domain similar to TLRs; a nucleotide-binding domain that is essential for oligomerization and subsequent signalling; and effector motifs such as the caspase-recruitment domain. PPRs are expressed in a wide variety of immune cells (neutrophils, macrophages, dendritic cells, natural killer cells, B cells) and non-immune cells (epithelial and endothelial cells). The engagement of PRRs leads to the activation of some of these cells which secretes cytokines and chemokines which in turn creates an inflammatory environment leading to the establishment of the adaptive immune response (Gordon, 2002).

1.3.3. Toll-like receptors (TLRs)

TLRs are transmembrane signalling proteins expressed by cells of the mammalian immune system. They have been shown to have high specific binding to different ligands which are evolutionary signatures of invading pathogens (PAMPs) (Table 1.2). TLRs have been derived from the Drosophila Toll and there are 10 of them described in humans. TLRs 1, 2 and 6 are triggered as homo or heterodimers by peptidoglicans and other bacterial products, TLR3 by dsRNA, TLR4 by LPS, TLR5 by flagellin, TLR7 and 8 by imidazoquinolines and ssDNA molecules and TLR9 by unmethylated CpG DNA motifs (Aguilar and Rodríguez, 2007).

Table 1.2: Toll like receptors (TLR) and their ligands (adapted from Aguilar and Rodríguez, 2007).

TLR	Ligand
TLR1	Bacterial/mycoplasmal lipopeptides (with TLR2)
TLR2	Peptidoglycan, Lipoteichoic acid
TLR3	Bacterial and viral dsRNA (double-stranded RNA)
TLR4	Host hsp60/70 (heat shock proteins 60 and 70), LPS (lipopolysaccharide), RSV (respiratory sincitial virus) fusion protein, MMTV (mouse mammary tumour virus) envelope protein
TLR5	Flagellin
TLR6	Bacterial/mycoplasmal lipopeptides (with TLR2)
TLR7	Bacterial, viral and host ssRNA (single-stranded RNA)
TLR8	Bacterial, viral and host ssRNA
TLR9	Bacterial and viral CpG DNA, DNA-IgG complexes, malarial hemozoine
TLR10	(not reported)

TLR sensing of their natural or synthetic agonists can orientate the immune response towards a Th1 or Th2 response and promote regulatory T (TReg) cell activity. In particular, LPS agonists, imidazoquinolines and unmethylated CpG oligonucleotides induce Th1 responses after sensing by TLR4, 7/8 and 9, respectively (Guy, 2007).

1.3.4. Signalling of innate immune responses by the receptors

TLRs are type I integral membrane glycoproteins similar to IL-1 receptors. TIR (Toll–IL-1 receptor domain), which is a cytoplasmic domain, associates it with the adapter proteins by linking the transmembrane receptors to their downstream signalling pathways (Figure 1.2).

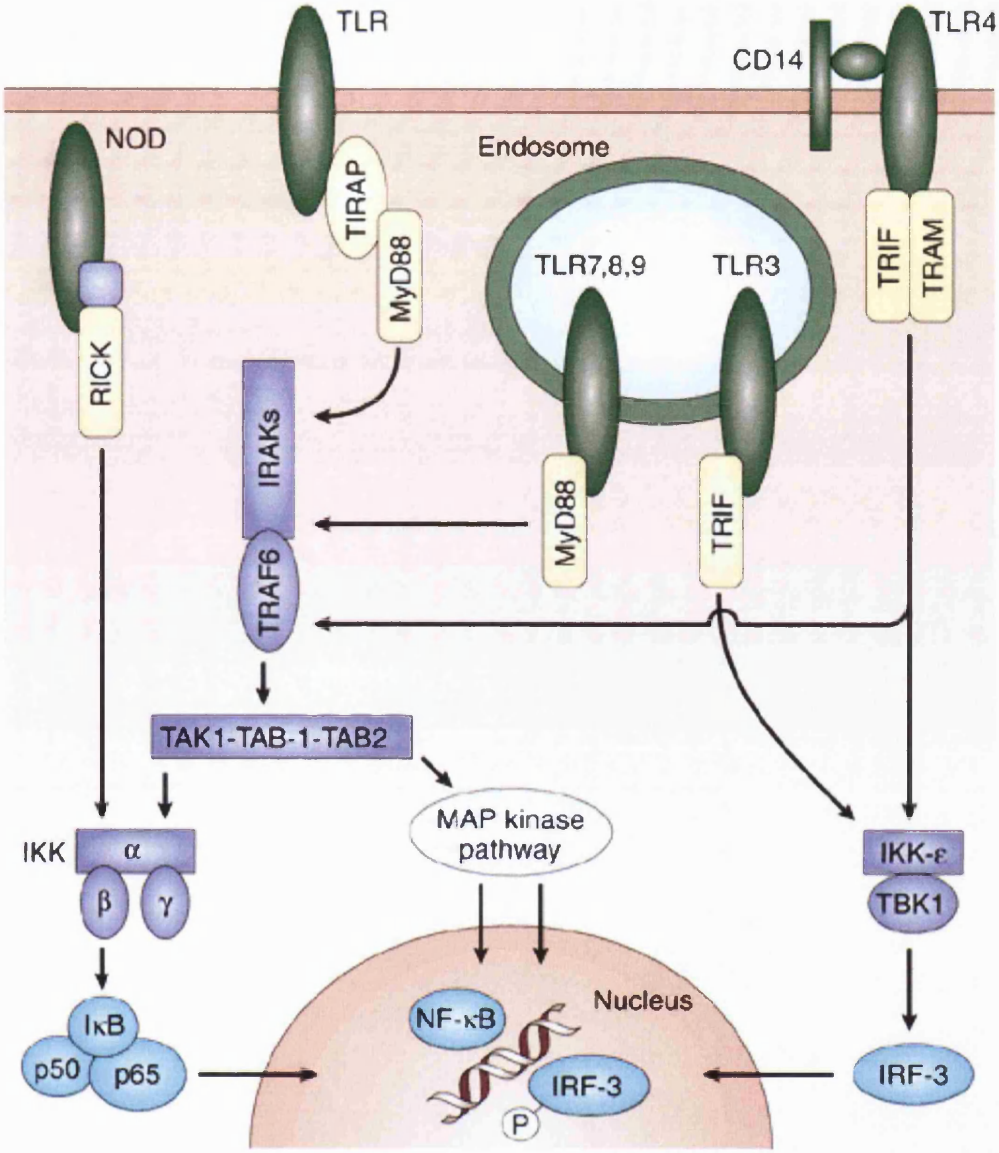

Figure 1.2: Signalling through the receptors of the innate immune system. Upon interaction with PAMPs, PRRs initiate signalling cascades leading to activation of transcription factors (such as NF-κB and IRF3), resulting in expression of inflammatory cytokines and other cellular activation events. A simplified pathway highlighting the main elements is shown, with receptors in green, adaptor proteins in yellow, kinases in purple and transcription factors in blue. Ligation of different TLRs may induce distinct gene expression patterns. These newly identified receptors and signalling moieties of the innate immune system provide a rich source for the discovery of targeted immune potentiators and development as vaccine adjuvants. TRIF, TIR domain–containing adaptor protein inducing IFN-β; TRAM, TRIF-related adaptor molecules; TBK1, TRAF family member–associated NF-κB activator binding kinase 1; IKK, IkB kinase complex; TAK1, transforming growth factor β–activated kinase; TAB1/2, TAK1 binding protein (from Pashine *et al.*, 2005, with permission from publishers).

All TLRs (other than TLR3) shares a common signalling pathway that uses an adapter protein called MyD88 (myeloid differentiation factor 88). In some cases (TLR5, TLR7, TLR9) MyD88 binds directly by TIR domain, whereas in TLR2 and TLR4 it binds with TIRAP (TIR domain–containing adapter protein). MyD88 binds to another family of signal transducers, known as IRAKs (IL-1 receptor–associated kinases) which in turn

binds to TRAF6. The subsequent activation of the transcription factors NF-B and AP-1 by TRAF6 involves yet other adaptor proteins known as TAK1 and TAB2. The NOD proteins recognise the intracellular pathogens and MDP from bacteria. When they associate with their ligands NOD1 and NOD2 activate NF-B by RICK (receptor-interacting serine/threonine kinase) pathway which leads to the expression of cytokines and chemokines. Thus the signalling of innate immune system involves a complex and rapidly growing set of transmembrane and intracellular proteins (Pashine *et al.*, 2005).

1.3.5. Conditioning of the adaptive immunity by the innate immunity

The transcription factors such as NF-κB and IRF3 (interferon regulatory factor 3) are activated due to the PRR signalling resulting in the inflammatory reactions of the host defences. The NF-κB pathway expresses the proinflammatory cytokines such as IL-1β and tumour necrosis factor-α and the IRF3 pathway leads to the production of antiviral type I interferons (IFN- α and IFN- β) (Akira and Takeda, 2004). During the release of these cytokines, chemokines like IL-8, monocyte chemo attractant proteins, macrophage inflammatory proteins and RANTES (*Regulated upon Activation, Normal T-cell Expressed, and Secreted*) are also released resulting in alteration of surface expression of selectins and intercellular cell adhesion molecules which ultimately lead to the extravasation and selective retention of leukocytes. This cellular infiltrate consists of activated monocytes, neutrophils, basophils, eosinophils and natural killer cells, many of which also express TLRs which gets activated creating an inflammatory microenvironment and monocytes which infiltrate the site differentiating into macrophages and dendritic cells (Figure 1.3). This activation of innate immunity ultimately conditions the inflamed site for the initiation of adaptive immune responses (Iwasaki and Medzhitov, 2004). The dendritic cells initiate the priming of naive $CD4^+$ helper T (Th) cells and also induces $CD8^+$ T cell into killer cells (Banchereau *et al.*, 2000).

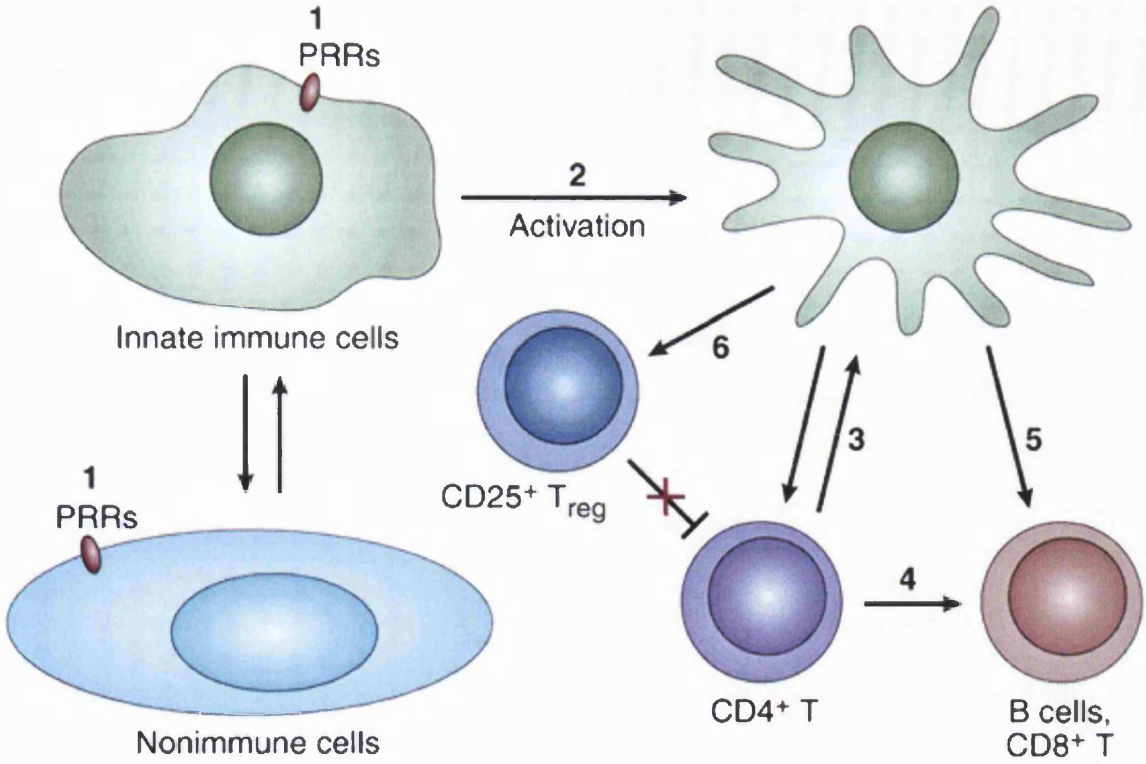

Figure 1.3: Initiation of the immune response. Upon interaction with PAMPs (1), resting innate immune cells and certain nonimmune cells, such as tissue epithelial cells, secrete cytokines and chemokines. Double arrows indicate cross-talk between cells. Key features of this early activation are an increase in APC function characterized by increased costimulatory capacity and upregulation of surface major histocompatibility complex molecules (2). Cells of the adaptive immune system, such as $CD4^+$ and $CD8^+$ T cells, then recognize presented antigen and undergo clonal expansion (3). Cognate interaction between T and B cells leads to B cell activation, expansion and conversion to plasma cells that secrete specific antibodies (4). Activated dendritic cells can also act directly on $CD8^+$ T cells to license them to become effector CTLs (5) and may also secrete soluble factors that block the immunosuppressive effects of $CD25^+$ T_{reg} (6). A proportion of adaptive immune cells also differentiate into memory cells that will be ready for a secondary encounter with the specific pathogen. Thus, complete elimination of pathogens is achieved by effector cells and soluble factors of both the innate and adaptive immune systems (from Pashine *et al.*, 2005, with permission from publishers).

When the immature dendritic cells recognise potential dangerous microbial signals through their PRRs, they mature and migrate to secondary lymphoid organs to prime naive T cells. Thus the activation of dendritic cells results in inducing distinctive immune responses, for example if the lymphoid dendritic cells which secrete IL-12 are activated it primes Th1 responses, whereas activation of myeloid dendritic cells leads to Th2 or Th0 responses (Pulendran, 2004). (Th0 cells are precursors of Th1 and Th2 cells, which produce both interferon-γ and interleukin-4 and have the capacity to become Th1 and/or Th2 cells). The TLR signalling also determines the type of response; TLR2 produces Th2 responses whereas TLR1 produces Th1 responses (Dabbagh and Lewis, 2003).

1.4. Mucosal Vaccination

Most human diseases start with the passage of pathogens through mucosal membranes and after colonization, some microorganisms release toxic factors; others penetrate into the tissues generating systemic or organ-specific infection (McGhee *et al.*, 1992). Knowing this mechanism, it is logical to presume that the stimulation of mucosal immunity by vaccination may be the most effective approach in preventing the initial infection (McGhee and Kiyono, 1993).

There are several reasons why mucosal immunization has got rapidly increasing attention. The first reason is related to the simplicity of this procedure by using non-invasive route of administration such as oral, nasal, rectal, vaginal, ocular and transdermal routes. The ease of administration results in increased compliance, no need for trained personnel and reduced risk of transmission of disease and problems of disposal of contaminated needles. The second reason is to localize immune responses to the site of potential pathogen invasion. The majority of infections occur at, or emanate from mucosal surfaces which consist of a surface of 400m² (Nugent *et al.*, 1998). These surfaces are protected by several non-specific physical barriers such as mucus secretion, stomach acids, enzymatic degradation, peristaltic movements, tight junctions and also by a specific immunological barrier, the mucosal immune system. The third reason is the existence of a Common Mucosal Immune System (CMIS). It has been found that immune cells stimulated by vaccination at one mucosal surface, especially in the gut or the nose may disseminate to some other mucosae as well, thus inducing immunity at several distant mucosal sites. The last reason is that mucosal immunization can efficiently stimulate a systemic immunity whereas a systemic immunization fails to protect mucosal surfaces. A comparison of the main characteristics of mucosal and systemic immunization illustrates that mucosal immunization is a better alternative for systemic immunization (Table 1.3). Many studies have established the presence of CMIS which enables administration of the vaccine at one site and also induces immunity at distant mucosae. The CMIS may be functionally divided into two interconnected sites: Inductive sites, where the antigen is encountered and B- and T-cell are activated; effector sites, having IgA plasma cells on their large surface areas and where the production of S-IgA antibody takes place leading to an immune protection at local and distal mucosal sites. Examples of these effector tissues are the lamina propria

regions of the intestine, the bronchi, the genitourinary tract and various secretory glands (i.e. lacrimal, nasal and salivary) (McGhee *et al.*, 1992).

Table 1.3: Comparison of the main characteristics of mucosal and systemic immunization (adapted from Nugent *et al*, 1998).

Mucosal Immunization	Parenteral Immunization
• Local and systemic immunity	• Systemic immunity only
• S-IgA (secretory immunity) and IgG produced	• IgG and IgA produced
• No age associated immune dysfunction	• Age associated immune dysfunction
• More antigen required to induce an immune response	• Responds to small amounts of antigen
• Injection not necessary	• Administered by injection
• Little training to administer vaccine	• Training required to administer vaccine

The surfaces of respiratory, gastrointestinal and urogenital tracts are covered by epithelial cells which excrete mucus, which forms a protective physical barrier and other substances such as lysozyme and lactoperoxidase which kill or inhibit the growth of microorganisms. Furthermore, the presence of the mucosa-associated lymphoid tissue (MALT) has the function to discriminate between food, other not dangerous antigens, commensal microorganisms and pathogens. Aggregates of MALT, occurring along the mucosae, act as inductive sites and include bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT) with Peyer's patches and nasal-associated lymphoid tissue (NALT) (Giudice and Campbell, 2006). In addition to the epithelial cells there are other types of cells controlling the access of potential antigens and pathogens through the intercellular tight junctions (Eldridge *et al.*, 1989; Eldridge *et al.*, 1993; Neutra *et al.*, 1996; Nagler-Anderson, 2001; Rescigno *et al.*, 2001; Cheroutre and Madakamutil, 2004; Cheroutre and Madakamutil, 2005; Kunisawa *et al.*, 2005):

- Microfold-cells (M-cells) or specialized follicle-associated epithelial cells (FAE) which are interspersed among polarized epithelial cells and responsible for the actual uptake of viruses, bacteria, toxins and microparticles smaller than 10 μm. This uptake is carried out by a vesicular transport from the lumen to the underlying lymphoid tissue, especially in the gut, the latter is organized in aggregates of lymphoid follicles called Peyer's patches
- Interdigitating Dendritic cells, a professional family of antigen presenting cells (APCs), which capture and sample luminal antigens forming tight junctions with polarized epithelial cells thanks to their dendritic-like processes.

- Macrophages, B Lymphocytes, CD4+ T helper (Th) cells, CD8+ cytotoxic T Lymphocytes (CTLs) and Plasma cells: immunocompetent cells in the lamina propria of the epithelia
- Mast cells and Natural-killers.

The most abundant antibody isotype produced in mucosa-associated tissues is IgA, which is usually not obtained by parenteral immunization, whose typical immunoglobulins are G and M (IgG and IgM). Two IgA monomers make a complex with a chain within sub-epithelial plasma cells which later release the immunoglobulin-chain complex permitting it to interact with a secretory component on the epithelial cells' surface. Then, the new compound is internalised in a vesicle, transported through the epithelial cell and finally extruded on the luminal surface by reverse endocytosis such as an S-IgA molecule. This immunoglobulin occurs in dimeric or tetrameric forms and has a greater avidity compared to the monomeric ones. Mucosal secretions contain an amount of this antibody larger than 80% of all other isotypes, but also IgG, IgM T lymphocytes and other immunity cells can be found. Secretory IgA have many functions in host defence: inhibition of the adherence of pathogens to mucosal surface and homologous antigen absorption from the mucosae; neutralization of bacteria, viruses, toxins and enzymes; enhancement of the antimicrobial activity of other immune effector systems such as lysozyme, peroxidise, lactoferrin and protection from their induced proteolysis; inhibition of complement IgM- or IgG-induced activation decreasing inflammatory reactions (Mestecky and McGhee, 1990; O'Hagan and Illum, 1990; Gilligan and Li Wan Po, 1991; McGhee *et al.*, 1992).

B- and T- lymphocytes, which have been primed in mucosal tissues, re-circulate through different mucosae and home because they express specific homing receptors. These receptors are very important for mucosal preventive vaccination because the immunization at one mucosal site allows the production of antigen-specific lymphocytes in distant mucosal districts (i.e. immunity against sexually transmitted diseases can be induced by oral or intranasal immunization). Following mucosal vaccine administration, all the intact antigens in lamina propria are transported through M cells to inductive sites in the MALT, where dendritic cells present antigens to T-cells. These T-cells induce the transformation of the B-cells in antibody secreting cells (ASCs) and migrate to the peripheral blood. The ASCs first return to the sites where they originated generating strong antigen-specific secretory IgA responses at the area of induction and

then they also reach remote mucosae. Mucosal immunization also provides the production of serum antibodies, lymphocytes proliferation, cytokine production and cytotoxic lymphocyte activity which are distinctive responses of the systemic immunity (Kantele, 1990; Kaul and Ogra, 1998; Mills *et al.*, 2003; Kiyono and Fukuyama, 2004; Giudice and Campbell, 2006).

1.4.1. Success of mucosal vaccines

The origin of mucosal vaccination is several thousands years old and originates from the time when Chinese medicine men allowed children to inhale powders made from dried crusts of pox scars. Major attention to this type of immunity was given in the late 1880s and mucosal immunology was born in the early 1900s. From then on, there was a decline in interest in this subject matter due to the spread of modern vaccinology focused mainly on parenteral immunization. Only since the early 1980s, with the advances in biotechnology, molecular biology and pharmaceutical delivery systems, attention on mucosal vaccination arose again. Hence, mucosal immunization is an attractive alternative to parenteral immunization to produce both humoral and cell-mediated immune responses and to induce mucosal and systemic immunity at the same time (Vajdy *et al.*, 2004).

However, in practice it has been proven difficult to stimulate strong SIgA immune responses and protection by mucosal administration of antigens irrespective of the attractive features. Only half a dozen of the approved vaccines are administered mucosally in humans (Holmgren and Czerkinsky, 2005). The list of these approved vaccines is summarized in the Table 1.4. Vaccination through the mucosal routes remains a challenging concept and has not yet been successfully commercialized for a wide range of products. A Switzerland based company Berna Biotech AG introduced an inactivated influenza virus vaccine marketed as Nasalflu in the year 2000 but it was subsequently withdrawn in 2001 because of the vaccine associated incidence of Bell's palsy, which was thought to originate from the use of E. coli LT as an adjuvant (Bell's palsy is a condition of facial paralysis triggered by viral infection wherein the facial nerve becomes oedematous) (Mutsch *et al.*, 2004). Almost all of the currently available vaccines are injected systemically, with a few exceptions (live attenuated Polio and Salmonella vaccines, which are administered orally and an adjuvanted flu vaccine, administered intranasally).

Table1.4: Internationally licensed vaccines against mucosal infections (adapted from Holmgren and Czerkinsky, 2005)

INFECTION AND VACCINE(S)	ROUTE	TRADE NAME (MANUFACTURER)
Polio		
Live attenuated vaccine (OPV)	Oral	Many
Cholera		
Cholera toxin B subunit + inactivated *V. cholerae* O1 whole cells	Oral	Dukoral (SBL Vaccine)
CVD 103.HgR live attenuated *V. cholerae* O1 strain	Oral	Orochol (Berna, SSVI)
Typhoid		
Vi polysaccharide	Deep subcutaneous or intramuscular	TyphimVi (Aventis)
Ty21a live attenuated vaccine	Oral	Vivotif (Berna, SSVI)
Rotavirus		
Live attenuated monovalent human rotavirus strain	Oral	RotaRix
Influenza		
Live attenuated cold-adapted influenza virus reassortant strains	Nasal	FluMist (MedImmune)

The oral polio vaccine (OPV) is considered as the classical oral-mucosal vaccine as it had a great impact in reducing polio in the world and has also served as a useful tool for elucidating fundamental aspects of mucosal immunity in humans (Zhaori *et al.*, 1988). However, there is a risk of reversion of OPV virus strains toward neurovirulence, which has led to the replacement of OPV by injectable inactivated polio vaccine (IPV) in most of the developed countries. An example of a nasally delivered vaccine, which was licensed by the FDA in 2003, is the live cold-adapted trivalent influenza vaccine (FluMist™ Medimmune, Gaithersburg, MD) for use in healthy persons aged 5 to 49 years old. It is administered directly into the nostrils with a prefilled single-use spray device. This vaccine induces an immune response that more closely resembles natural immunity than the response elicited by the injectable vaccine (Harper *et al.*, 2003).

The success of mucosal vaccines depends on the efficient antigen delivery systems. The requirements of such carriers are: protection of antigen from physical elimination and enzymatic digestion, targeting the mucosal inductive sites, stimulation of innate immune system to generate effective adaptive immunity (Holmgren and Czerkinsky, 2005).

1.5. Intranasal Route and Nasal Vaccines

The nasal route of administration has huge potential for the delivery of drugs and vaccines as it offers a large mucosal surface and accessibility. The mucosa is a highly vascularised leaky epithelium of large surface area, and after nasal absorption the blood reaches organs before reaching the liver and thereby avoids first-pass metabolism. Moreover there is a rapid onset of action providing better pharmacological benefits. The nasal route is also ideal for the administration of products that undergo degradation in the GIT since conditions in the nasal cavity are less aggressive than those present in the GI tract due to lower enzymatic activity and suitable pH (Almeida and Alpar, 1996). Anatomically, the nasal cavity is divided into five regions, nasal vestibule, atrium, respiratory area, olfactory region and the nasopharnyx. The two nasal cavities have three turbinates (inferior, middle and superior turbinate) (Figure 1.4). The nasal epithelium has non-ciliated, goblet, basal cells and the presence of microvilli on cells greatly influence and increase the available area for permeation.

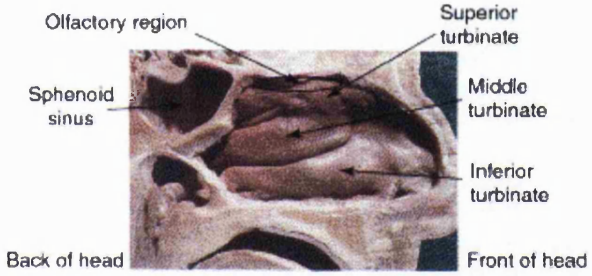

Figure 1.4: Anatomy of nasal cavity (Davis, 2001)

Numerous studies have shown that the i.n. route of immunization is more immunogenic than other mucosal routes for the induction of a local immune response in the respiratory tract and lung (Mestecky *et al.*, 1997). Intranasal immunization also facilitates immune response at distant mucosal sites and has been shown to enhance the efficiency of parenteral vaccines (Treanor *et al.*, 1992; VanCott *et al.*, 1998; Betts and Treanor, 2000; Treanor *et al.*, 2006).

Advantages of nasal vaccination

- Easily accessible, highly vascularised and has large absorption surface due to the presence of microvilli on the nasal epithelium.
- It is the most effective route to elicit optimal protective immunity in both mucosal and systemic immune compartments.

- Immune responses can be induced at distant sites (i.e. respiratory and genital tracts, lacrimal glands, gastrointestinal tract) due to the dissemination of effector immune cells in the common mucosal system.
- It can generate cross-protective immunity in the gut through the CMIS.
- It can avoid degradation of vaccine antigen caused by digestive enzymes, so requires a smaller dose of antigen than oral immunization.
- Do not require needles or syringes, which are potential sources of infection.
- Does not require trained medical personnel for delivery therefore vaccines can be easily administered to large population groups, such as in immunization campaigns in the developing world or in the event of natural pandemics or bioterrorism attacks.

Disadvantages of nasal vaccination

- Possible deposition of antigen in the central nervous system through the olfactory bulbs and olfactory nerves; this requires further investigation.
- Requires adjuvant safety to be clinically determined; clinical studies indicate that Bell's palsy is caused by influenza nasal vaccine that contains the native form of *Escherichia coli* heat-labile enterotoxin as a mucosal adjuvant.

Nasal associated lymphoid tissue (NALT)

In man, the target site for a nasal vaccine is the NALT, situated mainly in the pharynx as a ring of lymphoid tissue and also called Waldeyer's ring. The Waldeyer's ring consists of nasopharyngeal tonsil or adenoid (NT), the pair of palatine tonsils (PT), the pair of tubal tonsils (TT) and the linguinal tonsils (LT) (Figure 1.5). In addition, the mucosae of the pharynx contain smaller, subepithelial collections of lymphoid tissue, which complete the ring. APCs, B- and T-cells are abundant in these tissues (Davis, 2001).

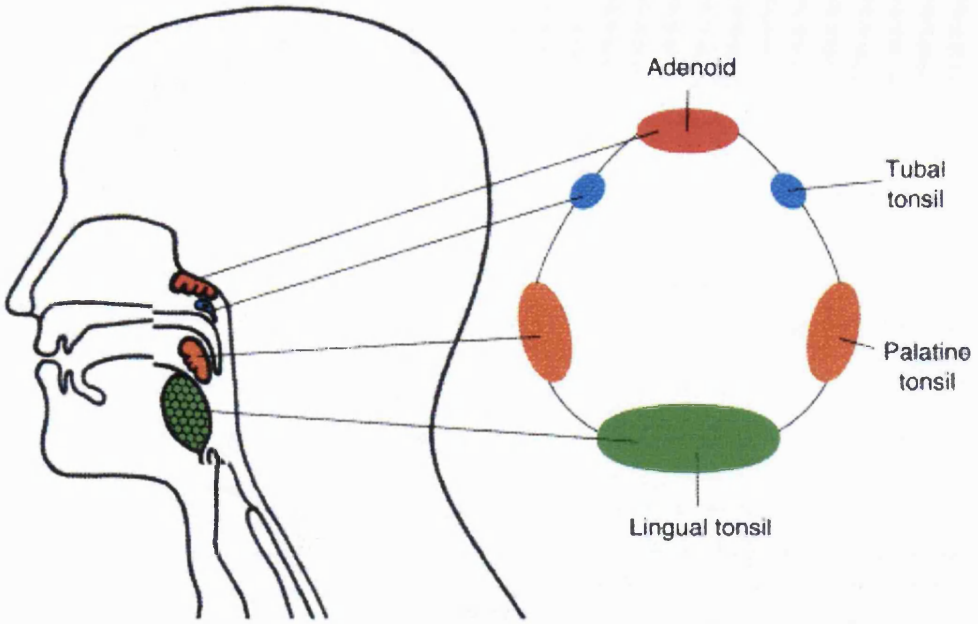

Figure 1.5: Pharyngeal lymphoid tissue of Waldeyer's ring (adapted from Davis, 2001).

The mucosal surfaces of the respiratory tract are the initial sites of contact of pathogenic viruses and bacteria such as influenza A virus, adenovirus, *Haemophilis influenza* and *Streptococcus pneumonia*. Some of these diseases can result in major pandemics and show high morbidity and significant mortality, thus a protective mucosal response in the upper respiratory tract is critical to control or prevent further spread of the diseases caused by such pathogenic organisms (McGhee and Kiyono, 1993; Liang *et al.*, 2001). The nose, upper respiratory tract (URT) and lower respiratory tract have an immune system that can be divided into three parts: (1) Epithelium, which has at the surface the epithelial compartment and an underlying connective tissue containing immunocompetent cells; (2) Lymphoid structures of the nose and bronchus-associated epithelium (nasal-associated lymphoid tissue, NALT), larynx-associated lymphoid tissue (LALT), bronchus-associated lymphoid tissue (BALT); (3) Lymph-nodes draining the respiratory system. The nasal mucosae present an epithelium with ciliated epithelial cells, mucous goblet cells and specialised non-ciliated cells similar to the M-cells in the Peyer's patches.

The NALT is situated below the epithelial surface and contains aggregates of lymphoid follicles (B-cell areas), inter-follicular areas (T-cell areas), macrophages and dendritic cells in a loose reticular network. In mice and rats, the NALT is represented by the

bilateral aggregates of non-encapsulated lymphoid tissue underlying the epithelium on the ventral aspect of the posterior nasal cavity. The nasal cavity of mice is composed of aggregates of B, CD4+ or CD8+ T cells, dendritic cells, and macrophages covered by a single epithelial monolayer where M cells are scattered (Figure 1.6) similar to the Peyers patches (Kuper *et al.*, 1992; Perry and Whyte, 1998).

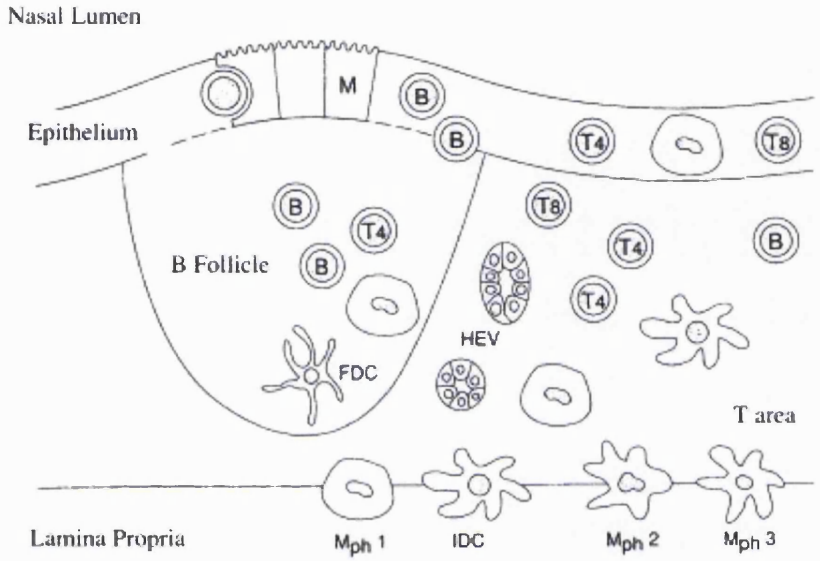

Figure 1.6: Schematic representation of compartments and cellular composition of NALT. B: B-cell, T4: CD4-positive (CD4+) T cell, T8: CD8-positive (CD8+) T cell, Mph1, Mph2, Mph3, macrophages positive for ED1, ED2 or ED3 (macrophage surface antigens), IDC: interdigitating dendritic cell, FDC: follicular dendritic cell, HEV: high endothelial venule, M: microfold cell (M-cell) (adapted from Davis, 2001)

The T-cell areas contain CD^{4+} and CD^{8+} T cells and numerous macrophages. The B-cell areas are populated by B cells predominated by the IgM and IgG isotypes, and T helper cells are scattered throughout along with macrophages. The mouse NALT is covered with a specialized epithelium, the lymph-epithelium or the follicle-associated epithelium, which differs from the surrounding respiratory epithelium by the presence of non-ciliated cells and infiltrations of lymphocytes and macrophages. The non-ciliated cells are M cells, identical to those seen in the bronchus and gut-associated lymphoid tissues (Kuper *et al.*, 1992).

After intranasal immunization, both humoral and cellular responses can occur. The nature of the antigen is indicative for the type of immunity. Small soluble antigens penetrate the epithelium and interact with dendritic cells, macrophages and lymphocytes (intraepithelial and submucosal). Subsequently, they are drained from the mucosa by the

superficial cervical lymph nodes and are in turn drained by the posterior cervical lymph nodes. Hence, they preferentially induce a systemic response leading to the production of IgG. In contrast, particulate antigens are either removed by the cilia in NALT or in great measure taken up by M-cells. Because the drainage occurs particularly to the cervical lymph-nodes, the antigens taken up by the M-cells can better elicit a local mucosal response or lead to tolerance (Tilney, 1971; Kuper *et al.*, 1992).

After these mechanisms, the antigens are transported to underlying lymphoid cells in the submucosa where they are presented and processed. Here T-cells activation takes place and the lymphocytes help B-cells to develop into IgA plasma cells. Precursors of mucosal IgA appear and their maturation takes place in the local lymph nodes. Then, they enter the circulation through the thoracic duct and undergo differentiation in the lamina propria of distant mucosal sites where S-IgAs appear. This phenomenon confirms the presence of a common mucosal immune system (Wu *et al.*, 2000; Wu and Russell, 1997; Partidos, 2000).

1.5. Adjuvants

Adjuvants are defined as a group of structurally heterogeneous compounds which can increase the immune response to an antigen. The famous immunologist Charles Janeway has called adjuvants as "the immunologist's dirty little secrets". It is appropriate to call adjuvants as 'secrets' because there has been a limited knowledge about their mode of action therefore development of vaccines was largely an empirical process (Schijns, 2000). The term adjuvant is derived from the latin word *adjuvare*, which means to help or to enhance (Vogel, 1998). This definition was first proposed by Ramon, who showed increased immune response to tetanus and diphtheria toxoids injected together with other compounds such as agar, tapioca, lecithin, starch oil, saponin or breadcrumbs in the year 1925 (Gupta and Siber, 1995). Traditionally the term adjuvant has been used to describe any molecule that improves the immune response to co-administered antigen. An adjuvant can be effective in more than one way, it can stimulate an elevated immune response or can also stimulate a depressed immune response, therefore the term "immunomodulating agent" can be considered to be more appropriate. Many compounds have been investigated as adjuvant formulations, but most of them have never been accepted for vaccination due to their toxicity and side effects, both local and systemic. Usually a balance between toxicity

and adjuvanticity is accepted based upon risk/benefit analysis (Gupta *et al.*, 1993). However, recent advances in various scientific disciplines has made the understanding of the mechanisms of 'adjuvanticity' easier which will be useful in the design of safer and more effective immune enhancers or potentiators. For an adjuvant to be claimed as ideal, it should have the following properties (Marciani, 2003):

- should be non-toxic or should have negligible toxicity;
- safety with regards to local reactions, systemic reactions, non-pyrogenic, autoimmune diseases, hypersensitivity reactions, carcinogenicity, teratogenicity;
- capable of stimulating a strong humoral and/or T cell immune response;
- good immunological memory or long-term immunity;
- defined chemically in order to be manufactured consistently;
- be able to elicit a protective immune response with weak antigens such as polysaccharide-protein conjugates with lower doses of antigens and with fewer injections;
- be effective in infants at birth and in young children;
- stable under broad ranges of storage time, temperature, and pH.

None of the adjuvants presently available meets all these criteria. Therefore there is a need for finding new adjuvants or make the existing adjuvants safer. At present the most challenging aspect in the field of adjuvant research is to find the 'perfect mix'; an optimal, safe formulation. This adjuvant based formulation will not only be additive but synergistic leading to better immune responses (Guy, 2007).

There are different types of classification for adjuvants based on their source, mechanism of action or physicochemical properties. The biologist Gerald Edelman classified adjuvants in three categories: (a) active immunostimulants; (b) carriers; (c) vehicle adjuvants. Alternatively they are classified based on the route of delivery such as parenteral and mucosal adjuvants (Petrovsky and Aguilar, 2004). A recently published review classifies adjuvants in the following categories (Aguilar and Rodríguez, 2007):

- Mineral salts
- Tensoactive compounds
- Microorganism-derived adjuvants

- Emulsions
- Particulate antigen delivery systems
- Cytokines
- Polysaccharides
- Nucleic acid-based adjuvants
- TLRs and adjuvanticity

1.6.1. Mineral salts

The most widely used adjuvants are aluminium salts. The salts of calcium, iron and zirconium have also been used to adsorb antigens, however not to the extent as alumunium salts (Petrovsky and Aguilar, 2004).

The adjuvant properties of aluminium salts were described for the first time in the year 1926 by Glenny who used diphtheria toxoid precipitated with the aluminium salt; the resulted compound was referred to as "alum precipitated toxoid". This suspension had much higher immunogenicity than the soluble toxoid. During the 1930s, the superior effectiveness of alum was established by several studies in humans using alum-precipitated tetanus and diphtheria toxoids in comparison with other adjuvants. Aluminium-adsorbed vaccines stimulate immune competent cells, activating the complement, eosinophilia and macrophages; they are also up-taken by APCs due to their particulate nature and optimal size (<10 μm) (Gupta *et al.*, 1993; Gupta *et al.*, 1995). They are effective with many antigens, but repeat administration is necessary to achieve protection against the infection. Their main mode of adjuvancy is due to their ability to provide a short-term depot effect for absorbed proteins, slowly leaking antigen to the body's immune system (Cleland *et al.*, 1997).

Aluminium compounds, generally referred as alum, include aluminium hydroxyphosphate commonly called aluminium phosphate, aluminium oxyhydroxide and hydroxide. The general term "alum" is not correct if used for all aluminium compounds, because aluminium hydroxide and aluminium phosphate have different physical and adjuvant properties. An excessive amount of adjuvant may suppress immunity by covering the antigen completely with mineral compounds or by being cytotoxic to macrophages. The dilution of aluminium-adsorbed vaccines lowers the immunogenicity/potency therefore it is preferable to administer these vaccines undiluted

or with a minimum dilution. The total amount of acceptable aluminium dose for human vaccines is 1-1.5 mg *per* vaccination (Gupta *et al.*, 1995).

1.6.2. Tensoactive compounds

Saponins like Quil A are tensoactive glycosides containing a hydrophobic nucleus of triterpenoid structure with carbohydrate chains. Quil A is derieved from the aqueous extract of *Quillaja saponaria.* Quil A is a widely used as a veterinary adjuvant and defined saponins like the purified extract QS-21 and ISCOPREP 703 are proposed as human adjuvants. Saponins induce strong Thl and Th2 responses and moderate CTL responses. It is assumed that after mixing the saponins with proteins there is formation protein-saponin micelles (Cox and Coulter, 1997). Studies have shown that these tensoactive compunds induces Th1 and Th2 responses including the induction of cytotoxic T-lymphocytes to soluble antigens (Kensil *et al.*, 1998; Mowat *et al.*, 1999).

1.6.3. Microorganism-derived adjuvants

Bacterial or fungal substances are good source of adjuvants due to their immunostimulatory properties. Bacterial cell wall peptidoglycan or lipolysaccharide (LPS) are not highly immunogenic but enhances the immune response by activating the Toll-like receptors (TLRs) that mediate the danger signals activating the host immune defence system. LPS can be administered at a site different from the antigen making it a usual adjuvant. Although LPS has good adjuvant properties it is toxic therefore its use in humans is limited. The active component of LPS is lipid A and there have been numerous attempts to prepare a modified lipid A to make it less toxic. Monophosphoryl lipid A has shown many of the adjuvant effects of lipid A and LPS and was found to be safe for human administration (Ogawa *et al.*, 1986). The adjuvant activity of bacterial substances is mediated by N-acetyl muramyl-l-alanyl-d-isoglutamine, also called muramyl dipeptide (MDP). MDP and its derivatives have been shown to act as effective parenteral and mucosal adjuvants for many antigens (Michalek *et al.*, 1983; Friedman and Warren 1984; Fukushima *et al.*, 1996). Trehalose dimycolate (TDM) is another extract from bacterial walls which has shown to stimulate both humoral and cellular responses (Aguilar and Rodríguez, 2007).

1.6.4. Emulsions

After aluminium, emulsions are the most widely used adjuvants. The prototype emulsions are Montanide 720 (water-in-oil emulsion) and MF59 (oil-in-water emulsion). Like aluminium, the adjuvanticity of emulsions was proposed to be linked in part to the depot effect but this does not apply to the water-in-oil emulsions (Guy, 2007).

1.6.5. Particulate antigen delivery systems

Particulate antigen delivery systems include the adjuvants like liposomes, polymeric microspheres, nano-beads, immunostimulating complexes (ISCOMs), virus-like particles (VLPs). The antigen is either encapsulated or adsorbed on the surface of liposomes and polymeric micro/nanoparticles (this is discussed in detail in section 1.7).

Liposomes are vesicles consisting of bilayered phospholipids surrounded by aqueous layered membranes which have been used as immunoadjuvant and antigen delivery systems (Alving, 1992; Gregoriadis, 1990). They are generally composed of phospholipids and cholesterol, and can range in size from 0.01 micron to 150 micron. The adjuvanticity of liposomes is independent of specific vesicle features (e.g. composition, size, surface charge) or mode of immunization in terms of antigen choice or route and frequency of dose (Gregoriadis *et al.*, 1996). Liposomes have the ability of protecting the antigen from acids and proteolytic enzymes of the gastrointestinal system. A number of studies have investigated the potential of liposomes as musocal delivery system (Vangala *et al.*, 2007; Perrie *et al.*, 2004; Kersten and Crommelin, 2003; Perrie *et al.*, 2001; Bramwell *et al.*, 1999; Gregoriadis *et al.*, 1999). Studies have shown that the mucosal adjuvancy of liposomal delivery systems can be enhanced by co-administrating with cationic molecules such as chitosan and dimethyl dioctadecylammonium bromide (Bramwell *et al.*, 1999; Vangala *et al.*, 2007).

Immunostimulating complexes (ISCOMs) comprise of a mixture of the saponin Quil-A and cholesterol to form the cage-like structure of an ISCOM matrix and were first described by Morein and co-workers (Morein *et al.*, 1984). Quil-A serves as a 'built in' adjuvant making the ISCOM highly immunogenic (Sjolander *et al.*, 1998). There are a number of studies describing the use of ISCOMs as potent adjuvants which can induce

cell-mediated and protective immunity (Kensil *et al.*, 1998, Brennan *et al.*, 1999, Mowat *et al.*, 1999, Mohamedi *et al.*, 2001, Crouch *et al.*, 2005, Angelos *et al.*, 2007).

Virus-like particles (VLP) are inert, empty capsids of viruses without DNA/RNA but have the intact structure of the virus. VLPs can be obtained by genetic engineering with an attached antigen. VLPs-displayed antigens are efficiently taken up by dendritic cells (DC) and induce potent immune responses after parenteral, mucosal and transcutaneous immunizations. Hepatitis B surface antigen, produced as VLPs in *Saccharomyces cerevisiae* and *Pichia pastoris* yeasts have been used for the past 15 years in human use. Recently the FDA has approved a human papilloma virus (HPV) vaccine for clinical use, which consists of the recombinant VLPs of HPV 6, 11, 16, 18 mixed with an aluminium adjuvant (Aguilar and Rodríguez, 2007).

1.6.6. Cytokines

Cytokines act as short range mediators and they are considered to have similarities with neurotransmitters. They can enhance the immunity of the antigens due to an over production of cytokine. The dose of cytokine in the formulation is very important as insufficient cytokine does not produce any response, excess dose results in loss of activity, suppression or activation of bystander reactions (Hughes, 1998). The granulocyte-macrophage colony stimulating factor (GM-CSF) enhances the primary immune response by activating and recruiting antigen presenting cells (APC). Other cytokines such as IL-12 have shown to be potent mucosal adjuvant (Aguilar and Rodríguez, 2007).

1.6.7. Polysaccharides

Gamma inulin, a naturally occurring carbohydrate from plants of *Compositae* family, is a humoral and cellular immune adjuvant which activates the macrophages. The advantage of inulin is that it can induce both Th1 and Th2 immune responses and does not have any significant local or systemic toxicity as it is metabolized into simple sugars fructose and glucose. Other polysaccharides which can be used as adjuvants are glucans, dextrans, lentinans, glucomannans and galactomannans (Petrovsky and Aguilar, 2004).

1.6.8. Nucleic acid-based adjuvants

The immunostimulatory capacity of DNA heralded in the development of immunostimulating DNA-based molecules which can be used as adjuvants. CpG motifs are six deoxynucleotides-long DNA sequences with a central CpG dinucleotide which normally occur in bacterial DNA. The bacterial DNA has been shown to induce B-cell proliferation, antibody secretion and Th1-type responses dominated by the IL-12. This was attributed to specific single-stranded oligonucleotide sequences containing unmethylated CpG dinucleotides (CpG motifs). A similar immuno modulating effect can be seen with synthetic oligodeoxynucleotides (ODS) containing CpG motifs. Given these immunostimulatory properties, synthetic CpG ODNs have been extensively tested for therapeutic applications and for immunopotentiating activity to co-administered vaccine antigens. Because CpGs can be tolerated even at high concentrations without any toxic effects, this enables the ease of production on a large scale at a minimum cost and can induce immune activation on human cells (Partidos, 2000).

1.6.9. TLRs and adjuvanticity

TLRs are transmembrane signalling proteins expressed by cells of the mammalian immune system. This has been discussed in detail in the previous section 1.3.

1.7. Antigen Delivery Systems

Generally soluble antigens and antigens synthesized by recombinant DNA techniques are weakly immunogenic and need protection against passage through the mucosal epithelium. The delivery systems that have shown potential for mucosal delivery of antigens include biodegradable micro/nanoparticles, liposomes, mucosal adjuvants, bacterial and viral vector systems. The mode of action of these particulate carriers is by promoting the uptake of the antigen by APC or by delivering the antigen directly to the lymph node (Eldridge *et al.*, 1991; O'Hagan *et al.*, 1991, 1993; Alpar *et al.*, 1994).

Microspheres are defined as porous microparticulate drug delivery systems representing a polymeric matrix system containing the drug/antigen uniformly distributed throughout the polymer matrix. Nanoparticles are submicron (less than 1 μm) colloidal systems made of biodegradable or non-degradable polymers. Depending on the technique used

for the preparation, nano/microspheres or micro/nanocapsules can be obtained. The biodegradable particulate systems can be classified as monolithic type where antigen is evenly dispersed throughout the polymeric matrix or reservoir type where antigen is located in solution cavities formed by the polymeric material surrounded by an outer polymer shell (Morris *et al.*, 1994). Nanoparticles were first developed in 1976 by Birrenbach and Speiser and later they were applied to design drug delivery systems using biodegradable polymers which were highly suitable for human applications (Fattal *et al.*, 1997). These systems for antigen delivery present four important advantages compared to the soluble molecules:

- the particles are absorbed with greater efficiency than soluble molecules in the mucosal epithelium;
- they provide a greater concentration of antigen at the inductive site;
- they have the potential to reduce the frequency of vaccination required to establish long term protection: the release properties of microspheres can be tailored for the antigen to produce a sustained immune response;
- it has been demonstrated that they induce cell-mediated immune responses.

The enhanced immunogenicity of particulate antigens is unsurprising, since pathogens are particulates of similar dimensions and the immune system has evolved to deal with them. Particulate delivery systems present multiple copies of antigens to the immune system and promote trapping and retention of antigens in local lymph nodes. Polymeric particles have the potential to reduce the frequency of vaccination required to establish long term protection. Moreover, particles are taken up by macrophages and dendritic cells, leading to enhanced antigen presentation and release of cytokines, to promote the induction of an immune response (O'Hagan *et al*, 1998).

The advantage of using microspheres is that the release properties of microspheres can be tailored for the antigen to produce a sustained immune response (Nugent *et al.*, 1998). Initially, it was proposed that the polymeric particles enhance the immunity by providing a long term suppository for the antigen similar to the depot theory of the adjuvant action. However it has to be noted that the polymeric particles can release the antigens for weeks to months, which is far superior to the depot effect of aluminium salts or water/oil emulsions such as Freund's adjuvants. Moreover they can deliver the antigen in a pulsatile fashion over several months which can mimic the traditional bolus primary and booster immunizations. In addition to the depot theory, polymeric particles

enhance the immune responses by several other mechanisms as well. They have the ability to target the phagocytosis by antigen presenting cells (APC's) resulting in the intracellular delivery of antigen for processing by the major histocompatibility complex (MHC) class I and class II pathway. The antigen presentation by the MHC class II molecules leads to enhanced antibody production (humoral immune response), whereas antigen presentation by the MHC class I molecules leads to a cytotoxic T lymphocyte (CTL) mediated immune response (Hanes *et al*, 1995).

In response to an infection, the innate mechanisms kill the infectious agents on the first contact as a defence mechanism. If an infectious agent is successfully infected the early non-adaptive responses try to moderate the infection until an adaptive immune response can be elicited. The T and B lymphocytes take several days to elicit the adaptive immunity. This does not happen until antigen specific T cells (T-helper cells) can proliferate and differentiate. The first appearance of a pathogen elicits the APCs such as macrophages dendritic cells which stimulate T cells by interacting the antigen bound to a MHC-molecule and the T-cell receptor. This results in the secretion of cytokines which controls the type of immune response (cellular responses by Th1 cells or antibody responses by Th2 cells). Upon a second contact with the pathogen the immunological memory which is the basic rationale behind the concept of vaccination plays a major role. The concept of immunological memory is not clear; however it has been it takes place switching of cells involved in a primary response from an effector to a long-lasting memory state or by continuous stimulation of the memory state. Therefore the concept of immunological memory is debatable. The sequence of events leading to immune response following release of antigen from microspheres is illustrated below in Figure 1.7 (Johansen *et al.*, 2000).

As evident from the Figure 1.7, these mechanisms involve a complex interplay between APC, lymphocytes, co-stimulatory signals and cytokines. The microparticles (MS) deliver the encapsulated antigen to APCs (e.g. macrophages or dendritic cells) via phagolysosomes or via cytosol, with the former mechanism supporting a MHC class II-restricted immune response, and the latter a MHC-class I response. Maturation of CD4-T helper (Th) cells is a direct function of the types of cytokines secreted during the primary immune response.

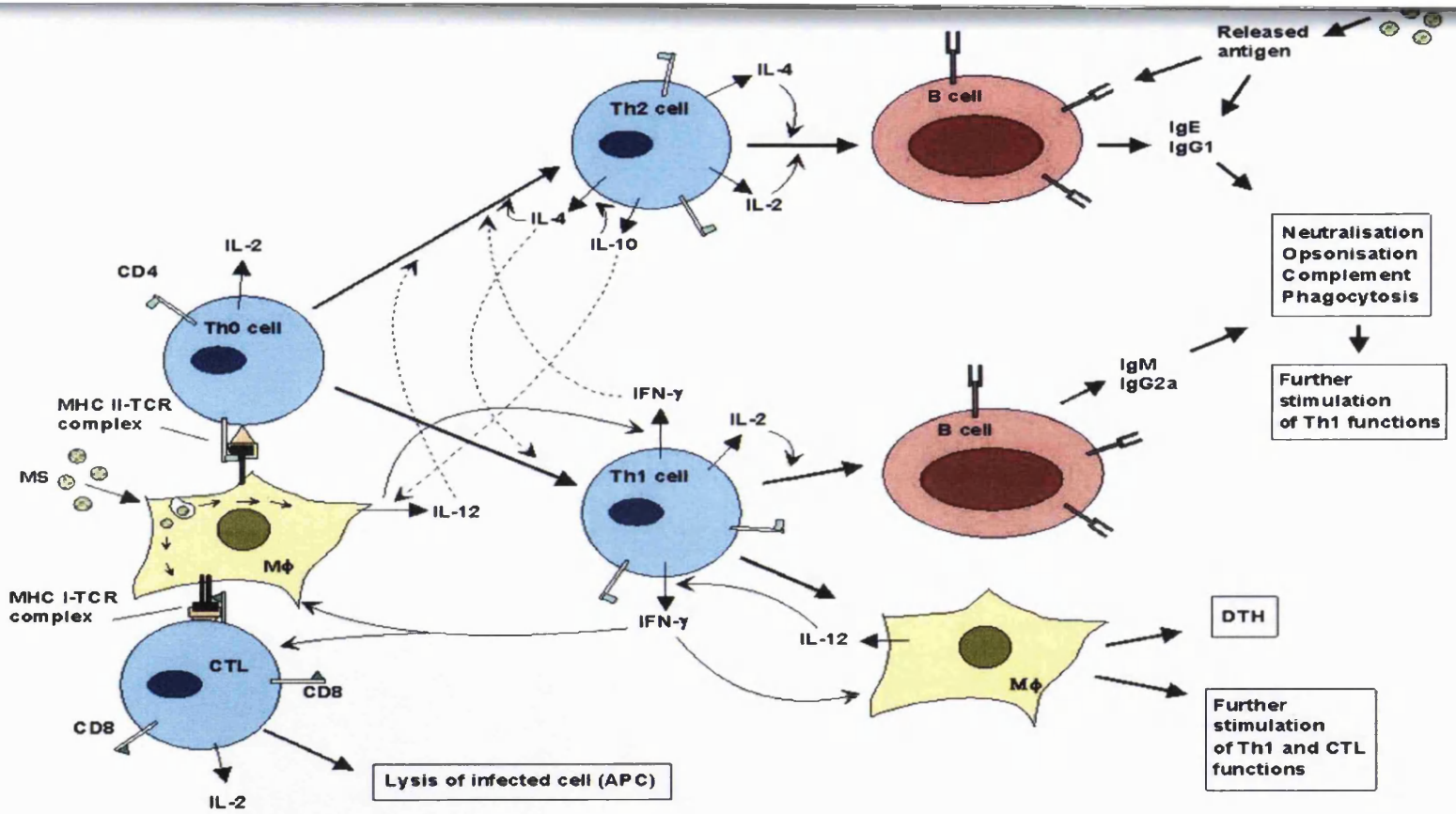

Figure 1.7: Mechanism cellular and molecular interactions in the generation of adaptive immune responses from antigen encapsulated polymeric particles. Mø, macrophage; MHC, major histocompatibility complex; TCR, T-cell receptor; DTH, delayed type hypersensitivity; CTL, cytotoxic T lymphocyte (Johansen *et al.*, 2000)

Typically, interleukin (IL)-4 and IL-10 (also IL-5 and IL-6) drive Th-cell polarization towards Th2 cells and related humoral mediated defence mechanism, whereas IL-12 and IFN-γ (also IL-18) support a Th1- or a cell-mediated response (solid curved feedback arrows). Th1- and Th2-associated cytokines tend to regulate reciprocally, as IFN-g and IL-12 inhibit Th2-cell functions, while IL-4 and IL-10 inhibit Th1-cell functions (dashed curved feedback arrows). IL-2 is important for general T-cell proliferation without influencing Th polarization. MS can release extracellular antigens which stimulate secondary immune responses through interactions with circulating or B-cell bound immunoglobulins. Th1-cells release IFN-γ, which co-stimulates Mø and cytotoxic T cells. The size of the microparticles has been shown to be a determinant of the nature of immune response, with larger particles more effective for inducing cellular immunity, and small particles being capable of inducing antibody responses as well as primary and secondary cellular-type immunity (McNeela and Mills, 2001).

Microencapsulation using either synthetic or biodegradable polymers has the advantages of protecting the antigen from the acidic and enzymatic environment of the mucosa; moreover the obtained dry powder of the polymeric particles has an extended shelf life (Morris *et al.*, 1994). In addition the main advantage is the degradability of the polymer, which can be tailored to release antigen over extended periods (by altering several conditions such as the size of the particles, ratio of monomers in matrix, copolymer weight and the nature of antigen and its loading. Also, certain polymers have demonstrated the ability to induce immune response in terms of IgA and IgG responses and hence serve as vaccine adjuvant (Chen, 2000). Different biodegradable polymers are available for the purpose of antigen entrapment and several polymers have been shown to have potential for single-dose vaccines. The selection of the polymer depends upon specific requirements, but the most important criteria are the biodegradable nature of the polymer, biocompatibility and low toxicity. Eventually, the adjuvant effect of polymeric particles can be further enhanced by their administration in vehicles with additional adjuvant activity or by microencapsulation of adjuvants (Singh *et al.*, 1998; Johansen *et al.*, 2000; Raghuvanshi *et al.*, 2002; Singh *et al.*, 2006; Singh *et al.*, 2007).

1.8. Preparation of polymeric particulate carriers

There are various techniques used for the preparation of the antigen/drug encapsulated polymeric micro/nanoparticles. These techniques include solvent evaporation method,

spray drying, polymerization, super critical fluid technology, etc. The most popular method is the water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation.

1.8.1. Solvent evaporation method

The water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation method (Figure 1.8) consists of four major steps: (a) dissolution of the polymer in an organic solvent (b) emulsification of this organic phase in a aqueous phase containing protein immiscible in polymer solution and subsequently emulsifying this primary emulsion into a second aqueous phase to form a $w_1/o/w_2$ emulsion (c) evaporation of the solvent from the dispersed phase transforming the droplets into solid particles (d) harvesting and drying of the particles (Freitas *et al.*, 2005).

In general, primary aqueous phase (w_1) consists of the antigen and a stabilizing agent the polymers like PLA, PLGA and PCL are dissolved in organic solvents like dichloromethane or chloroform or ethyl acetate making the oil phase (o). The aqueous phase is then poured into the oil phase and the mixture is emulsified using a using high speed or high pressure homogenization or ultrasonication or by vortex mixing yielding a primary (w_1) emulsion. This primary emulsion is then poured under vigorous mixing, into a large excess of an aqueous phase containing a macromolecular viscosity-enhancing stabilizing agent like poly (vinyl alcohol) (PVA) or poly (vinyl pyrrolidon) (PVP), alginates, methylcellulose or gelatin which prevents coalescence of the antigen/matrix. After the second homogenization, a transient $w_1/o/w_2$ double emulsion is formed which is stirred continuously at room temperature for 3 to 5 hours. The solvent evaporation induces polymer precipitation and thereby the formation of solid antigen-loaded particles. The solid particles are collected by centrifugation or filtration, washed several times to remove non-encapsulated antigen and the stabilising agent and dried under vacuum or freeze-dried.

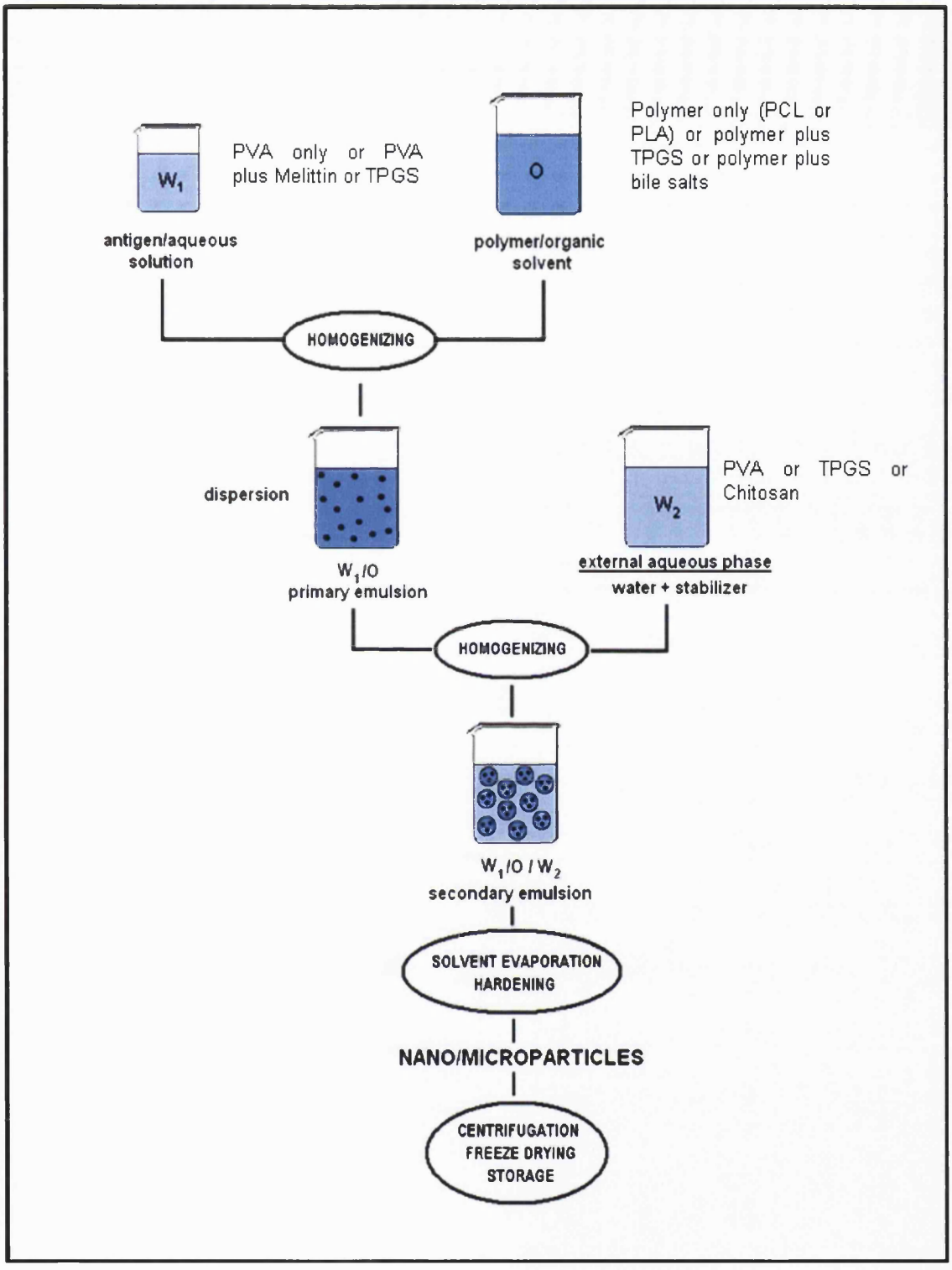

Figure 1.8: Schematic representation of the water-in-oil-in-water ($w_1/o/w_2$) double emulsion solvent evaporation method. The primary aqueous phase containing the drug/antigen (w_1) is homogenised in the oil phase (o) containing the polymer dissolved in organic solvent to form the primary (w_1/o) emulsion. This is homogenized with a secondary aqueous phase (w_2) to form a ($w_1/o/w_2$) double emulsion. The emulsion is left to evaporate off the solvent and to allow the polymer to precipitate out and form the micro/nanoparticles. Finally this is filtered or centrifuged and freeze-dried to obtain dry micro/nanoparticles.

The size of the particles can be controlled by adjusting the stir rate, type and amount of dispersing agent, viscosity of organic and aqueous phases and temperature. The rate of

volatile solvent removal from the solidifying particles can be controlled by temperature and pressure, high temperatures and reduced pressure promote solvent evaporation. There are number of factors which affect the physicochemical properties of the polymeric particles, these are discussed in section 1.8.3 (Fattal., *et al.*, 1997; Kissel *et al.*, 1997; Freitas *et al.*, 2005).

1.8.2. Spray drying

There has been enormous effort focussed on spray drying of pharmaceuticals from the early 1990s when the potential of the pulmonary route for therapeutic protein delivery was recognized. It was explored in the 1980s as an alternative means of making fine particles (Yu *et al.*, 2001). During this project, antigen encapsulated water-in-oil-in-water (w1/o/w2) double emulsion was spray dried to obtain the dry microparticles.

Spray drying involves atomisation of a solution or suspension or emulsion to fine droplets, which are evaporated in a warm air current to form dry particles (Figure 1.9). The feed (suspension or solution or emulsion) is introduced into the spray dryer through the nozzle wherein it gets atomized. The liquid feed can be atomized by different types of nozzles, depending on the droplet size required. The atomization is an important step as the production of homogenous spray results in optimum evaporation and the production of the desired particle size, shape and residual moisture. The atomized droplets come in contact with the air in the drying chamber resulting in the evaporation of the solvent. The dried particles or powder is separated by the cyclone and the particles are collected in the collector vessel. There is concern for the thermal degradation of the active ingredient as the drying air temperature can be relatively high (e.g., >100 °C) but damage can be minimal as the actual temperature of the evaporating droplets is significantly lower due to cooling by the latent heat of vaporization. The properties of the spray dried powders are controlled by the process and formulation parameters. A collector or a filter bag collects the product. Filter bags are not preferred since particulate of the filter material may contaminate the powder (Masters, 1991). Drying is controlled by the water content and the difference in the inlet and outlet temperatures of the drying air. Drying time of the droplets depends on the residence time of the droplets in the spray.

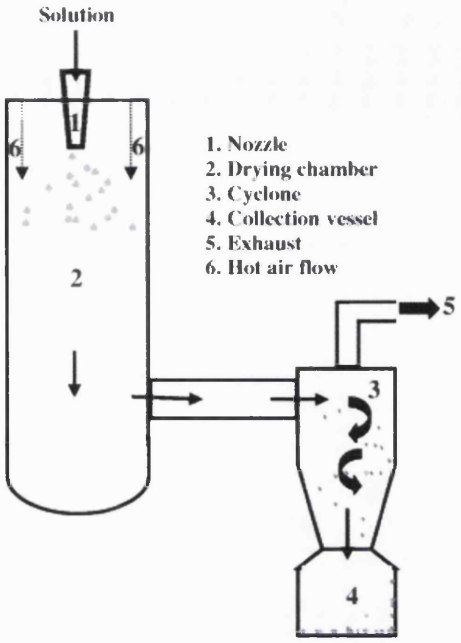

Figure 1.9: Schematic diagram of the spray drying process (Chan and Chew, 2003)

Reducing the airflow will lengthen the time for the droplets to evaporate and the drying efficiency will also be reduced, as less air is available to evaporate the droplets. Moreover the collection efficiency of the cyclone is decreased. On the other hand, higher airflow will evaporate the droplets more rapidly, resulting in a less crystalline product, hampering the production of stable fine particles. The problems of processing drugs susceptible to oxidation can be minimized by using an inert gas instead of air, or if feasible, use of an anti-oxidant. It should be noted that the product yield by spray drying is very low, depending on the particle size range and the cyclone collection efficiency (Chan and Chew, 2003).

1.8.3. Factors affecting the physicochemical properties of the polymeric particles

The physicochemical factors like the particle size, surface charge and the encapsulation efficiency of the polymeric particles is affected by a number of factors.

1.8.3.1. Particle size

Particle size is an important parameter affecting the immunogenicity of microparticles, since smaller microparticles (<10 μm) can be significantly more immunogenic than larger ones. The effect of particle size on immunogenicity is likely to be a consequence of enhanced uptake into lymphatics and greater access to antigen-presenting cells for the

smaller sized particles. Tabata *et al* (1996) have evaluated OVA loaded PLA microparticles with a range of mean sizes (0.6-26 μm) and concluded that 4 μm microparticles were optimal for the induction of serum immune responses, however, 7 μm particles were optimal for mucosal IgA responses. However, this does not mean that the particles less than 4 μm are not effective. The role of smaller sized particles has been discussed in subsequent chapters.

The droplet formation step determines the size and size distribution of the resulting particles. This parameter may affect the antigen/drug release, antigen encapsulation efficacy, *in vivo* uptake by phagocytic cells and biodistribution of the particles after subcutaneous injection or intranasal administration (Freitas *et al.*, 2005). The size tends to decrease by increasing stabilizer concentration and mixing speed, as it produces smaller droplets through stronger shear forces and increased turbulence. In contrast, the size increases with high polymer concentrations because the viscosity of the polymer solution is enhanced. The particle mean diameter also depends upon the mixing methods used to prepare the first and the second emulsions (Jeffery *et al.*, 1993; Yan *et al.*, 1994; Prieto *et al.*, 1994; Fattal *et al.*, 1997). Morphological observation of the particles revealed that the size is temperature dependent; when low and high temperatures were used the particle size was found to be larger where as intermediate temperature yielded smaller particles (Yang *et al.*, 2000). In another study it was observed that the low temperature increased the viscosity of the polymer solution therefore yielded larger sized particles (Jeyanthi *et al.*, 1997).

1.8.3.2. Surface charge

The surface charge of the particles can be influenced by the type of polymer and the stabilizing agent used. The modification of surface characteristics such as the surface charge and mucoadhesive properties, in the form of chemical modifications of the polymer or in the form of coatings, may profoundly affect the efficiency with which the microparticles target the delivery of bioactive agents to mucosally associated lymphoid tissues and to antigen presenting cells. Many coatings have been used and examples include chemicals, polymers, antibodies, bioadhesives, proteins, peptides, carbohydrates and lectins (Baras *et al.* 1999; Delgado *et al.* 1999; Kaiserlian and Etchart 1999).

1.8.3.3. Encapsulation efficiency

The primary emulsification has an important role in the final encapsulation efficiency and for controlling the internal structure of microparticles prepared using the w/o/w solvent evaporation technique (Rafati *et al.*, 1997; Blanco and Alanso 1998; Tamber *et al* 2005; Vlugt-Wensink *et al.*, 2007). Ghaderi *et al.*, (1996) showed that lipids can lower the encapsulation efficiency. In this study phosphatidylcholine (PC) has shown to decrease the encapsulation of mannitol. This can be attributed to the PC which enhances the emulsification resulting in the decrease of the droplets formed during the primary emulsion resulting in the diffusion of mannitol to the external phase subsequently decreasing the encapsulation. The type of polymer used can also affect the encapsulation. It was observed that PGA has higher encapsulation efficiency compared to PLA although similar conditions of preparation were used. This can be attributed to the faster precipitation of the PGAs at the sphere interface. Several publications have stressed the importance of the stage for increasing entrapment efficiency.

1.8.4. Biodegradable polymers for particulate carriers

Biodegradable polymers are composed of monomers which degrade in the body by enzymatically or non-enzymatically producing non toxic bye products while releasing the dissolved/ dispersed antigen or drug. The non-toxic bye products are released from the body by physiological pathways. These polymers have high tensile strength, controlled degradation rates, hydrophilicity and proven non toxicity making them the most preferred polymers for the preparation of polymeric particulate carriers (Sinha and Trehan, 2003). They can be broadly classified as following:

- Natural polymers
    - Proteins (albumin, globulin, gelatine)
    - Polysaccharides (chitosan, dextran, starch)
- Synthetic polymers
    - Polyorthoesters
    - Polyanhydrides
    - Polyamides
    - Polyalkylcyanoacrylates
    - Polyesters (lactides/glycolides, polycaprolactones)

- Polyphosphazenes
- Polyaminoacids

Natural polymers such as starch, alginate, chitosan, albumin, gelatin or cellulose are widely used in the encapsulation of antigens/drugs. The advantage of natural polymers is that they do not require the harsh conditions of heat and/or organic solvents such as dichloromethane (DCM) for encapsulation of antigen. However, the natural polymers may also be limited in their use due to the presence of extraneous contaminants, variability from lot to lot and low hydrophobicity. In contrast, synthetic polymers are more reproducible and can be prepared with the desired degradation rates, molecular weights and co-polymer compositions (Babiuk *et al*, 2000).

Among the synthetic biodegradable polyester polymers (Figure 1.10) are the most widely and extensively used polymers for drug delivery.

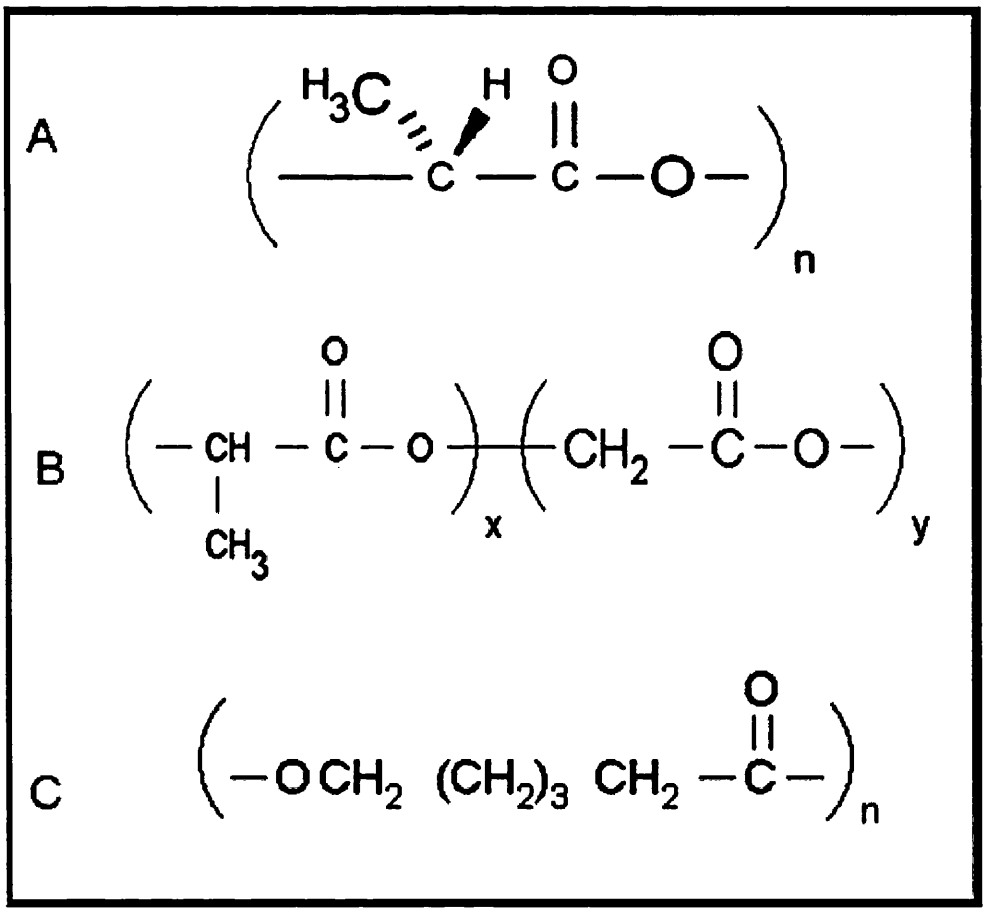

Figure 1.10: Chemical structure of polyester polymers. A: Poly (lactide) acid; B: poly (glycolic-lactide) acid; C: Polycaprolactone (from SigmaAldrich Inc catalogue).

During this project the polyesters PLA and PCL were used for the preparation polymeric particles, therefore only these polymers are discussed here. There are a number of reviews for further information on polymers used in the encapsulation of antigens/drugs.

1.8.4.1. Poly (lactide) acid

Poly (lactide) (PLA) and poly (lactide-co-glycolide) (PLGA) are the most popular polymeric candidates for the development of micro/nanoparticles as vaccine delivery systems. PLA is known for biocompatibility simple biodegradability (by hydrolysis) and is available in L, D and DL isomers. PLGA can be formed by adding glycolic acid to lactic acid. Depending upon its molecular weight and composition of the copolymers the degradation kinetics and physical properties can be modified. (L) PLA is crystalline therefore has better mechanical properties and higher degradation time compared to the amorphous (D) PLA. The humoral adjuvant effect achieved by the entrapment of antigens in biodegradable PLGA microparticles has been demonstrated in early 1990's. Even microparticles with adsorbed antigens were effective adjuvants, leading to the possibility of using these polymers for encapsulating the antigens with the potential for the development of single-dose vaccines (Gupta and Kumar, 2007).

The major drawback of PLGA polymers is that their degradation generates extreme acid environment (pH 2-3) leading to the concerns of antigens loosing their structural integrity and antigenicity. The biodegradation of PLGA occurs by homogenous bulk erosion, the polymer chains are cleaved by hydrolysis until water-soluble fragments are produced, followed by the monomeric acids (lactic acid and glycolic acid), which are eventually eliminated from the body as CO_2. Similarly, the degradation product of PLA polymer is lactic acid which also renders an acidic environment compromising the structural integrity of the antigen. Polymer degradation kinetics is determined by the hydrophilicity, morphology, molecular weight, porosity of the microspheres and polymer end groups of the polymer. The more hydrophilic PLGA has the fastest degradation rate (PLGA 50:50 low molecular weights degrades in about 50-90 days), while PLA 100 KDa requires about 12-14 months to completely degrade. Polymer erosion and antigen diffusion determine the release rate of the encapsulated material. First of all, the release rate increases with a decrease in particle size as a result of the increase in surface area. The rate of drug release also tends to increase with increased

drug loading. Moreover, the release rate depends upon factors such as aqueous solubility of the drug and the macroscopic porosity of the matrix. Eventually, additives included in the matrix may enhance drug release rate (Gander *et al*, 1993; Göpferich, 1996; Göpferich *et al.*, 1996; Ruan and Feng, 2003; Vlugt-Wensink *et al.*, 2007). Another significant concern is the possibility of antigen denaturation as a consequence of exposure to organic solvents, high shear, aqueous-organic interfaces and localized elevated temperatures during the encapsulation. However, a number of proteins have been successfully entrapped in PLGA microparticles with an intact full structural and immunological integrity.

1.8.4.2. Polycaprolactone

Polycaprolactone (PCL) is a semi-crystalline biodegradable polymer. The molecular formula is $C_6H_{10}O_2$ and physically it appears as white beads. Comparatively it is less expensive and offers many advantages over other biodegradable polyesters like polyglycolide, polylactide and their copolymers (Lin *et al,* 1999; Lin and Lu, 2002). It has a low glass transition temperature (Tg) of -60°C and melting point of about 60°C. It is permeable to low molecular weight drugs (<400Da), thus can be used in diffusion controlled delivery systems. PCL is non-toxic, non-mutagenic and shows minimal or insignificant tissue reaction (Cha and Pitt, 1990). The degradation of PCL is a two step bulk process. Initially there is autocatalysis wherein the liberated carboxylic acid end groups catalyze the hydrolysis of additional ester groups. This is followed by decrease in the rate of chain scission and the onset of weight loss resulting in low molecular weight polymer fragment which undergoes phagocytosis (Sinha *et al.*, 2004).

The main drawback with the PLA and PLGA polymers is their high cost which limits their use in providing a cost-effective controlled delivery system. Therefore, PCL being a cheaper polymer can be an alternative to PLA and PLGA polymers. PCL is under clinical evaluation worldwide for sustained delivery of levonorgestrel (Capronor^{TM}), a one year contraceptive (Baras *et al,* 2000). In optimum conditions, smooth and spherical PCL microparticles with encapsulation efficiency of approximately 50-55% were prepared. In this study it was stated that PCL, with numerous desirable characteristics could be an exciting alternative for the controlled release of proteinaceous compounds. In another study Baras *et al,* has investigated various technological parameters on spray-dried BSA loaded PCL microparticles (Baras *et al,* 2000). Here the maximum

entrapment efficiency of ~ 95% was achieved with particle mean size ranging from 9 to 24 μm. Benoit *et al,* have prepared PCL microparticles with a mean size between 5 and 10 μm by double emulsion method, using BSA as a model antigen with maximum entrapment efficiency of 30% (Benoit *et al,* 1999). Baras *et al,* have compared PCL and PLGA microparticles loaded with same antigen, and showed that PCL is capable of delaying the systemic immune responses after oral or nasal administration (Baras *et al,* 1999). PCL based nanocapsules encapsulating spironolactone were prepared with a high encapsulation efficiency and stable for 6 months. This formulation was prepared with an intention to use in the paediatric population (Blouza *et al.*, 2006).

1.9. Diphtheria toxoid

Diphtheria is a communicable disease caused by a Gram-positive bacillus *Corynebacterium diphtheriae* that produces diphtheria toxin, which causes systemic toxaemia (www.nih.go.jp). Two types of diphtheria can be distinguished: tonsillar or pharyngeal. It spreads by air and is characterised by a pseudo membrane covering the posterior pharynx (Illum *et al*, 2001). Diphtheria starts with non-specific symptoms like low-grade fever, vomiting, cough and sore throat. The disease develops because diphtheria toxin is absorbed on various organs, particularly the myocardium. The main cause of death from diphtheria is cardiovascular collapse with cardiovascular damage in the early stage, followed by myocardial disorder within 2-4 weeks (www.nih.go.jp). The mortality rate is 10 times lower when an immunised person develops diphtheria, compared with non-immunised patients. The available vaccines are given by parenteral route (Illum *et al*, 2001). Diphtheria vaccine (diphtheria toxoid) is manufactured from diphtheria toxin treated with formalin to inactivate the toxicity and to maintain the immunogenicity (www.nih.go.jp). Diphtheria toxin, secreted by certain strains of *Corynebacterium diphtheriae*, catalyzes the ADP-ribosylation of eukaryotic aminoacyl-transferase II (EF-2) using NAD as a substrate. This reaction forms the basis for its toxicity towards eukaryotic organisms. Diphtheria toxin is composed of a single polypeptide chain having an approximate molecular weight of 63,000 Da (Collier and Kandel, 1971).

In most industrialized countries, diphtheria has disappeared or become extremely rare thanks to the vaccination programme. However, recent outbreaks of diphtheria in the former Soviet Union have shown the importance of immunizing children worldwide and

to maintain high rate of vaccination among adults in endemic areas. Moreover, the current UK Department of Health guidelines recommend a minimum of five doses of DT vaccine regardless of age at which immunization was started (Cameron *et al*, 2007). Therefore, a biodegradable polymeric carrier which can decrease the number of immunizations for the DT can be of enormous benefit.

1.10. Hepatitis B

Hepatitis B is one of the major diseases of mankind and is a serious global public health problem (World Health Organization, 2000). More than 2 billion people alive today have been infected at some time in their lives, about 350 million people worldwide are chronically infected by the virus and one million people a year die from chronic active hepatitis, cirrhosis or primary liver cancer (World Health Organization and Vaccines and Biologicals Department, 2001). The severe pathological consequences of chronic infection are hepatic insufficiency, cirrhosis and hepatocellular carcinoma (HCC) (Robinson, 1994; Vierlong, 2007).

Hepatitis B immunization strategies and programs vary in several aspects from country to country and each country develops an immunization program tailored specifically to suit its population. In the Western Europe and USA, vaccination efforts have focused on prevention of prenatal infections and vaccination of adolescents, with less stress on infant immunization, while in Eastern European countries, the impetus is on immunization of infants. Thus, the immunization programs are dictated by patterns and variation in the incidence of the disease. The first vaccination programs were aimed initially only at high risk groups such as newborns of carrier mothers and drug users, but this strategy was marked by several drawbacks, the most important being that in almost 25% of hepatitis B cases, there are no identifiable risk factors. This has led to the understanding that proper control of the disease cannot be successfully and adequately achieved by targeting only certain high-risk groups and there is a need for a universal immunization strategy targeting the general population including infants as well as adolescents (Alter, 2003).

The hepatitis B surface antigen (HBsAg) is a 24,000 Da glycoprotein having an isoelectric point of 4.5. The currently used adjuvant in the marketed product of the hepatitis B vaccine is alum (Iyer *et al.*, 2004). Despite the availability of an effective

vaccine, the high cost of the vaccine, the geographical location of the population at risk and the multiple injection schedule (the commonly used immunization schedule is three injections given at 0, 1 and 6 months at either 10 or 20 μg dose) of the currently available vaccines have resulted in under utilization of the vaccine. The development of a delivery system for the hepatitis B vaccine (HBV) which could include the desired antibody response from a single injection would be an enormous benefit.

1.11. Overall Project Aims

The overall aim of the project was to combine the advantages of the biodegradable polymeric particles with various adjuvants to formulate a carrier system for antigens (DT and HBsAg) for nasal and parenteral delivery. The project is divided into three sections:

- Preparation of PLA or PCL micro/nanoparticles co-encapsulating the antigen and adjuvants; the different types of adjuvants used were aluminium hydroxide, chitosan, melittin, cholic acid, deoxy cholic acid, melittin, tocopherol-PEG-succinate, tocopherol acetate and tocopherol nicotinate.

- *In vitro* physicochemical characterization of the particulate carriers with respect to size, charge, morphology, antigen integrity, encapsulation efficiency, uptake and toxicity in the cell lines.

- *In vivo* studies of the prepared formulations in mouse model and subsequent analysis of the immunological responses.

A detailed outline of the aims and objectives is outlined in the subsequent chapters.

Chapter 2

Materials and Methods

2. Materials and Methods

A general outline of the materials and methods used for this project is presented below. Any changes or specific methods are outlined in the relevant chapters.

2.1 Materials

The following chemicals and reagents were used in the present work (Table 2.1); all the other solvents were of analytical grade.

Table 2.1: General materials used during the project

Name	Molecular weight	Lot number	Manufacturer
Diphtheria toxoid	63K	FA 006807	Pasteur merieux, Connaugent, France.
Hepatitis B Surface Antigen	24K	19D04	Shantha Biotechnics, India.
Albumin Chicken Egg (OVA) Grade V	45K	71K7028	Sigma-Aldrich Co., USA
TPGS 1000	-	80003000	Eastman
(±)-α-Tocopherol nicotinate	-	064K0766	Sigma-Aldrich Inc. USA
DL-α-Tocopherol acetate	-	074K1330	Sigma-Aldrich Inc. USA
Poly L lactide	2K	B00577	Lactel, Birmingham Polymers Inc., UK
Poly L lactide	80K	L00483	Lactel, Birmingham Polymers Inc., UK
Poly L lactide	120K	D00075	Lakeshore Biomaterials Inc, USA
Poly L lactide	147K	L42700	Birmingham polymers Inc, USA
Polycaprolactone	10K	03218 AF	Aldrich Chemical company, Inc, UK
Polycaprolactone	40K	78428 SE	Aldrich Chemical company, Inc, UK
Polycaprolactone	80K	45684 SA	Aldrich Chemical company, Inc, UK
Zinc sulfate 0.3 N solution,	-	48309 Z	Sigma Aldrich, UK
Zinc oxide	-	3856 PE	Sigma Aldrich, UK
Poly (vinyl alcohol) (PVA)	13-23K	BO 11725 BI	Sigma-Aldrich Co.
Chitosan	Low MW	407568/1	Fluka
Kollicoat®	45K	0573414	Basf
Cholic Acid ($C_{24}H_{40}O_5$)	408.6K	128F0819	Sigma-Aldrich Co. USA
Lithocholic Acid ($C_{24}H_{40}O_3$)	376.6K	101K1109	Sigma-Aldrich Co. USA
Alhydrogel® 2.0% Aluminium Hydroxide Gel	-	2949	Superfos Biosector, Denmark
QuantiPro® BCA Assay kit	-	125K6803	Sigma-Aldrich Co. USA

SimplyBlue™ SafeStain (Commassie® G-250)	-	1300019-1	Invitrogen UK
Coomassie Brilliant Blue	-	83641A	Bio-Rad Labs, USA
Silverquest® (silver stain)	-	1208653	Invitrogen life technologies, USA
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]	-	Various	Sigma-Aldrich, UK
L-glutamine and penicillin/streptomycin	-	Various	Sigma Aldrich, UK
RPMI media	-	Various	Gibco, UK
Fetal bovine serum	-	Various	Gibco, UK
DuoSet® sandwich ELISA kits	-	Various	R&D Systems, UK
Horseradish peroxidase conjugated Goat anti-mouse isotype specific immunoglobulin	-	Various	Serotec, UK
Dichloromethane	-	Various	BDH, UK
Acetic acid	-	Various	BDH, UK

2.2. Methods

2.2.1. Preparation of micro/nanoparticles

2.2.1.1. Preparation of micro/nanoparticles by double emulsion (w/o/w) solvent evaporation method

The theory and principle of the double emulsion (w/o/w) solvent evaporation method is explained in chapter 1 and further reading can be found in a number of review articles (Watts *et al.*, 1990, Conti *et al.*, 1992, O'Donnell and McGinity., 1997, Ogawa., 1997., Crotts and Park., 1998., Freitas *et al.*, 2005, Singh *et al.*, 2006). Unless otherwise stated, poly (vinyl alcohol) (PVA) with an average MW of 13,000-23,000 Da and 87-89% hydrolyzed was used as the stabilizing agent in the preparation of the micro/nanoparticles. PVA is an amphiphilic water-soluble polymer which attaches onto the polymer particles with its hydrophobic groups while the hydrophilic groups stretch out in the water phase to provide stability to the dispersion. PVA has a relatively simple chemical structure with a pendant hydroxyl group (Figure 2.1). Its monomer, vinyl alcohol, does not exist in a stable form but it rearranges itself to its tautomer, acetaldehyde.

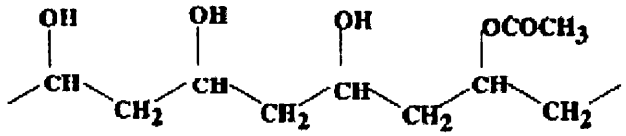

Figure 2.1: Chemical structure of poly (vinyl alcohol) chain (Rafati *et al.*, 1997)

PVA is produced by the polymerization of vinyl acetate to poly (vinyl acetate) (PVAc), followed by hydrolysis of PVAc to PVA. The hydrolysis is not complete and the resulting polymers show a degree of hydrolysis which depends on the extent of reaction. Therefore, PVA is always a copolymer of PVA and PVAc. The commercially available PVA has high degree of hydrolysis (above 98.5%). The degree of hydrolysis or the content of acetate groups in the polymer has an overall effect on its chemical properties, solubility and crystallizability. A high degree of hydrolysis decreases the solubility of PVA in water and the rate of crystallization (Hassan *et al.*, 2000).

The general method of preparation of protein/antigen loaded polymeric micro/nanoparticles is described below; the detailed process is described in the subsequent chapters. The theoretical loading of the protein in the double emulsification solvent evaporation process used was 1 or 2% m/m of the polymer. Approximately 100 mg of the polymer was dissolved in 6 ml of dichloromethane (DCM) to prepare the organic phase. The primary water-in-oil (w/o) emulsion was formed by dispersing 10% m/v of PVA along with the protein solution (internal phase) in the organic phase using an Ultra-turrax at 24,000 rpm for two minutes. This w/o emulsion was added drop by drop to 30 ml of 2.5% m/v of PVA solution and homogenized for six minutes at 10,000 rpm using the Silverson homogeniser (Silverson, model L4RT). The formed water-in-oil-in-water (w/o/w) emulsion was left stirring for 4 hours using a magnetic stirrer under the fume hood to evaporate the organic solvent. This emulsion was centrifuged (20,000 rpm, 45 minutes, 4 °C) to obtain a pellet of micro/nanoparticles which was resuspended in 4 ml of water and freeze-dried (Virtis, England) to obtain a fine powder of micro/nanoparticles.

2.2.2. Preparation of microparticles by the spray drying method

The theory and principle of preparation of microparticles by the spray drying method is explained in chapter 1. The microparticles were prepared using a double emulsification process followed by spray drying of the emulsion. Water-in-oil (w/o) emulsion was

produced and then further emulsified to get a water-in-oil-in-water (w/o/w) emulsion. The protein/antigen solution in 750 µl of 7.5% m/v of PVA was emulsified in a solution of 250 mg polymer dissolved in 5 ml dichloromethane for 2 minutes at 24,000 rpm (Ultra turrax, T25 Janke & Kunkel, IKA-Labortechnik). This primary emulsion was poured into 30ml of 2.5% m/v of PVA (MW = 13,000-23,000 Da and 87-89% hydrolyzed) to produce a water-in-oil-in-water (w/o/w) emulsion using a Silverson homogeniser. The homogenization was carried out for 5 minutes at 10,000 rpm. This secondary emulsion was spray-dried with a Mini Büchi B-191 laboratory spray-dryer (Büchi Laboratorium AG, Switzerland) using a 0.5 mm nozzle. The process parameters were set as follows: inlet temperature (40 °C ±2 °C); outlet temperature (26 °C ±2 °C); aspirator setting (50%); pump setting (5 ml/minutes) and spray flow (600 litres/minutes). These process parameters were optimised with respect to the product yield and encapsulation efficiency of the particles. Microparticles in powder form were stored at room temperature in a desiccator until characterization.

2.2.2. Particle Characterization

2.2.2.1. Determination of nanoparticle size by photon correlation spectroscopy (PCS) method

Photon correlation spectroscopy (PCS) is also known as Dynamic Light Scattering (DLS) or Quasi-Elastic Light Scattering (QELS) which is used to measure the particle sizes in sub micron size. It involves measuring the variation in the scattered light intensities over time. PCS measures the Brownian motion of the particles relating it with the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules. The scattered intensity can be superposition of waves scattered from individual scattering centres subjected to Brownian motion. As the scattering particles move towards or away from the detector there is a shift in the scattered light frequency which generates frequencies proportional to the magnitude of the shift. The lesser the particle size the more it moves resulting in greater Doppler shifts and frequency fluctuations (van Wuyckhuyse *et al*, 1996). The velocity of the Brownian motion is defined as the translational diffusion coefficient (D) from which the size of the particle is calculated by using the Stokes-Einstein equation;

$$d(H) = \frac{kT}{3\Pi\eta D}$$

where, d (H) = hydrodynamic diameter, k = Boltzmann's constant, T = absolute temperature, η = viscosity and D = translational diffusion coefficient

The diameter measured refers to how the particle diffuses in the fluid so it is referred to as hydrodynamic diameter. The diameter of the particle has the same translational diffusion coefficient as the particle. The translational diffusion coefficient of the particle is affected by the surface of the particle and ionic strength of the medium. The ions and the ionic strength of the medium change the thickness of the electric double layer (Debye length, K^{-1}) around the particle therefore affecting the particle diffusion speed. Lowering the conductivity of the medium increases the double layer and therefore reduces the diffusion speed resulting in a higher hydrodynamic diameter. If the particle shape is not spherical but it does not affect the diffusion speed then there is no change in the hydrodynamic size. For example, changes in the diameter of a rod shaped particle, do not affect the diffusion speed whereas a change in the rod length will affect the diffusion speed and therefore the hydrodynamic diameter (Malvern, Technical sheet, 2006). The PCS apparatus involves shining a polarized laser beam through the sample and measuring the scattered radiation. An autocorrelator stores the scattered light intensities at different times and calculates a correlation function that decays exponentially with time. These correlation functions are deconvolved using a computer program called CONTIN. The results are obtained as average particle size and polydispersity. An example of a report output obtained from the Malvern ZetaMaster (Malvern Instruments, Malvern, UK) is shown in Figure 2.2.

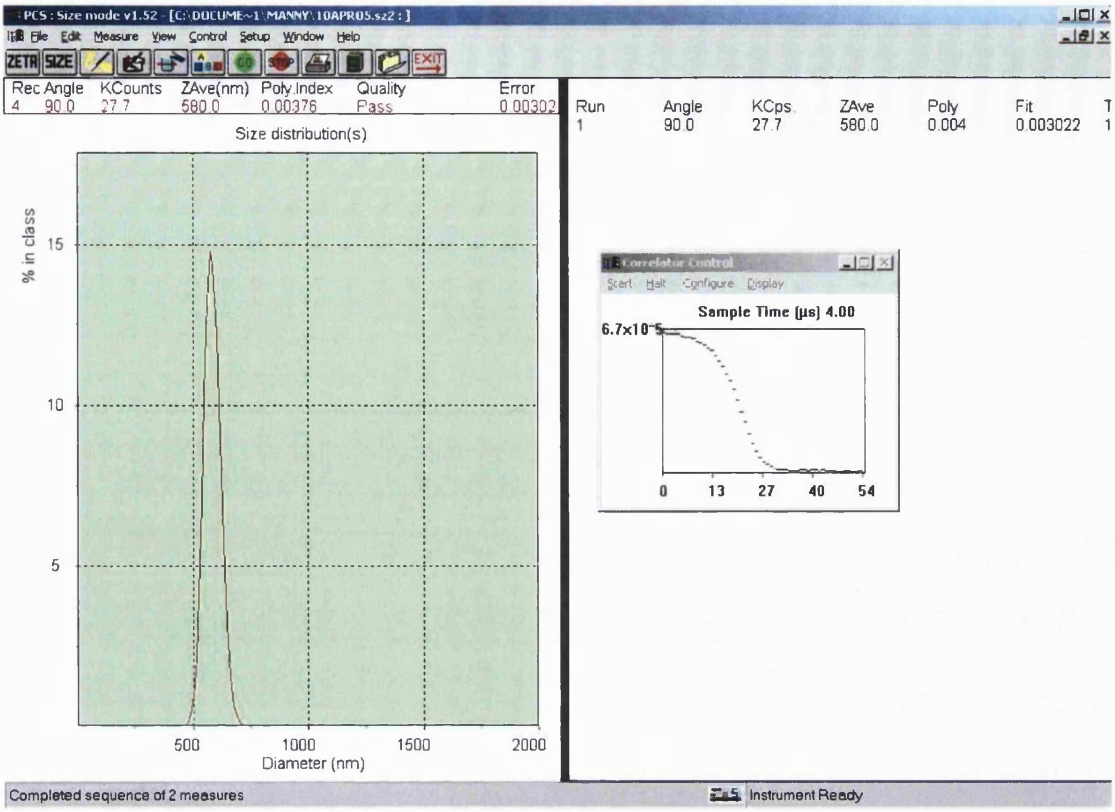

Figure 2.2: Example of a report output for the measurement of particle diameter from ZetaMaster (Malvern, UK). The Z-ave (nm) and the polydispersity of the samples were recorded.

Approximately 5 mg of the nanoparticles were dispersed in 5 ml of ddH2O and was placed into a clear-sided acrylic cuvette for analysis. It was ensured that all samples passed the minimum criteria set by Malvern instruments with regard to polydispersity, count rate, merit (signal to noise ratio). Values were represented as Z-average diameter.

2.2.2.2. Determination of microparticle sizes by laser diffraction

The mean volume diameter (MVD) of the particles was measured with a Malvern Instruments MasterSizer which is equipped with a 5mW Helium-Neon laser (632.8nm). Depending on the type of dispersion used, this instrument measures particles with a diameter range of between 0.02 µm to 2,000 µm using the Mie theory. The software used to collect the data was MasterSizer-S v2.18.

Briefly, a drop of each sample was dispersed with 15 ml of 0.2 µm filtered, distilled water in the small volume magnetically stirred cell. The 300F lens with a sizing range of 0.5-900 µm was selected along with an active beam length of 14.3 mm; percentage obscuration (20% was ensured for each sample to enhance reproducibility). Results were analysed by the Fraunhofer model and are presented as MVD (µm). The width of

the distribution can be calculated (termed Span) by the following equation:

$$\text{Span} = \frac{D\,[v, 0.9] - D\,[v, 0.1]}{D\,[v, 0.5]}$$

Where, D [v, 0.9] is the point at which the diameters are cut off at 90% of the distribution; D [v, 0.1] is the point at which the diameters are cut off at 10% of the distribution and D [v, 0.5] is the volume median diameters. Span does not have any units; however it gives an idea of whether the distribution is narrow or wide. An example of a report output is shown in Figure 2.3.

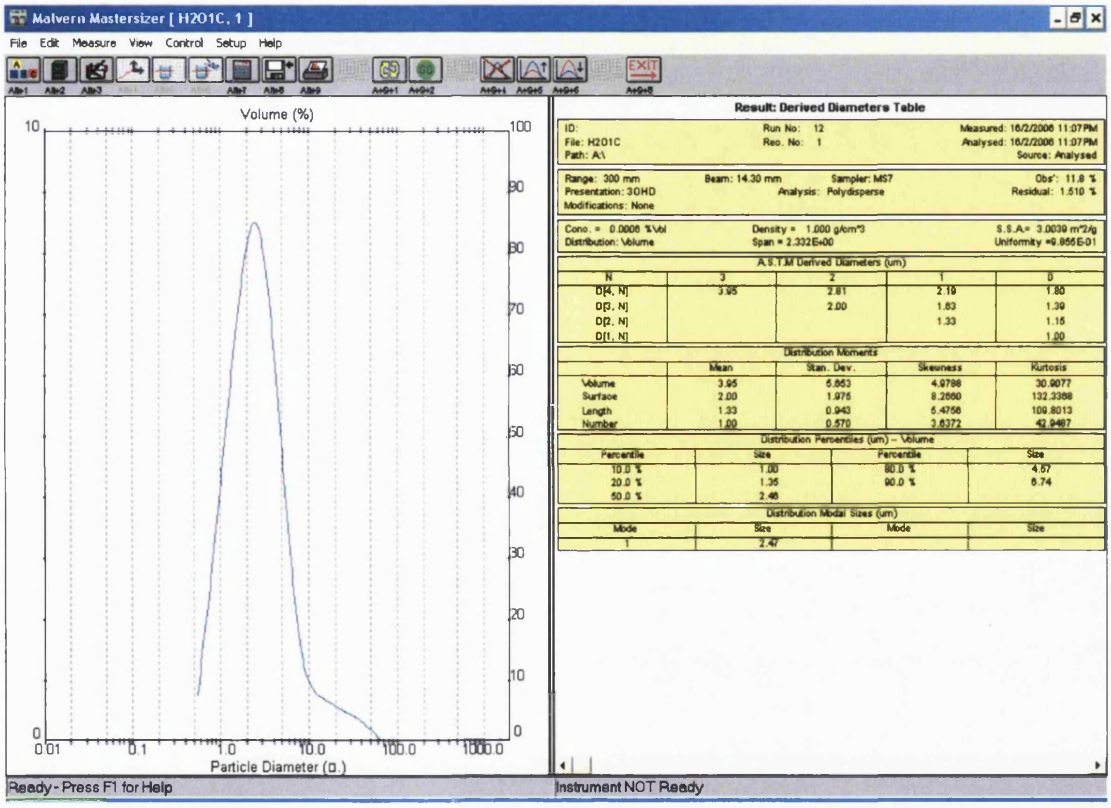

Figure 2.3: Example of a report output for the measurement of particle diameter using MasterSizer (Malvern, UK). The mean volume diameter (MVD) [D 4, 3], and the D [v 0.1], D [v 0.5] and D [v 0.9] were recorded.

2.2.2.3. Measurement of zeta-potential of micro/nanoparticles

Colloidal systems are generally not stable and the surface charge of particles plays an important role in the colloidal behaviour and their cellular interaction. The zeta potential of a particle is a measure of the surface charge on that particle and is also an indication of the likelihood of aggregation between the particles. If the zeta potential is highly negative or highly positive then the likelihood of the particles aggregating will be lower because they will repel each other. Zeta potential represents an index for particle

stability. High zeta equals stable particles. Physically stable nanosuspensions solely stabilized by electrostatic repulsion will have a minimum zeta potential of -30 mV. This stability is important in preventing aggregation. Zeta potential measurements utilise electrophoresis which involves the application of an electric field across an electrolyte, leading to the attraction of ions/particles to the oppositely charged electrode. The viscosity of the medium opposes this mobility and on achieving equilibrium between the two forces the ions/particles move with constant velocity. This is known as the electrophoretic mobility. The electrophoretic mobility is calculated and converted to a zeta potential by the software (PCS v.1.52) using Henry's equation (or Smoluchowski's approximation to Henry's equation if a polar solvent is used). The suspending medium and pH affects the zeta potential measurements. Ionic liquids suppress the electrical bilayer and therefore reduce the zeta potential. Similarly the pH as it contributes to the extent of ionisation of the particles. An acidic pH renders more positive charge where as alkaline pH renders more negative charge.

A sample (2-3 mg) of micro/nanoparticles was suspended in 10^{-3} M potassium chloride to produce a dilute suspension and the average zeta potential (mV) was determined in triplicate. Example of a report output obtained from ZetaSizer (Malvern Instruments Ltd, UK) is shown in Figure 2.4.

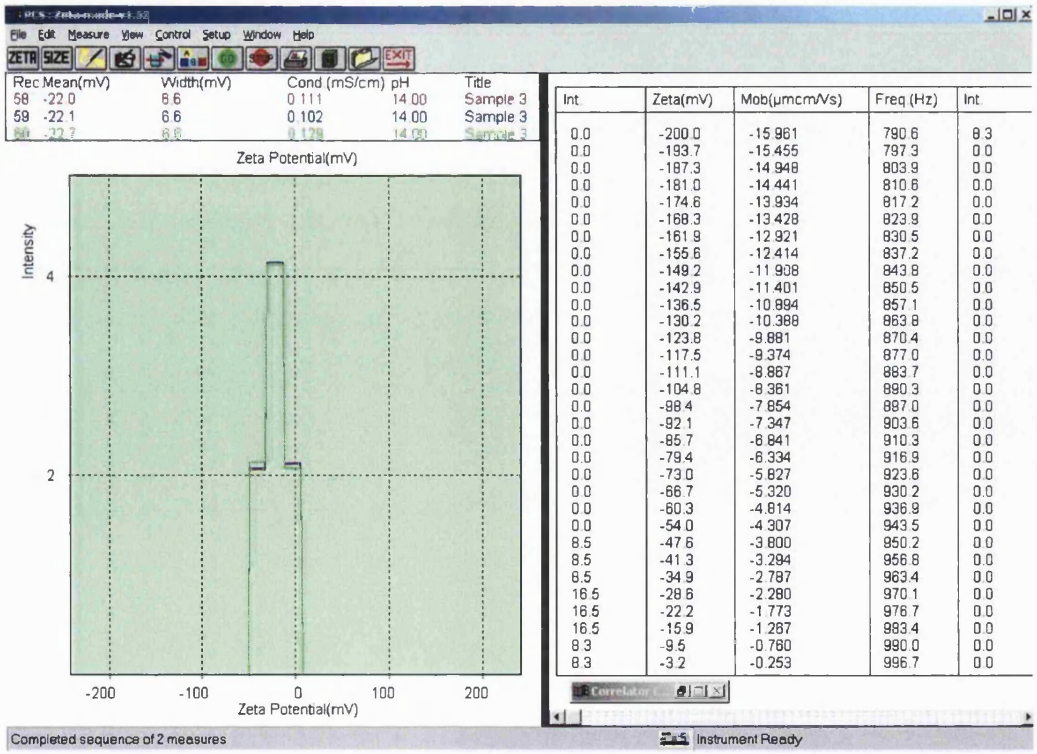

Figure 2.4: Example of a report output from for the measurement of zeta potential using ZetaMaster (Malvern, UK). The zeta potential (mV) was recorded.

2.2.2.4. Determination of surface morphology of micro/nanoparticles by scanning electron microscopy (SEM)

Scanning electron microscopy (SEM, Phillips/FEI XL30) was used to analyse the surface morphology of the micro/nanoparticles. A thin layer of micro/nanoparticles was mounted onto aluminium stubs using adhesive carbon pads either as a dry powder or in a dilute suspension and dried onto the surface of the support. The samples were then coated with a thin layer (approximately 20 nm) of gold under vacuum in an argon atmosphere using a sputter coater (Emscope SC500).

2.2.2.5. Freeze-drying of polymeric particles

Aliquots of diluted solutions of washed polymeric particles were dispersed into glass vials and frozen at -70 °C for 2 hours. After freezing, the micro/nanoparticle samples were freeze-dried using a Virtis advantage freeze dryer (Virtis, UK). The thermal stage was set at -20 °C for 2 hours and the primary drying was carried on for 14 hours at 200 psi. The secondary drying was set at 10 °C for 8 hours at a reduced pressure of 50 psi. Dried polymeric particles with encapsulated antigen were stored at room temperature in a desiccator.

2.2.2.5. Quantification of protein/antigen in polymeric particles using the bicinchoninic acid protein assay (BCA)

The quantification of protein/antigen in polymeric particles to determine the encapsulation efficiency was performed using the bicinchoninic acid (BCA) protein assay. The BCA assay is a rapid, sensitive and reproducible method of protein determination. This technique offers manipulative simplifications, more tolerance toward interfering substances, greater working reagent stability, increased sensitivity, and greater protocol flexibility when compared to the standard Lowry assay (Smith *et al.*, 1985).

The principle of the assay relies on the ability of protein, placed under an alkaline system containing copper II (Cu^{2+}), to form a Cu^{2+} - protein coloured complex followed by reduction of the Cu^{2+} to copper I (Cu^{1+}). The amount of reduction is proportional to the amount of protein present in the solution. Protein concentration also determines the

intensity of colour changing from green to purple. Substances such as uric acid, glucose, cysteine, cystine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+}. Bicinchoninic acid forms a 2:1 complex with the Cu^{1+} ion in alkaline environments (Figure 2.5), resulting in a stable, highly purple-blue coloured chromophore with a spectrophotometric maximum absorbance at λ 562 nm.

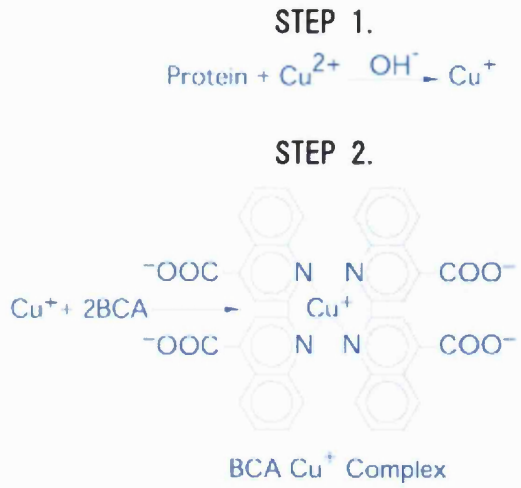

Figure 2.5: Bicinchoninic acid protein assay. Two-step formation of 2:1 purple complex between BCA and cuprous ion (Cu^{1+}) (Sigma Aldrich Inc catalogue)

This reaction can provide a basis to monitor the reduction of alkaline Cu^{2+} by proteins and therefore their quantification by measuring the absorption spectra and comparing them to protein solutions with known concentrations (Sigma-Aldrich catalogue, 2005; Smith *et al.*, 1985).

Depending on the protein concentration, two different types of BCA Assay Kits are available from the manufacturer Sigma Aldrich, UK: (a) The Bicinchoninic Acid Protein Assay Kit, which gives a linear response at high concentrations of protein, from 200 to 1000 µg/ml (b) The QuantiPro® Bicinchoninic Acid Assay Kit, which gives a linear response from 0.5 to 30 µg/ml.

As the protein concentration in the micro/nanoparticles was low (0-10 µg/mg of the particles), the QuantiPro® BCA Assay Kit was used in these experiments. This kit consists of three reagents, Buffer QA (an aqueous solution of 8% sodium carbonate ($Na_2CO_3 \cdot H_2O$), 1.6% of sodium tartrate, sufficient sodium bicarbonate ($NaHCO_3$) to adjust the pH to 11.25 and 1.6% of 0.2 M sodium hydroxide (NaOH)), Reagent QB (4% m/v BCA-Na_2 (water-soluble sodium salt of bicinchoninic acid) in deionized water, pH 8.5) and Reagent QC (pentahydrate solution of 4% m/v Copper(II) sulfate

[$CuSO_4 \cdot 5H_2O$]). The QuantiPro® Working Reagent is prepared by mixing 25 parts of Reagent QA with 25 parts of Reagent QB. After the two reagents are combined, 1 part of Reagent QC is added and the mixture is gently shaken until it appears uniform in colour.

Approximately 5 mg of the micro/nanoparticles were digested in 2 ml of 5% m/v SDS in 0.1 M sodium hydroxide solution in glass vials and left under stirring overnight in order to extract the encapsulated antigens. Placebo micro/nanoparticles (without antigen) were used as control. The assay was performed in a 96-well microplate and each well was filled with 150 µl of the standard/protein sample solutions and 150 µl of the prepared QuantiPro® Working Reagent. The microplate was then covered and incubated at 60 °C for 1 hour or at 37 °C for 2 hours. The optical density was read at 570 nm using a Dynax MRX microplate reader (Dynax Technologies, UK). The standard curve is created by plotting the Absorbance (y axis) versus the protein standards concentrations (x axis). Concentrations of 0 to 40 µg/ml were used. The mathematical equation obtained from the standard curve was "y=mx+c" and the concentration of the unknown samples was calculated.

The amount of antigen was determined as percentage according to the manufacturer's instructions. Actual protein/antigen content (AC) and encapsulation efficiency (EE) were calculated using the equations 1 and 2. All analyses were carried out in triplicates and blank micro/nanoparticles were used as control.

$$AC\,(\%) = \frac{M_{act}}{M_{ms}} \times 100 \qquad \text{Eq 1}$$

$$EE\,(\%) = \frac{M_{act}}{M_{the}} \times 100 \qquad \text{Eq2}$$

Where M_{act} is the actual protein/antigen content in weighed quantity of micro/nanoparticles, M_{ms} is the weighed quantity of powder of micro/nanoparticles and M_{the} is the theoretical amount of protein/antigen in micro/nanoparticles calculated from the quantity added in the process.

2.2.2.6. Determination of antigen integrity by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The integrity of the antigen encapsulated in the micro/nanoparticles was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis is based on migration of charged molecules in solution in response to an electric field and it can also be used as a macromolecules separation technique. The rate of migration depends on the strength of the field, on the net charge, size and shape of the molecules and on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. A common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulphate (SDS). SDS is an anionic detergent which denatures proteins and binds them, conferring a negative charge. The disulphide bridges are reduced using 2-mercaptoethanol therefore the migration of the gel is determined by the molecular weight of the protein rather than the electrical charge of the polypeptide. The molecular weight is determined by a pre-stained marker.

Preparation of the gels: The gel casting plates were secured on the casting rig. Water was then poured between the plates to check that they are secure and the gel will not run out when it is cast. The water was removed and 12% separating gel was poured in between the plates and allowed to set. The stacking gel (5%) was then prepared and poured on top of the separating gel. The combs, which mould the wells for the samples, were added and the gel was allowed to set. If necessary, the gels were then placed in the fridge overnight to be run the next day.

Preparation of the samples and the running of the gel: The samples were prepared by adding 40 µl of sample to 40 µl of loading buffer (loading buffer composition: 6 ml of 10% m/v SDS, 4 ml of 50% v/v glycerol, 8.72 ml of double distilled water, 1.24 ml of 0.062M Tris (pH6.8), 40 µl of 5% m/v bromophenol blue). For SDS-PAGE analysis of the microparticles before freeze drying, 20 µl of micro/nanoparticle suspension was used and diluted when it was necessary. These samples were vortexed and heated to 100 °C for 5 minutes. The combs were removed from the plates leaving wells for the addition of samples. The gel-running rig was filled with Tris-Glycine running buffer and the samples (20 µl) were loaded onto the gel. The gel was then run at 150V for 45 minutes using a Bio-Rad 300 power pack.

Staining of the gels: The gels were stained to visualise the proteins using commercially available stainers. If the concentration of protein present is high then the coomassie blue staining method may be used. The gels were fixed for 3 x 20 minutes in a destain solution (40% methanol, 10% glacial acetic acid, 50% water). The gels were then stained with coomassie blue stain (0.1% coomassie blue R250 stain, 50% methanol, 10% glacial acetic acid, 40% water) for 30 minutes. For some of the formulations when coomassie blue stain was unable to reveal any bands, silver staining was used. The de-staining procedure was then performed with the same mixture used for fixation and the gels were stored in distilled water and photographed using a white light transilluminator.

2.2.3. Cell Culture Studies of micro/nanoparticle formulations

2.2.3.1. Culturing of J774A.1 cells (Mouse BALB/c monocyte macrophage)

J774A.1 cells were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). The cells were maintained at 37 °C, 5% CO_2 and 100% humidity levels. The culturing of cells was carried out according to the following procedure.

Growth Media: A 500 ml bottle of Dulbecco's Modified Eagle's Medium (DMEM, Sigma) contains 50 ml foetal calf serum (FCS), an aliquot of Penicillin/Streptomycin antibiotic (100U each) and 2mM of glutamine.

Thawing of cells: An appropriate amount of pre-warmed growth medium was pipetted into a cell culture flask (e.g. 25 cm^2 or 75 cm^2). Frozen cells from the ampoule were pre-warmed and cells were placed drop-wise into the pre-warmed growth medium. The cell culture flask was transferred to a humidified 37 °C incubator, gassed with 5% CO_2. The cell culture was assessed after 24 hours and sub-cultured as necessary.

Sub-culturing (passaging/splitting) cells: When cells achieved 70-90% confluence, they were sub-cultured to avoid the culture dying. Confluence was assessed using an inverted microscope. The growth media was removed, transferred to a sterile tube and centrifuged at 1,500 rpm for 5 minutes to pellet the cells. A cell scraper was used to remove cells that have adhered onto the surface of the flask. Once scraped, a small

amount of medium was added into the flask and cells were assessed under a microscope to ensure that all cells have detached. The centrifuged cell pellet was combined with scraped cells, seeded into new flask at 1:3 to 1:4 split ratios and fresh growth medium was added as required. (e.g. for a 75 cm^2 flask, 15-20 ml was added). Sub-culturing of cells was carried as necessary according to growth characteristics of the cell line, e.g. every 2-3 days. Cells that had grown to a confluency of 70-90% were frozen down in DMEM medium supplemented with 50% serum or 10% dimethyl sulphoxide (DMSO).

2.2.3.2. Uptake studies of nanoparticles by J774A.1 macrophage cells

The J774A.1 macrophage cells were cultured as described in section 2.2.3.1 and subsequently used for the uptake studies of the nanoparticles. The media was removed from wells using a disposable pipette and 1 ml of HBSS media (pH 7.4) (with penicillin/streptomycin) was added to wash the cells and sucked out straight away. Serum free media HBSS (900 µl) was added into each well. A sample of BSA-FITC loaded polymeric nanoparticles, (100 µl of 5 mg/ml) was added up to a total volume of 1 ml, shaken gently and left for incubation for 2 hours at 37 °C in a 5% CO_2 and 90% relative humidity atmosphere. After incubation the culture medium was collected, and the cells were washed twice with HBSS. The cells were lysed with 900 µl of hypotonic media for 2 minutes. Further 100 µl of lysis solution was added and left for 2 more minutes. The solution was removed and saved. The study determined the interaction of nanoparticles with cells, whether they were internalized or strongly surface bound by observing under confocal microscopy.

2.2.3.3. Uptake studies of nanoparticles by Caco-2 cells

Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Further information regarding cell culture techniques in general and for specific cell lines can be found in European Collection of Cell Cultures (http://www.ecacc.org.uk). The uptake of polymeric biodegradable nanoparticles by Caco-2 cells was determined using FITC–BSA to assess the binding and uptake of nanoparticles to the intestinal epithelium. Caco-2 cells were maintained in 75 cm^2 vented tissue culture flasks, in Dulbecco's modified Eagle's medium with Glutamax plus 10% fetal calf serum, 1% non-essential amino acids, 1% sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin. The Caco-2 cells were harvested with trypsin–

EDTA and seeded in 12-well plates at 200,000 cells/well and allowed to grow until about 70% confluency. For uptake studies (performed in triplicate), the culture medium was removed and cells were washed with HBSS media followed by addition of serum free media. Samples of BSA–FITC loaded polymeric nanoparticles were loaded onto the cells, shaken gently and left for 2h at 37 °C in 5% CO_2 and 90% relative humidity. Before the culture medium was collected, the cells were washed with HBSS and lysed with hypotonic media.

2.2.3.4. MTT assay of cell viability and evaluation of cytotoxicity of nanoparticles

The MTT Cell Assay is a colorimetric assay system which measures the reduction of a tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into an insoluble formazan product by the mitochondria of viable cells. When the cells are incubated with the MTT reagent and a detergent is added to lyse the cells, the coloured formazan crystals solubilize and the reaction determined spectrophotometrically at 570nm. The amount of colour produced is directly proportional to the number of viable cells. The MTT is taken up into cells and reduced by a mitochondrial dehydrogenase enzyme to yield a purple formazan product which accumulates in the healthy cells because they are impermeable. The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity which, in turn, is interpreted as a measure of viability of cells.

Chinese Hamster Ovary (CHO-K1) cells were seeded in flat bottom 96 well sterile plates to a density of 10^4 cells/well and incubated at 37 °C with 5% CO_2 for 24 hours. The nanoparticles were dispersed in the cell culture media F-12 (Hams) to give a concentration of 5 mg/ml and then added to the cells to give a concentration range of 0 to 5 mg/ml. Each formulation was prepared in triplicate and incubated for 72 hours and the viability of the cells was determined by the MTT assay. MTT (20 µl of 5 mg/ml solution in PBS) was added to each well and the plates were incubated for 2-5 hours at 37°C with 5% CO_2. The plates were tapped gently and the media was removed without disturbing the formazan crystals layers at the base of each well. DMSO (100 µl) was added to each well and the plates were incubated at 37 °C with 5% CO_2 for 30 minutes to dissolve the formazan crystals. The plates were read at 570 nm using a Wallac Victor II (PerkinElmer, Inc). Cells incubated in cell culture medium without any formulation were used as a control to assay 100% viability. Viability of cells exposed to the

formulations was expressed as percentage of the viability seen in control untreated cells (n=3). In general the ID_{50} value (i.e. concentration of chemical resulting in 50% inhibition of cell growth or reducing cell survival by 50%) can be obtained from the viability plots.

2.2.4. *In vivo* animal studies

In vivo animal studies were performed in accordance with the Animals Act 1986 (Scientific Protocol).

2.2.4.1. Immunization of the antigen loaded micro/nanoparticle formulations in mice by intranasal route

The specific details about the formulations and the dosage are explained in the subsequent chapters. In general, female BALB/c mice (25g, 4-6 weeks old) were anaesthetized with an inhaled gaseous mixture of 3% v/v halothane (RMB Animal Health, UK) in oxygen (300 cm^3 min^{-1}) and nitrous oxide (100 cm^3 min^{-1}) for intranasal dosing procedures. The particles were administered intranasally to the mice using a micropipette. Each mouse received a 20 or 50 µl volume (10 or 25 µl *per* nostril), of antigen loaded particle suspension or protein solution alone as appropriate. Tail vein blood samples were taken to assess the extent of the anti-IgG titres by ELISA as outlined in section 2.2.4.5.

2.2.4.2. Immunization of the antigen loaded micro/nanoparticle formulations in mice by intramuscular or subcutaneous route

The specific details about the formulations and the dosage are explained in the subsequent chapters. In general, female BALB/c mice (25g, 4-6 weeks old) were administered intramuscularly (at the hind-legs) or subcutaneously with the antigen formulations using a hypodermic syringe. Each mouse received a volume of 50 µl antigen loaded particle suspension or protein solution alone as appropriate. Tail vein blood samples were taken to assess the extent of the anti-IgG titres by ELISA as outlined in section 2.2.4.5.

2.2.4.3. Preparation of mice lung washes to measure the mucosal immune response

On termination of the experiments, the animals were sacrificed by CO_2 (schedule I method). To measure the mucosal immune responses (SIgA and IgG), the respiratory tract and lung secretions were collected by washing with the lung lavage solution. The lung lavage solution consists of Phenylmethylsulphonylfluoride (PMSF) 1mM, Sodium chloride 0.9% m/v, Tween 20 0.5% v/v and Sodium azide 1% m/v. The trachea and lungs were exposed by removing the thymus and 2 ml of gavage solution was gently inserted into the trachea to inflate the lungs. The liquid was immediately extracted and the samples centrifuged at 20,000 rpm for 30 minutes. The supernatant was stored at -70 °C and subsequently freeze-dried. These freeze dried samples were reconstituted in 100 µl of PBS and analysed by ELISA as outlined in section 2.2.4.5.

2.2.4.4. Preparation of mice gut washes to measure the mucosal immune response

On termination of the experiments, the animals were sacrificed by CO_2 (schedule I method). To measure the mucosal immune responses (SIgA and IgG), the gut secretions were collected from the sacrificed animals by washing with the gut lavage solution consisting of Iodoacetic acid 1Mm, Trypsin Inhibitor 0.1% v/v and sodium EDTA 10mM. The abdomen was exposed and 2 inches of small intestine incorporating the Payer's patches was isolated and transferred into a petri dish. The gut was sectioned longitudinally and 2 ml of lavage solution was added. The gut was scraped through and the exposed mucus and the liquid collected. This was sonicated for 30 sec, the samples were centrifuged and freeze dried. These freeze dried samples were reconstituted in 100 µl of PBS and analysed by ELISA as outlined in section 2.2.4.5.

2.2.4.5. Enzyme linked immunosorbent assay (ELISA) for the determination of IgG antibodies

Antibody responses in serum of the immunized animals were monitored using a microplate ELISA. The 96-well ELISA flat bottom microtiter plates (Dynatech, U.K) were coated with 100 µl *per* well of a 10 µg/ml DT or HBsAg in PBS solution (pH 7.4) overnight at 4° C. The plates were washed three times with 0.05% v/v solution of Tween-20 in PBS (PBST). The serum samples were serially diluted (1 in 16 was the initial dilution) in PBS and 50 µl of each sample was added to each well of the coated

ELISA plates. These were covered and incubated for one hour at 37°C. The plates were again washed three times with the PBST solution. 50 µl of goat anti-mouse IgG horseradish peroxide conjugate, diluted 1 in 1000 using PBS, was added to each well and the plates covered and incubated again for one hour at 37 °C. The plates were again washed three times in PBST and 50 µl of the substrate (2, 2'azino-bis) 3-ethylbenzthiazoline-6-sulphonic acid (ABTS), at a concentration of 3 tablets *per* 50 ml of citrate buffer (pH4.2) plus 5 µl hydrogen peroxide, was added to each well and incubated at 37 °C for 30 minutes for colour development (citrate buffer was freshly prepared using 44% 0.1M citric acid 56% 0.2M disodium hydrogen orthophosphate). The optical densities of the plates were then read at 405 nm using the Dynax MRX microplate reader. End-point titres were expressed as the last dilution, which gave an optical density (at OD_{405}) above the OD_{405} of negative controls after the 30 minutes incubation. Serum samples from individual animals were studied to determine the titre range and the immune responses of the different groups of animals at each time point were compared.

2.2.4.6. IL-4, IL-6 and IFN-γ assessment using cytokine ELISA

Cytokine sandwich ELISA is an enzymatic immunoassay which detects and quantitates the concentration of soluble cytokine and chemokine proteins. This method makes use of highly-purified anti-cytokine antibodies, capture antibodies, which are non-covalently adsorbed onto plastic microwell plates. The plate is washed with buffer after which the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples which were applied to the plate. The unbound material is washed off and the captured proteins are detected by biotin-conjugated anti-cytokine antibodies. This is followed by the addition of a streptavidin conjugated enzyme (horse radish peroxidase). The streptavidin conjugate binds strongly to the biotinylated antibody and following the addition of a chromogenic substrate, the level of coloured product generated by the bound detection reagents can be measured spectrophotometrically using an ELISA-plate reader at optical density 405 nm. A standard curve is plotted by making serial dilutions of a standard cytokine protein solution of known concentration. Standard curves are generally plotted as the standard cytokine protein concentration (pg of cytokine/ml) versus the corresponding mean OD value of replicates.

The spleens from the mice were collected in PBS solution after termination of the experiment. They were washed with the media containing RPMI (Sigma Aldrich, UK) supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), L-glutamine and penicillin/streptomycin (Sigma Aldrich, UK). This step was followed by crushing the spleens in the media using an inverted syringe and filtering the splenocytes. The spleen cells were centrifuged at 2,000 rpm for 10 minutes and washed twice with the media. Equal amounts of spleen cells (counted using a hemacytometer) suspended in 100 µl working media were systemically added to each flat bottom Nunc Maxisorp well, in a 96 well cell culture plate. 20 µg of antigen, working media or con A solution in 100 µl of media was added systematically to the wells containing the spleen cells. The cells were incubated for 48 hours at 37 °C and 90% humidity. The supernatants were taken for analysis of IL-4, IL-6 and IFN-γ using DuoSet® sandwich ELISA kits (R&D Systems, Oxon, UK) in accordance with the manufacturer's instructions. Due to logistical reasons, only IL-4, IL-6 and secretions were evaluated during these experiments, with IFN-γ giving the Th1 orientation and IL-4 and IL-6 giving the Th2 orientation

2.2.5. Statistics

Statistical analysis was preformed using an analysis of variance (ANOVA) general linear model with SPSS software (Version 17, Microsoft) assuming P values of $P \leq 0.05$ as significant.

Chapter 3

Tocopherol Derivatives as Adjuvants

3. Co-encapsulation of tocopherol derivatives with HBsAg or DT in PLA or PCL micro/nanoparticles for improved adjuvancy

3.1. Tocopherol derivatives as adjuvants

As discussed in chapter 1, the polymeric micro/nanoparticulate systems have a great potential as vaccine carriers. There is a further scope of improving these systems by co-encapsulating adjuvants along with the antigens. The focus of this study is to use tocopherol derivatives such as D α-tocopheryl polyethylene glycol 1000 succinate (TPGS), tocopherol acetate (TA) and tocopherol nicotinate (TN) in the polymeric carriers to increase the adjuvanticity of the antigens such as diphtheria toxoid (DT) and hepatitis B surface antigen (HBsAg). TPGS has been used in our laboratory as an adjuvant in the past and to the best of our knowledge TA and TN has not been used as adjuvants. When TPGS solution was co-administered with TT/DT solution, it evoked many folds higher specific serum IgG titres compared to the free antigens. This adjuvant effect of TPGS was owed to the anti-oxidant properties of tocopherol (Alpar *et al.*, 2001). In the past studies TPGS was only used as solution along with the antigens; however it will be interesting to investigate the adjuvant effects of TPGS if co-encapsulated in the polymeric carriers. We anticipate that the duration and intensity of the adjuvant effects of tocopherol derivatives can be improved if they are co-encapsulated in the micro/nanoparticulate systems. This is based on the hypothesis that the adjuvancy will be improved due to the synergistic effects of tocopherol derivatives which has already shown adjuvant properties and the depot formation of the polymeric carriers.

3.1.1. D α-tocopheryl polyethylene glycol 1000 succinate (TPGS)

TPGS (Figure 3.1) is a water-soluble derivative of natural-source vitamin E, which is formed by esterification of vitamin E succinate with polyethylene glycol 1000. The chemical structure of vitamin E TPGS comprises both lipophilicity and hydrophilicity, resulting in amphiphilic properties. Moreover, its lipophilic alkyl tail (tocopherol) and hydrophilic polar head portion (polyethylene glycol) are bulky and have a large surface area. Such characteristics allow TPGS to be used in a wide range of water-oil immiscible systems.

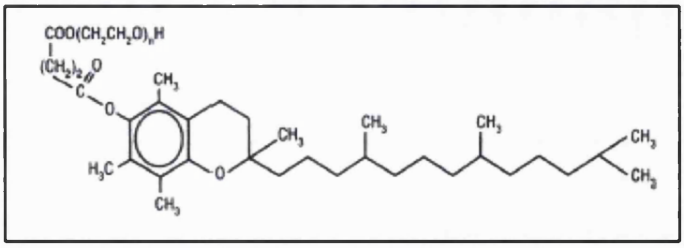

Figure 3.1: Chemical structure of D α-tocopheryl polyethylene glycol 1000 succinate (TPGS) (Eastman Chemical Co. catalogue)

TPGS is miscible with water and forms solutions with water at concentrations up to 20% m/v beyond which liquid crystalline phases may form. Above its critical micellar concentration (CMC), TPGS begins to form micelles and continues to form relatively low viscosity solutions with water until a concentration of 20% m/v is obtained. TPGS does not act as a good surfactant at both higher and lower concentrations. Acids and bases may facilitate hydrolysis of the ester linkages; however as the pH approaches 7 in buffered solutions, TPGS exhibits < 10% hydrolysis during three months' storage (Eastman Chemical Co, datasheet). The properties of TPGS are described in the Table 3.1.

Table 3.1: Properties of TPGS (Eastman Chemical Co. datasheet)

Molecular weight	1513
Physical form	Waxy solid
Melting temperature	37-41 °C
Solubility in water	Miscible in all parts
Hydrophile/lipophile balance	13.2
Critical Micellar Concentration at 37 °C	0.02% by weight
Bulk stability	Stable to air; reacts with alkali
Stability in aqueous solutions	Stable at pH 4.5-7.5
Stability to heat	Stable until 200 °C

TPGS is a safe and effective form of vitamin E that is used for reversing and preventing vitamin E deficiency, which results in varying degrees of neurological degeneration. It improves the vitamin E oral bioavailability in animals and humans; TPGS delivers α-tocopherol to the enterocytes in the absence of bile salts, whereas α and γ tocopherols normally require bile salts for absorption. It is thus very useful in patients suffering from vitamin E deficiency due to chronic cholestasis (a lipid malabsorption syndrome). The mechanism by which TPGS delivers tocopherol (vitamin E) has been studied in human fibroblasts and erythrocytes and in human intestinal cell lines. The results

demonstrated that intact uptake of the TPGS molecule occurs followed by intracellular hydrolysis of ester bonds (Sokol *et al.*, 1987; Traber *et al.*, 1988; Sokol *et al.*, 1993).

TPGS is used as a safe and effective absorption enhancer for poorly water soluble drugs such as cyclosporine and protease inhibitors. It acts as a surfactant increasing epithelial membrane permeability by affecting mucus and membrane proteins and lipids. Moreover, enhanced oral bioavailability of drugs co-administrated with TPGS may in part be due to the inhibition of P-glycoprotein in the intestine, a membrane protein that functions as an ATP dependent drug efflux pump. One activity of this pump is to lower the intracellular concentration of drugs thereby reducing the cytotoxic activity of drugs and causing drug resistance (anticancer drugs, steroids, antihistamines, antibiotics, calcium channel blockers and anti-HIV peptidomimetics). This membrane protein is primarily expressed on the luminal surface of epithelial cells from several tissues including the intestine, liver, kidney and endothelial cells comprising the blood-brain and blood-testes barriers (Sokol *et al.*, 1991; Argao *et al.*, 1992; Yu *et al.*, 1999; Dintaman and Silverman, 1999).

TPGS has also been used as absorption enhancer for vaccines. It has been used as a mucosal adjuvant for nasal vaccination applied to Diphtheria toxoid (DT) and Tetanus toxoid (TT). Solutions of the TPGS were co administered with TT/DT and were found to markedly improve the humoral responses to nasally delivered TT and DT (Alpar *et al.*, 2001).

TPGS has been used as an emulsifier in the single emulsion solvent evaporation/extraction technique for fabrication of polymeric nanoparticles for controlled release of paclitaxel, one of the best antineoplastic drugs characterized by an extremely poor solubility (Mu and Feng, 2002). The potential applications of TPGS are summarized in the Table 3.2.

Table 3.2: Potential applications of TPGS in drug delivery systems (Eastman Chemical Co. datasheet)

Potential applications of TPGS in drug delivery systems
Drug solubilizer
Emulsion vehicle
Vehicle in a semisolid dosage form
Prevent drugs from crystallization
Protect drugs during the absorption process
Enhance bioavailability of poorly adsorbed drugs
Reduce drug sensitivity on skin tissues
Functional ingredient in self-emulsifiable formulations
Provide vitamin E in water soluble form

3.1.2. Tocopherol acetate and Tocopherol nicotinate

Tocopherols are methyl-substituted hydroxychromans with a phytyl side chain and are the predominant form of vitamin E in plasma and tissues. Tocopherol acetate (TA) and tocopherol nicotinate (TN) are the semi-synthetic acetate ester or nicotinate ester of naturally occurring α-tocopherol (Figure 3.2). Natural vitamin E is composed of two homologous series namely, the tocopherols with a saturated side chain and the tocotrienols with an unsaturated side chain. Tocopherol and its ester derivatives are used as antioxidants and skin conditioning in the cosmetic formulations.

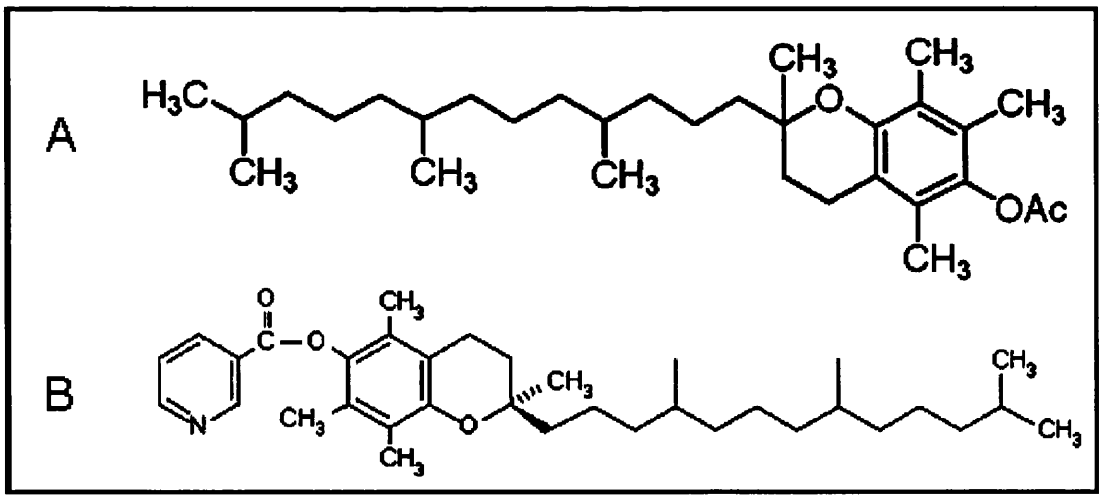

Figure 3.2: Chemical structure of (A) DL-α-Tocopherol acetate; (B) (±)-α-Tocopherol nicotinate (Sigma Aldrich Inc. catalogue).

In a bioavailability study conducted in sheep, it was found that the availability of TA was significantly greater than TN. Here TN or TA was administered as a single dose, either into the rumen or the peritoneal cavity (Hidiroglou and Charmley, 1990; 1991; Toutain *et al.*, 1995). There has been no evidence of the use of TN or TA in the formulation of micro/nanoparticles. The reason for using these ingredients is based on

assumption that these ingredients might act as adjuvants in a similar manner to TPGS, mainly due to their antioxidant properties.

3.1.3. Aims and Objectives

As mentioned earlier, TPGS has been used in the past as an adjuvant in our laboratory; it was given as a solution together with the antigens. One way to increase immunogenicity is to incorporate adjuvants in the vaccine formulation or vaccine carriers such as micro/nanoparticles. In the present work the effect of co-encapsulating TPGS or TA or TN with antigens and also using TPGS as a stabilizing agent in the preparation of antigen loaded PLA or PCL micro/nanoparticles for improving the immunogenicity of the carriers was examined. The formulations were prepared to investigate the following parameters:

- Optimisation studies using TPGS along with the polymer in the preparation of nanoparticles using OVA as model antigen

- Effect of TPGS when used as a matrix with PLA or as stabilizing agent in the internal phase emulsion on HBsAg loaded PLA microparticles

- Effect of TA or TN concentration when used as a matrix on HBsAg loaded PLA nanoparticles

- Effect of TPGS when used as a matrix on DT loaded PLA microparticles

- Effect of varying concentration of TPGS used in the internal phase stabilizer on DT loaded nanoparticles

- Effect of varying concentration of TPGS in the organic phase of DT loaded PCL microparticles prepared by spray drying technique

These formulations were characterized with respect to size, yield, surface charge, loading efficiency and uptake by cell lines. The best formulations were chosen for *in vivo* animal studies (in the mouse model) and the antibody responses were measured together with the antigen specific cytokine responses. The important aspect of the *in*

vivo studies was to recognize the best formulation which can improve the adjuvancy of the antigens for which the following studies were carried:

- Long term immune response (over 400 days) of HBsAg loaded PLA nanoparticles (TPGS in organic or aqueous phase) administered subcutaneously

- Intramuscular *versus* intranasal immune response of HBsAg loaded PLA nanoparticles containing TA or TN in the organic phase

- Intramuscular *versus* intranasal immune response of DT loaded PLA microparticles with TPGS in organic phase or aqueous phase

- Intranasal immune response of DT loaded PCL microparticles prepared by spray drying method with varying concentration of TPGS in the organic phase

3.2. Materials and methods

3.2.1. Materials

Diphtheria Toxoid (DT) was from Pasteur Merieux, Connaugent, France, and Hepatitis B surface antigen (HBsAg) at a concentration of 1.68 mg/ml was provided as a gift by Shantha Biotechnics, India. Poly (L-lactic acid) (120 K) was from Lakeshore Biomaterials Inc, USA. Albumin Chicken Egg (OVA) Grade V, (±)-α-tocopherol nicotinate, DL-α-tocopherol acetate, polyvinyl alcohol (13,000-23,000 K and 87-89% hydrolyzed), L-glutamine and penicillin/streptomycin, QuantiPro® Bicinchoninic acid assay kit was from Sigma Aldrich, UK. Alhydrogel® 2% (Aluminium Hydroxide Gel Adjuvant) was from Superfos Biosector, Denmark. RPMI and fetal bovine serum were from Gibco, UK. Horseradish peroxidase conjugated Goat anti-mouse isotype specific immunoglobulin from Serotec, UK. DuoSet® sandwich ELISA kits was from R&D Systems, UK. Dichloromethane (DCM) was from BDH laboratory suppliers, UK. All other reagents used were of analytical grade.

3.2.2. Methods

3.2.2.1. Preparation of micro/nanoparticles

The micro/nanoparticles were prepared by the w/o/w double emulsification solvent evaporation method and w/o/w double emulsification-spray drying method. The theory and principle of these techniques are explained in chapter 1.

3.2.2.1.1. Preparation of OVA loaded PLA plus TPGS nanoparticles

Microparticles loaded with 2% OVA were prepared using the w/o/w double emulsion solvent evaporation technique. Varying concentrations of TPGS was dissolved in the solvent dichloromethane (DCM), thereby using TPGS as a matrix material along with the polymer (Table 3.3). For each formulation, approximately 125 mg of PLA and varying concentrations of TPGS (0, 2.5, 5, 7.5, 10 and 15% m/m of polymer) were dissolved in 5 ml of DCM to form the organic phase. One millilitre of 10% m/v PVA containing OVA was dispersed in the organic phase using an Ultraturrax homogenizer (T25 Janke & Kunkel, IKA-Labortechnik) for 2 minutes at 24,000 rpm to make the w/o emulsion. This primary emulsions were then added drop wise to 30 ml 2.5% m/v PVA and homogenized for 6 minutes at 10,000 rpm using the Silverson® L4RT homogenizer to form the w/o/w emulsion. The resultant double emulsion was gently stirred at room temperature by a magnetic stirrer overnight to evaporate the DCM. The produced microparticles were collected by centrifugation (35,000 rpm, 15 minutes, 4 °C) (Du Pont Sorvall Combi plus Model) and washed twice with deionised water to remove excess PVA. The product was then suspended in 4 ml deionised water and freeze-dried (Virtis Advantage, England) to obtain a fine powder.

Table 3.3: Formulations containing TPGS in varying concentrations in OVA loaded PLA nanoparticles. The internal and external phase stabilizing agent was 1ml of 10% m/v PVA and 30 ml of 2.5% m/v PVA, respectively

Code	% mass (m/m) of TPGS dissolved in the organic phase with PLA
A1	-
A2	2.5
A3	5
A4	7.5
A5	10
A6	15

3.2.2.1.2. Formulation of 0.5% m/m HBsAg loaded PLA plus TPGS or Pluronic F-127 microparticles

Four formulations of 0.5% m/m HBsAg loaded PLA microparticles were prepared using the w/o/w double emulsification solvent evaporation method. TPGS was used as a matrix or an emulsifier in two formulations. In one formulation Pluronic F-127 was blended with PLA and dissolved in DCM. The last formulation was the control; PVA was used as emulsifier without any excipient (Table 3.4).

Table 3.4: Formulations of 0.5% m/m HBsAg loaded PLA plus TPGS or Pluronic F-127 microparticles. The external phase stabilizing agent was 30 ml of 2.5% m/v PVA.

Code	% mass (m/m) of TPGS or Pluronic F-127 dissolved in the organic phase with PLA	Internal phase stabilizer along with HBsAg (total volume 1.25 ml)
B1	-	PVA 10% m/v
B2	TPGS10% m/m	PVA 10% m/v
B3	-	TPGS 10% m/v
B4	Pluronic F-127 10% m/m	PVA 10% m/v

PLA and TPGS or Pluronic F-127 was dissolved in 4 ml DCM. HBsAg (0.75 ml) was mixed with 10% m/v PVA or TPGS. It has to be noted that the DCM volume has been reduced here (4 ml) so as to increase the viscosity of the emulsion system, thereby increasing the particle size in microparticle range rather than keeping it at submicron level. These two solutions were dispersed by homogenization using Ultraturrax homogenizer (T25 Janke & Kunkel, IKA-Labortechnik) for 2 minutes at 24,000 rpm. The w/o emulsion was added drop wise to 30 ml of 2.5% m/v PVA solution and homogenized for 6 min at 10,000 rpm (Silverson® L4RT). The secondary emulsion was stirred for four hours with a magnetic stirrer and subjected to the same conditions as in section 3.2.2.1.1.

3.2.2.1.3. Formulation of 1% m/m HBsAg loaded PLA microparticles – TPGS as matrix with PLA or as stabilizing agent in the internal phase emulsion

Three formulations of 1% m/m HBsAg loaded PLA microparticles were prepared using TPGS as a matrix and an emulsifier by the double emulsion technique (Table 3.5). One formulation was TPGS emulsified, in another TPGS was blended with PLA, and a control which is prepared without using TPGS. For the internal phase, 10% m/v TPGS solution was prepared by melting 10 mg of TPGS and pouring into 100 ml of hot deionised water (90 °C). This solution was stirred for approximately 1 hour until complete dissolution of the surfactant.

Table 3.5: Formulation of 1% m/m HBsAg loaded PLA microparticles containing TPGS. The particles were made by solvent evaporation method. TPGS was used as matrix with PLA or as stabilizing agent in the internal phase emulsion. The external phase stabilizing agent was 30 ml of 1.25% m/v of PVA.

Code	% mass of TPGS dissolved in the organic phase with PLA	Internal phase stabilizer along with HBsAg (total volume 1.25 ml)
C1	-	PVA 10% m/v
C2	TPGS 10% m/m	PVA 10% m/v
C3	-	TPGS 10% m/v

PLA (and TPGS when used as a matrix) were dissolved in 4 ml DCM. HBsAg (0.75 ml) was mixed with 0.5 ml of aqueous solution containing 10% m/v PVA or TPGS. The primary emulsion was formed by dispersing these two immiscible solutions with Ultraturrax (T25 Janke & Kunkel, IKA-Labortechnik) at 11,500 rpm for 2 minutes. This emulsion was added drop wise to 30 ml of 1.25% m/v PVA solution and homogenized for 6 minutes at 8,000 rpm with the Silverson® L4RT. The formed secondary emulsion was stirred for four hours with a magnetic stirrer and subjected to the same conditions as in section 3.2.2.1.1.

3.2.2.1.4. Formulation of 1% m/m HBsAg loaded PLA plus tocopherol acetate or tocopherol nicotinate nanoparticles

HBsAg loaded PLA nanoparticles were prepared using PVA as the stabilising agent by the w/o/w emulsification solvent evaporation method. The theoretical loading of the HBsAg was 1% m/m of the polymer. Approximately 100 mg of PLA with 20% m/m of either TA or TN was dissolved in 6 ml of DCM to form the organic phase (Table 3.6). The primary emulsion (w/o) was formed by dispersing 10% m/v PVA together with HBsAg (internal phase) in the organic phase using an Ultraturrax at 24,000 rpm for 2 minutes. This w/o emulsion was added drop wise to 30 ml of 1.25% PVA m/v solution and homogenized for 6 minutes at 10,000 rpm using a Silverson® homogeniser (Silverson, model L4RT). The formed secondary emulsion was stirred for four hours with a magnetic stirrer and subjected to the same conditions as in section 3.2.2.1.1.

Table 3.6: Formulation of HBsAg loaded PLA nanoparticles with varying concentration of TA or TN in polymer matrix. The nanoparticles were prepared by solvent evaporation method, internal and external phase stabilizing agent was 1 ml of 10% m/v of PVA and 30 ml of 1.25% m/v PVA respectively.

Code	% mass of TA or TN dissolved in the organic phase with PLA
I1	-
I2	TA 20% m/m
I3	TN 20% m/m

3.2.2.1.5. Formulation of 2% m/m DT loaded PLA microparticles – TPGS as matrix with PLA or as stabilizing agent in the internal or external phase of the emulsion

DT 2% m/m loaded microparticles were prepared using the double emulsion technique. TPGS was used as a matrix or as an emulsifier (Table 3.7).

Table 3.7: Formulation of 2% m/m DT loaded PLA microparticles containing TPGS. The microparticles were prepared by solvent evaporation method; TPGS was used as a matrix with PLA or as stabilizing agent in the internal or external aqueous phase of the emulsion

Code	% mass of TPGS dissolved in the organic phase with PLA	Internal phase stabilizer along with 0.26 ml DT (conc. 19.3mg/ml) (the total volume of aqueous phase is 1.26 ml)	External phase stabilizer (30ml) m/v
D1	-	PVA 10% m/v	PVA 2.5% m/v
D2	10	PVA 10% m/v	PVA 2.5% m/v
D3	25	PVA 10% m/v	PVA 2.5% m/v
D4	5	PVA 10% m/v	PVA 2.5% m/v
D5	-	TPGS 10% m/v	PVA 2.5% m/v
D6	-	TPGS 10% m/v	TPGS 10% m/v

One formulation did not contain TPGS (control), in three formulations different percentages of TPGS were blended with PLA, in another formulation TPGS was used as internal phase emulsifier and in the last one it was used as internal and external phase emulsifier. PLA 120 K (and TPGS when used as a matrix) was dissolved in 5 ml DCM to form the organic phase. The internal phase was made of 0.26 ml DT (19.3 mg/ml) plus 1 ml of 10% m/v PVA or TPGS solution. The internal phase aqueous solution was dispersed in the organic phase using Ultraturrax (T25 Janke & Kunkel, IKA-Labortechnik) for 2 minutes at 24,000 rpm. The formed primary emulsion was poured in 30 ml of 2.5% m/v PVA solution or 10% m/v TPGS solution using the Silverson® homogenizer (L4RT) at 10,000 rpm, to obtain a w/o/w emulsion. The formed secondary emulsion was stirred for four hours with a magnetic stirrer and subjected to the same conditions as in section 3.2.2.1.1.

3.2.2.1.6. Formulation of 2% m/m DT loaded PLA microparticles – varying concentration of TPGS as internal phase stabilizing agent

DT (2% m/m) loaded microspheres were prepared using the double emulsion w/o/w technique. Varying concentrations of TPGS were used as internal phase stabilizing agent (Table 3.8). For all the formulations PVA 2.5% m/v was used as external phase stabilizing agent. Two hundred and fifty milligrams of PLA (120 K) was dissolved in 5

ml DCM to prepare the organic phase. DT (0.26 ml, 19.3 mg/ml) was mixed with 1 ml aqueous solution of PVA or TPGS making 1.26 ml of internal aqueous phase. The two solutions were dispersed using Ultraturrax (T25 Janke & Kunkel, IKA-Labortecnik) homogenizer at 24,000 rpm for 2 minutes. The formed o/w primary emulsion was added drop wise to 30 ml of 2.5% m/v PVA solution and homogenized for 6 minutes at 10,000 rpm with the Silverson® homogenizer (L4RT). The formed w/o/w double emulsion was stirred for 4 hours with a magnetic stirrer and subjected to the same conditions as in section 3.2.2.1.1.

Table 3.8: Formulation of 2% m/m DT loaded PLA microparticles with varying concentration of TPGS in the internal phase. A 30ml of 2.5% m/v of PVA was used as external phase stabilizing agent.

Code	Internal phase stabilizer along with 0.26 ml DT (conc. 19.3 mg/ml) (the total volume of aqueous phase is 1.26 ml)
E1	PVA 10% m/v
E2	TPGS 20% m/v
E3	TPGS 10% m/v
E4	TPGS 5% m/v
E5	TPGS 2.5% m/v

3.2.2.1.7. Preparation of DT encapsulating PCL plus microparticles by spray drying technique - varying concentration of TPGS in the organic phase

DT 2% m/m loaded microparticles were prepared using the double emulsification process followed by spray drying of the emulsion. Polycaprolactone (PCL 20 K) (and TPGS when used as a matrix) was dissolved in 5 ml DCM to make the organic phase (Table 3.9).

Table 3.9: Formulation of 2% m/m DT loaded PCL microparticles by spray drying method. Varying concentration of TPGS was used in the organic phase. (750 µl of 7.5% m/v of PVA + 0.26 ml of DT 19.3 mg/ml) was used as internal phase stabilizing agent and 30 ml of 2.5% m/v of PVA was used as external phase stabilizing agent, in some formulations Kollicoat® was used as stabilizing agent)

Code	% mass of TPGS dissolved in the organic phase with PCL	% m/v PVA in internal phase, 750 µl	% m/v PVA in external phase, 30 ml	% m/v Kollicoat® in internal phase, 750 µl	% m/v Kollicoat® in external phase, 30 ml
F1	-	7.5	2.5	-	-
F2	10	7.5	2.5	-	-
F3	20	7.5	2.5	-	-
F4	40	7.5	2.5	-	-
F5	-	-	-	7.5	2.5
F6	-	-	-	7.5	5
F7	-	-	-	15	10
F8	-	-	-	15	20

The internal phase was made of 0.26 ml DT (19.3 mg/ml) plus 1 ml of 7.5% m/v PVA. The internal phase aqueous solution was dispersed in the organic phase using Ultraturrax (T25 Janke & Kunkel, IKA-Labortechnik) for 2 minutes at 24,000 rpm. The formed primary emulsion was poured into 30 ml of PVA or Kollicoat® solution (a graft copolymer of Polyvinyl alcohol-polyethylene) using the Silverson® homogenizer (L4RT) at 10,000 rpm, to obtain a w/o/w emulsion. This double emulsion was spray-dried with a Büchi Mini B-191 laboratory spray-dryer (Büchi Laboratorium AG, Switzerland) using a 0.5 mm nozzle. The process parameters were set as follows: inlet temperature (40 °C ± 2 °C); outlet temperature (26 °C ± 2 °C); aspirator setting (50%); pump setting (5 ml/min) and spray flow (600 L/min). Microparticles in powder form were stored at -4 °C until characterization.

3.2.2.2. Characterization of micro/nanoparticles

The micro/nanoparticles obtained were characterized with respect to their size distribution, surface charge, encapsulation efficiency and morphology as described in sections 2.2.2.1 to 2.2.2.5. The integrity of the protein or antigen was determined using SDS-PAGE as described in section 2.2.2.6. The MTT assay for PLA plus TPGS or TA or TN nanoparticles encapsulated with HBsAg was carried out as described in section 2.2.3.4. Statistical analysis was preformed using an analysis of variance (ANOVA) general linear model with SPSS software (Version 17, Microsoft) assuming P values of $P \leq 0.05$ as significant.

3.2.2.3. Immunization schedule of antigen loaded formulations in mice

3.2.2.3.1. Immunization schedule used for HBsAg loaded PLA microparticles by subcutaneous route – TPGS as matrix with PLA or as stabilizing agent in the internal phase emulsion

Five groups ($n = 4$) of female BALB/c mice (25g, 4-6 weeks old) were subcutaneously administered with the formulations using a hypodermic syringe (Table 3.10).

Table 3.10: Immunization schedule of HBsAg loaded PLA microparticles by subcutaneous route – TPGS as matrix with PLA or as stabilizing agent in the internal phase emulsion. The priming dose was 1 μg of HBsAg *per* mice on day 1 followed by one booster dose of 0.5 μg of HBsAg *per* mice on day 21.

No	Formulations	Route
1	HBsAg loaded PLA microparticles	Subcutaneous
2	HBsAg loaded PLA + TPGS (in organic phase) microparticles	
3	HBsAg loaded PLA microparticles (TPGS used as internal phase stabilizing agent)	
4	Free HBsAg plus Alum	
5	Free HBsAg	

Each mouse received a volume of 50 μl antigen loaded particle suspension dispersed in PBS (pH 7.4) or antigen alone as appropriate. Tail vein blood samples were taken on days 15, 60, 250, 300 and 400, to assess the serum-specific antibody responses by ELISA as described in section 2.2.4.5.

3.1.2.3.2. Immunization schedule used for HBsAg loaded PLA plus TA or TN nanoparticles by intramuscular or intranasal route

Four groups (n = 4) of female BALB/c mice (25g, 4-6 weeks old) were anaesthetized with an inhaled gaseous mixture of 3% v/v halothane (RMB Animal Health, UK) in oxygen (300 cm^3 min^{-1}) and nitrous oxide (100 cm^3 min^{-1}) for intranasal dosing procedures (Table 3.11).

Table 3.11: Intramuscular *versus* intranasal immunization schedule of HBsAg loaded PLA nanoparticles containing TA or TN in the organic phase. The priming dose was 1 μg of HBsAg *per* mice on day 1 followed by one booster dose of 0.5 μg of HBsAg *per* mice on day 21.

No	Formulations	Route
1	HBsAg loaded PLA nanoparticles	Intramuscular
2	HBsAg loaded PLA + 20% m/m TA (in organic phase) nanoparticles	
3	HBsAg loaded PLA + 20% m/m TN (in organic phase) nanoparticles	
4	Free HBsAg	
5	HBsAg loaded PLA nanoparticles	Intranasal
6	HBsAg loaded PLA + 20% m/m TA (in organic phase) nanoparticles	
7	HBsAg loaded PLA + 20% m/m TN (in organic phase) nanoparticles	
8	Free HBsAg	

The particles were administered intranasally to the mice using a micropipette. Four groups (n = 4) of female BALB/c mice (25g, 4-6 weeks old) were intramuscularly (at the hind-legs) administered with the antigen formulations using a hypodermic syringe. Each mouse received a volume of 50 μl antigen loaded particle suspension dispersed in PBS or antigen alone as appropriate. Tail vein blood samples were taken on days 15, 30, 45 and 90, to assess the serum specific antibody responses by ELISA as described in section 2.2.4.5.

3.1.2.3.3. Immunization schedule of DT loaded PLA microparticles – TPGS as matrix with PLA or as stabilizing agent in the internal or external phase emulsion

Eight groups (n = 4) of female BALB/c mice (25g, 4-6 weeks old) were administered intramuscularly or intranasally with the antigen formulations as described in the section 3.1.2.3.1.2. Each mouse received a volume of 50 µl antigen loaded particle suspension dispersed in PBS or antigen alone as appropriate (Table 3.12). Tail vein blood samples were taken on days 15, 30, 45 and 90, to assess the serum specific antibody responses by ELISA as described in section 2.2.4.5.

Table 3.12: Immunization schedule used for DT loaded PLA microparticles – TPGS as matrix with PLA or as stabilizing agent in the internal or external phase emulsion. The priming dose was 10 LF of DT *per* mice on day 1 followed by one booster dose of 5 LF of DT *per* mice on day 21.

No	Formulations	Route
1	DT loaded PLA microparticles	Intramuscular
2	DT loaded PLA + TPGS (in organic phase) microparticles	
3	DT loaded PLA microparticles (TPGS used as internal phase stabilizing agent)	
4	Free DT	
5	DT loaded PLA microparticles	Intranasal
6	DT loaded PLA + TPGS (in organic phase) microparticles	
7	DT loaded PLA microparticles (TPGS used as internal phase stabilizing agent)	
8	Free DT	

3.1.2.3.4. Immunization schedule used for nasal delivery of DT loaded PCL microparticles prepared by spray drying technique

Four groups (n = 4) of female BALB/c mice (25g, 4-6 weeks old) were administered intranasally with the antigen formulations as described in section 3.1.2.3.1.2. Each mouse received a volume of 20 µl antigen loaded particle suspension dispersed in PBS or antigen alone as appropriate (Table 3.13). Tail vein blood samples were taken on days 15 and 45, to assess the serum specific antibody responses by ELISA as described in section 2.2.4.5.

Table 3.13: Intranasal immunization schedule of DT loaded PCL microparticles prepared by spray drying method with varying concentration of TPGS in the organic phase. The priming dose was 10 µg of DT *per* mice on day 1 followed by one booster dose of 5 µg of DT *per* mice on day 28.

No	Formulations	Route
1	DT loaded PCL microparticles	Intranasal
2	DT loaded PCL + 10% m/m TPGS (in organic phase) microparticles	
3	DT loaded PCL + 10% m/m TPGS (in organic phase) microparticles	
4	Free DT	

3.2.2.4. Enzyme linked immunosorbent assay (ELISA) for the determination of IgG antibodies and cytokine analysis

Antibody responses in serum of the immunized animals were monitored using a microplate ELISA as described in chapter 2 (section 2.2.4.5). The cytokine analysis was carried out as described in chapter 2 (section 2.2.4.6) for the determination of endogenous cytokines levels, interleukin-2 (IL-4), interleukin-6 (IL-6) and interferon-γ (IFN-γ).

3.3. Results and Discussion

3.3.1. Characterization of micro/nanoparticles

3.3.1.1. Effect of TPGS concentration on OVA loaded PLA nanoparticles

In a study conducted by Mu and Feng (2003), it was observed that TPGS, blended with hydrophobic polymers such as PLGA and PLA, can be used as a component of the matrix material to improve the controlled release properties of paclitaxel-loaded nanoparticles prepared by the single emulsion solvent evaporation technique. In the present work the effect of TPGS on the particle size, zeta potential, loading and release characteristics of OVA-loaded PLA microspheres were investigated; the results are described in the Table 3.14. In this study, formulations containing TPGS have slightly higher yield. However, the difference compared to the TPGS free formulation (A1) is not high. The size of particles was found to be less than 1 μm and the surface charge was negative for all the formulations. As the ratio of TPGS increases, the zeta potential becomes slightly more negative. However, the difference is not statistically significant.

Table 3.14: Optimisation studies using TPGS along with the polymer in the preparation of OVA loaded nanoparticles. Particles were characterized for size, loading and zeta potential of the nanoparticles. The internal and external phase stabilizing agents were 1 ml of 10% m/v PVA and 30ml of 2.5% m/v PVA respectively (n=3, mean ±s.d).

Code	% mass of TPGS dissolved in PLA	Yield % ±s.d	Z average particle size (nm) ±s.d	Poly-dispersity index ±s.d	Zeta potential, mV ±s.d	Encapsulation efficiency % ±s.d	Actual protein content (μg/mg) ±s.d
A1	-	46.7 ±12.2	971.4 ±7	0.534 ±0.024	-2.4 ±0.5	52.6 ±1.6	2.25 ±0.69
A2	2.5	61.5 ±2.6	803.7 ±3	0.546 ±0.027	-2.8 ±0.2	45.8 ±4.5	1.48 ±1.46
A3	5	62.4 ±6.3	832.2 ±8	0.529 ±0.037	-3 ±0.1	45.3 ±2.6	1.45 ±0.83
A4	7.5	59.7 ±8.2	828.8 ±3	0.586 ±0.022	-3.4 ±0.2	54.7 ±0.3	1.83 ±0.10
A5	10	68.5 ±10.5	837.4 ±6	0.447 ±0.038	-3.6 ±0.1	44.0 ±0.1	1.28 ±0.03
A6	15	61.1 ±3.1	891.6 ±5	0.587 ±0.034	-4.1 ±0.6	48.4 ±7.5	1.58 ±2.45

The formulations A3, A4, A5 and A6 were difficult to disperse compared to A1 and A2. This may be attributed to the particles forming aggregates during the freeze-drying process or to the increased particle hydrophobicity due to the presence of TPGS, leading to a difficulty in dispersion and consequently to an anomaly in the results with the photon correlation spectroscopy. The loading efficiency of all the formulations was similar with no appreciable differences. All these results confirm that there was no effect on the loading efficiency when TPGS is used as a matrix in the double emulsion technique, in accordance with the results observed by Ruan *et al* (2002). Encapsulation of proteins and peptides within polymeric microspheres is a complex phenomenon subject to variations attributed by several factors. However, the tensioactive properties of proteins and the stability of the primary emulsion are considered key points for the entrapment of hydrophilic compounds (Nihant *et al.*, 1994). Rosa *et al* (2000) observed that co encapsulation of surfactants reduced encapsulation efficiency of insulin in PLGA microspheres. They attributed the results to the displacement of the protein from the w/o emulsion interface by the surfactants, eventually decreasing the amount of insulin located within the w/o emulsion. More precisely, when the surfactant is added to the organic phase, they assume that it could limit the location of protein at w/o interface, when arranging its hydrophilic moieties toward the internal aqueous phase, and thereby facilitate protein leakage into the external aqueous phase.

For all the formulations, the scanning electron microscopy (SEM) showed particles of homogeneous size and smooth surface (Figure 3.3). From the SEM, TPGS did not affect particle size of the nanoparticles contrary to other studies in the literature. Mu and Feng (2003) studied the effect of TPGS on the size of paclitaxel-loaded nanoparticles prepared by the single emulsion technique. It may be interesting to observe that Ruan *et al* (2002) evaluated the size of human serum albumin loaded microspheres prepared by the double emulsion technique using the SEM only.

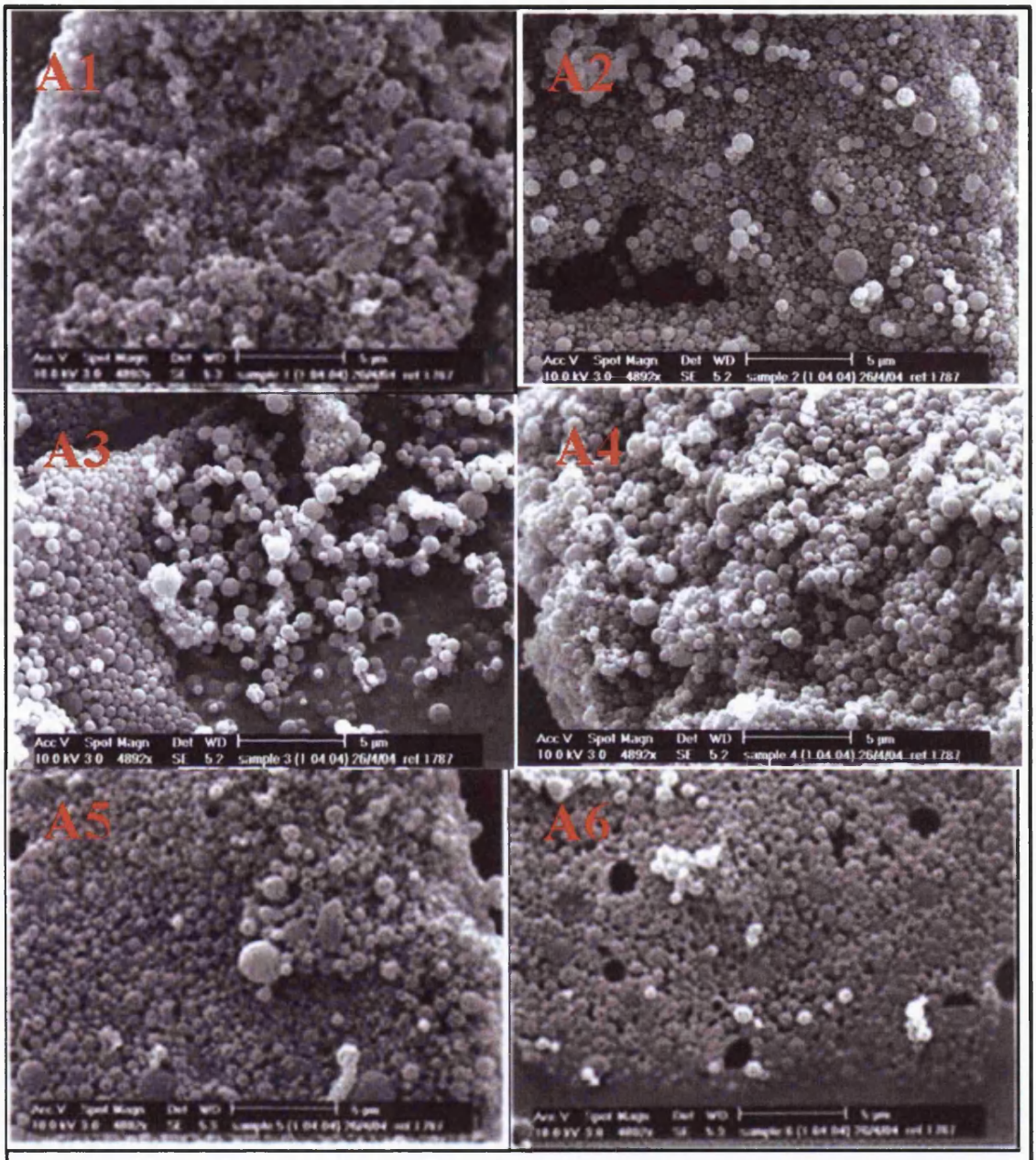

Figure 3.3: Scanning electron micrograph of OVA loaded PLA nanoparticles prepared by a w/o/w solvent evaporation method with varying concentrations of TPGS. (A1) PLA only; (A2) PLA plus 2.5% m/m TPGS; (A3) PLA plus 5% m/m TPGS; (A4) PLA plus 7.5% m/m TPGS; (A5) PLA plus 10% m/m TPGS; (A6) PLA plus 15% m/m TPGS.

SDS-PAGE is a useful method to evaluate the integrity of the protein/antigen after the process of microencapsulation. SDS–PAGE was carried out on the particles after freeze-drying to check the integrity of the protein (Figure 3.4). It can be observed that OVA do not lose its integrity during the process of encapsulation (only one band is observed for each well, whereas two bands would have indicated a degradation of the protein). It can also be observed that the intensity of the bands was not similar for all the

formulations, mainly lanes 7 and 8 which had OVA loaded PLA plus 5% TPGS and PLA only nanoparticles. This is might be due to the extraction/digestions technique used in this study. It might be possible that the nanoparticles were not completely digested when PBS and NaOH were used. Nevertheless, only single bands were shown for all the formulations implying that there was no degradation of the protein.

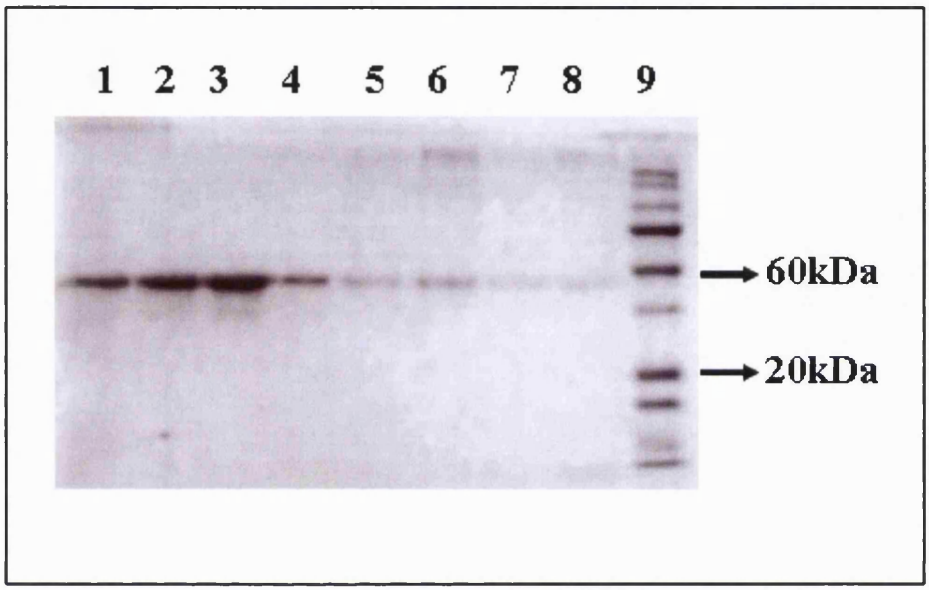

Figure 3.4: SDS–PAGE of OVA, nanoparticles were digested in PBS and NaCl Lanes are as follows: (1) 125 µg/ml of free OVA; (2) 175 µg/ml of free OVA; (3) 250 µg/ml of free OVA (4) 75 µg/ml of free OVA (5) OVA extracted from PLA nanoparticles – 15% m/m TPGS dissolved in organic phase; (6) OVA extracted from PLA nanoparticles – 7.5% m/m TPGS dissolved in organic phase; (7) OVA extracted from PLA nanoparticles – 5% m/m TPGS dissolved in organic phase; (8) OVA extracted from PLA nanoparticles; (9) Molecular Weight Marker (Broad Range).

In vitro burst release results for OVA-loaded PLA nanoparticles at the time intervals of 1, 8, 24 and 72 hours are shown in the Figure 3.5. A carrier system for antigen needs to release the antigen as soon as it is administered. It is not desirable to have a long delay in release of the antigen from the polymeric system. However, it is also not desirable if the entire antigen is released with a short time. Therefore the burst release was carried out to ascertain the percentage of OVA being released from the nanoparticles in the initial stages.

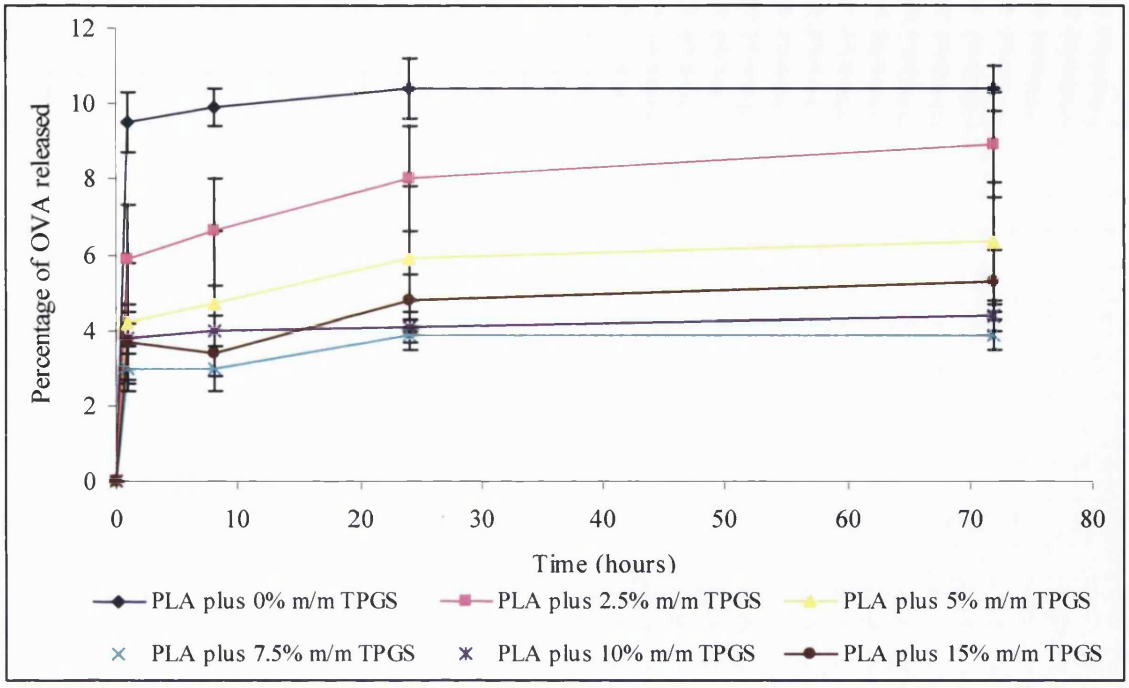

Figure 3.5: *In vitro* burst release of OVA from PLA nanoparticles prepared with varying concentrations of TPGS. The release was carried at the time intervals of 1, 8, 24 and 72 hours (n=3 ±s.d).

The degradation time for PLA 147 K is 12 to 16 months, due to its high hydrophobicity and molecular weight. It prevents the drug from diffusing from the polymer matrix into the aqueous solution; a low burst release is thus expected. For all the formulations the burst release after 24 hours is approximately 10% and does not significantly increase over 72 hours. Formulations containing TPGS have a lower burst release. For percentages of TPGS higher than 2.5% m/m the release is even less than 6%. This is probably caused by an enhanced interaction between OVA and PLA in the matrix due to the presence of the amphiphilic molecule. These observations are encouraging for the development of a formulation which can be administered as a single dose or double dose vaccine rather than multiple doses. A long-lasting antigen release is required for a single or double dose vaccine.

3.3.1.2. HBsAg 0.5% m/m loaded PLA plus TPGS or Pluronic F-127 microparticles

In this study Hepatitis B surface antigen was encapsulated in PLA microparticles. The HBsAg dose required in humans for the vaccination is very low (10-20 µg *per* dose), therefore 0.5% m/m of HBsAg is the theoretical loading used for the formulations. From the precedent study two formulations were selected and prepared using10% m/m

TPGS as a matrix in one formulation and 10% m/v TPGS as internal phase emulsifier for another formulation. A fourth formulation containing Pluronic F-127 dissolved in the organic phase was also prepared. Pluronic F-127 is a non-ionic, hydrophilic polyoxyethylene–polyoxypropylene (POE-POP) block co-polymer; with surfactant and protein stabilizer properties (proteins contained within the pluronic matrix at high concentrations have shown to retain their native configuration). Pluronic F-127-based matrices are characterized by a phenomenon known as reverse thermoregulation: they undergo a phase transition from liquid to gel upon reaching physiological temperatures. Thereby, they potentially act as sustained release depot systems. It has been used in a number of studies to improve the immunogenicity of the antigens. When Pluronic F-127 was combined with chitosan and TT, it significantly increased the antibody responses to intranasally delivered antigen (MacLean *et al.*, 2001; Kang *et al.*, 2007). In another study it was demonstrated that Pluronic F-127 combined with chitosan or CpG with TT, DT or anthrax recombinant protective antigen, there was a significant increase in the antibody responses when delivered systemically, compared to antigen/alum formulations where the adjuvants is used alone with the antigen. The results showed a long lasting immune response after a single administration due to the depot property of Pluronic F-127 (Coeshott *et al.*, 2004).

In this study Pluronic F-127 is used to impart adjuvancy for HBsAg loaded PLA microparticles with the aim to formulate a single dose vaccine. The influence of TPGS and Pluronic F-127 on particle size, zeta potential, yield and loading efficiency was investigated (Table 3.15).

Table 3.15: Characterisation of size, loading and zeta potential for 0.5% m/m HBsAg loaded PLA plus TPGS or Pluronic F-127 microparticles. 30 ml of 2.5% m/v PVA was used as stabilizing agent for secondary emulsion. (n=3, mean ±s.d).

Code	% mass of adjuvant in organic phase	Internal phase stabilizer with HBsAg	Yield (%)	Volume Mean Diameter (μm) ±s.d	Span	Zeta potential mV ±s.d	Actual protein content μg/mg ±s.d	Encapsulation Efficiency % ±s.d
B1	-	PVA 10%	76.3 ±4.2	2.8 ±0.04	2.3	-4.1 ±0.1	1.14 ±0.16	17.4 ±2.4
B2	TPGS 10%	PVA 10%	81.2 ±6.4	5.5 ±1.59	4.4	-4.6 ±0.2	3.96 ±0.14	64.2 ±2.3
B3	-	TPGS 10%	66.8 ±6.2	13.8 ±9.55	3.4	-9.6 ±1.3	5.33 ±0.13	71.2 ±1.8
B4	F-27 10%	PVA 10%	76.2 ±4.8	1.8 ±0.01	1.5	-4.0 ±0.1	1.58 ±0.31	24.1 ±4.8

The yield is high for all the formulations, especially when TPGS is used as a matrix, whereas it is lower when it is used as emulsifier. The yield is similar for the formulation (B4) containing Pluronic F-127 when compared with the control formulation (B1). Contrary to the preceding study, the size is bigger for TPGS emulsified microparticles (B3) compared to the microparticles where TPGS was used as a matrix (B2). In both cases the size is bigger than the size of the control microparticles, whereas for the particles containing Pluronic F-127 the size is smaller. Moreover, these particles are very easy to disperse in water, which can be attributed to the high hydrophilicity of the molecule. The zeta potential is slightly more negative compared to the control for the microspheres containing TPGS as a matrix, whereas it considerably decreases for TPGS emulsified microparticles. Pluronic F-127 did not have any influence on the zeta potential which is in accordance to the chemical structure of the block polymer. From the SEM pictures (Figure 3.6) we can observe that all the particles have a smooth surface and the size was more monodispersed unlike as indicated by the laser diffraction. There is not much difference in particle size between different formulations.

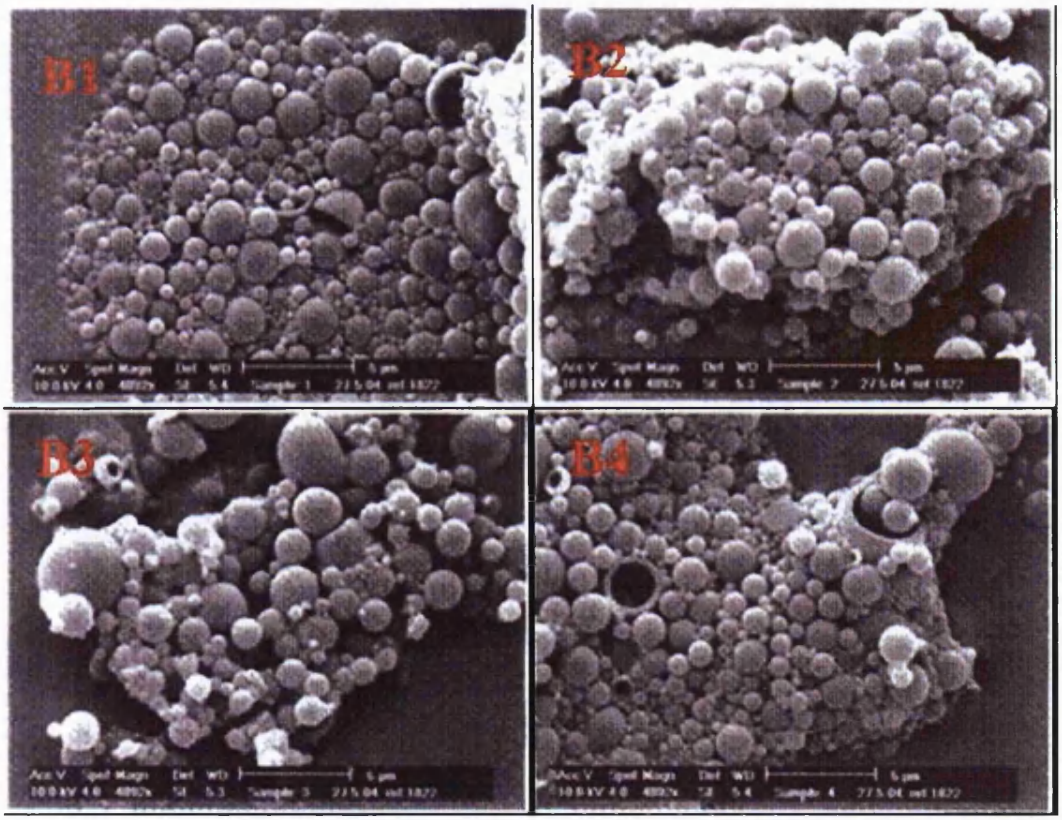

Figure 3.6: SEM of 0.5% m/m HBsAg loaded PLA plus TPGS or Pluronic F-127 nanoparticles. The external phase stabilizing agent was 30 ml of 2.5% m/v PVA. (B1) HBsAg loaded PLA nanoparticles – 10% m/m PVA as internal phase stabilizer; (B2) HBsAg loaded PLA nanoparticles – 10% m/m TPGS dissolved in organic phase and 10% m/m PVA as internal phase stabilizer; (B3) HBsAg loaded PLA nanoparticles – 10% m/m TPGS as internal phase stabilizer; (B4) HBsAg loaded PLA nanoparticles – 10% m/m Pluronic F-127 dissolved in organic phase and 10% m/m PVA as internal phase stabilizer.

The loading efficiency of the HBsAg in the microparticles was significantly influenced by the presence of TPGS ($P < 0.01$). These results clearly show the positive effect of TPGS as internal emulsifier on the loading efficiency. TPGS had a very different effect on the loading efficiency according to whether it is dissolved in the organic phase (and used as a matrix) or in the aqueous phase (and used as an internal phase emulsifier). When TPGS is added to the organic phase, it probably concentrates at the interfaces (as expected from its chemical nature) and facilitates protein leakage from the internal to the external aqueous phase. Whereas when TPGS is dissolved in the aqueous phase, TPGS molecules still concentrate at the interface, but this time they possibly form a thick film between the two phases which increases the stability of the primary emulsion and prevents protein leakage.

The particles were digested in PBS (pH 7.4) and NaOH overnight and subsequently the SDS-PAGE was carried on staining with Silver Stain (Figure 3.7). The integrity of the protein was inconclusive with the presence of a thin band appearing at the 30KDa range.

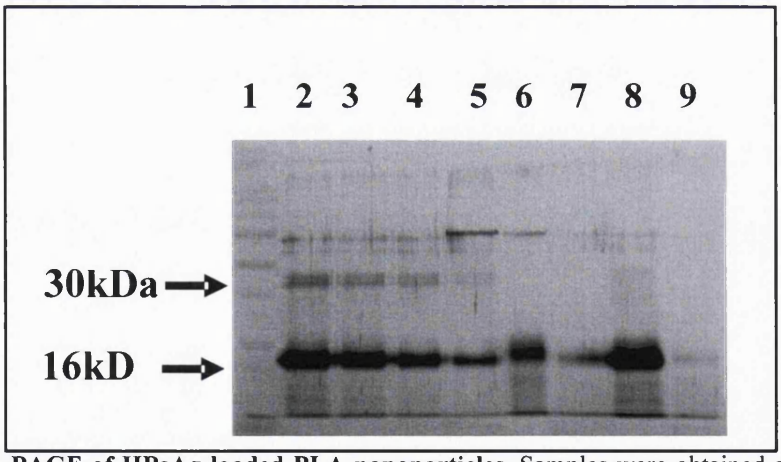

Figure 3.7: SDS–PAGE of HBsAg loaded PLA nanoparticles. Samples were obtained after digesting the nanoparticles in PBS and NaOH. . Lanes are as follows: (1) Molecular Weight Marker; (2) 400 µg /ml of free HBsAg; (3) 300 µg/ml of free HBsAg (4) 200 µg/ml of free HBsAg; (5) 100 µg/ml of free HBsAg; (6) HBsAg extracted from PLA nanoparticles – 10% m/m PVA as internal phase stabilizer; (7) HBsAg extracted from PLA nanoparticles – 10% m/m TPGS dissolved in organic phase and 10% m/m PVA as internal phase stabilizer; (8) HBsAg extracted from PLA nanoparticles – 10% m/m TPGS as internal phase stabilizer; (9) HBsAg extracted from PLA nanoparticles – 10% m/m Pluronic F-127 dissolved in organic phase and 10% m/m PVA as internal phase stabilizer

The alternative method to examine the protein integrity is either size exclusion chromatography or mass spectroscopy, although this was not done during this project. However, it was observed that the loading of the protein is definitely higher for TPGS emulsified particles (B3) as observed by the high intensity of the bands. Whereas as for

the particles containing TPGS (B2) and Pluronic F-127 (B3) in the matrix of the polymer the band intensity is comparatively lower than that of the control formulation (B1).

3.3.1.3. HBsAg 1% m/m loaded PLA microparticles with TPGS as matrix with PLA or as stabilizing agent in the internal phase emulsion

A carrier system containing antigen should be able to contain high concentrations of the antigen so that minimum quantity of the particles can be administered in the animal models. In the earlier experiment the theoretical loading of HBsAg was 0.5% m/m, in order to increase the antigen content in the microparticles 1% m/m HBsAg was used in preparing these formulations. The yield obtained is high for all three formulations and the results do not show any difference between the formulations (Table 3.16).

Table 3.16: Effect of TPGS when used as a matrix with PLA or as stabilizing agent in the internal phase emulsion on HBsAg loaded PLA microparticles. Particles were characterized for size, loading and zeta potential of nanoparticles. TPGS was used either in the organic phase dissolved with PLA or as an internal phase stabilizing agent. The external phase stabilizing agent was 30 ml of 2.5% m/v PVA. (n=1, analysis was done 3 times on same formulation, mean ±s.d).

Code	% mass of TPGS - organic phase	Internal phase stabilizer along with HBsAg	Yield (%)	Volume Mean Diameter (μm) ±s.d	Span	Zeta potential, mV ±s.d	Actual protein content (μg/mg) ±s.d	Encapsulation efficiency % ±s.d
C1	-	PVA 10%	71.1 ±2.4	0.35 ±0.01	0.2	-5.4 ±0.3	2.45 ±0.14	17.4 ±1.02
C2	10	PVA 10%	67.6 ±3.8	33 ±2.3	2.2	-8.0 ±0.4	11.86 ±0.04	80.2 ±0.28
C3	-	TPGS 10%	71.1 ±2.6	30.7 ±1.8	1.9	-15.8 ±0.8	13.32 ±0.23	94.7 ±1.67

TPGS has been used instead of the traditional and most popular emulsifier PVA. It is difficult to completely remove PVA from the surface of the nanoparticles, which may have side effects for health care products and as well hinder the nanoparticle uptake into cells. TPGS has few side effects and appears to be a better surface active agent in the preparation of polymeric nanoparticles. One advantage of TPGS against PVA is its unique property of being able to dissolve both in water and oil (with a hydrophobic ability greater than hydrophilic capability). In a study carried by Mu and Feng (2002) using the same fabrication conditions, the required amount of TPGS is 67 times less than the PVA. The particle characteristics (size, morphology) were similar to those of PVA emulsified nanoparticles. However, the excess TPGS on the surface of the nanoparticles could be cleaned relatively thoroughly. Moreover, it was found that, in comparison with the traditional chemical emulsifier PVA, TPGS could significantly

improve the encapsulation efficiency of the drug in the PLGA nanoparticles, which could be as high as 100%.

TPGS has also been used as a surfactant or as a matrix material blended with other biodegradable polymers (PLGA) to improve the physicochemical properties of paclitaxel loaded nanoparticles. The mixture of PLGA and TPGS has a self-emulsifying effect, which can form nanoparticles without the need to add another surfactant as stabilizer. The results showed that nanoparticles prepared with this mixture had smaller size and narrow polydispersity. The yield was decreased when the ratio of TPGS was increased due to the high solubility of TPGS in water. It was shown that when the theoretical drug loading was low the amount of drug entrapped in the nanoparticles decreased, but when the drug loading ratio was increased to 10% the encapsulation efficiency could reach 100% (Mu and Feng, 2003). In another study, however, using the w/o/w technique, TPGS was mixed to the polymer (PLGA or PELA) and human serum albumin (HSA) was encapsulated. The results demonstrated that the effect of TPGS on the particle size of microspheres was not significant; whereas the experimental data showed that vitamin E TPGS had negative effect on the encapsulation efficiency when w/o/w emulsion solvent extraction/evaporation process was used to encapsulate the hydrophilic compound HSA (Ruan *et al*, 2002).

In the present study, the encapsulation efficiency of the particles in absence of TPGS (C1) was found to be 17.4% whereas the encapsulation efficiency increased to 80.2 % and 94.7% when TPGS was used in the organic phase or TPGS was used in the internal phase stabilizing agent (C2 and C3). This is contradictory to the observation of Rosa *et al* (2000), where co-encapsulation of surfactants reduced encapsulation efficiency of insulin in PLGA microspheres. However, in a recent study it was observed that when TPGS-PLA copolymer was used for the encapsulation of bovine serum albumin (BSA), the encapsulation efficiency of the protein increased (Lee *et al*, 2007). The authors observed that the presence of TPGS in the polymer is advantageous and it does not displace the protein from the w/o emulsion during the w/o/w double emulsion solvent evaporation method. In our study PLA and TPGS were physically mixed rather than chemically as a copolymer of TPGS-PLA. However, the presence of TPGS might have increased the loading efficiency by preventing the HBsAg displacement from the w/o emulsion during the formulation process.

The difference in particle size of the control (C1) and the particles prepared using TPGS as a matrix (C2) and emulsifier (C3) is larger than the preceding study. The particles with PLA only (C1) were 346 nm whereas the particles containing TPGS (C2 and C3) have been demonstrated to have larger particle size (> 30 μm). However, these particles were easy to disperse in water evidently due to the high hydrophilicity of the molecule. The zeta potential is slightly more negative compared to the control for the microparticles containing TPGS as a matrix, whereas it has decreased for TPGS emulsified microparticles (C3). The SEM showed that all the particles have smooth surface and their size is homogeneous (Figure 3.8).

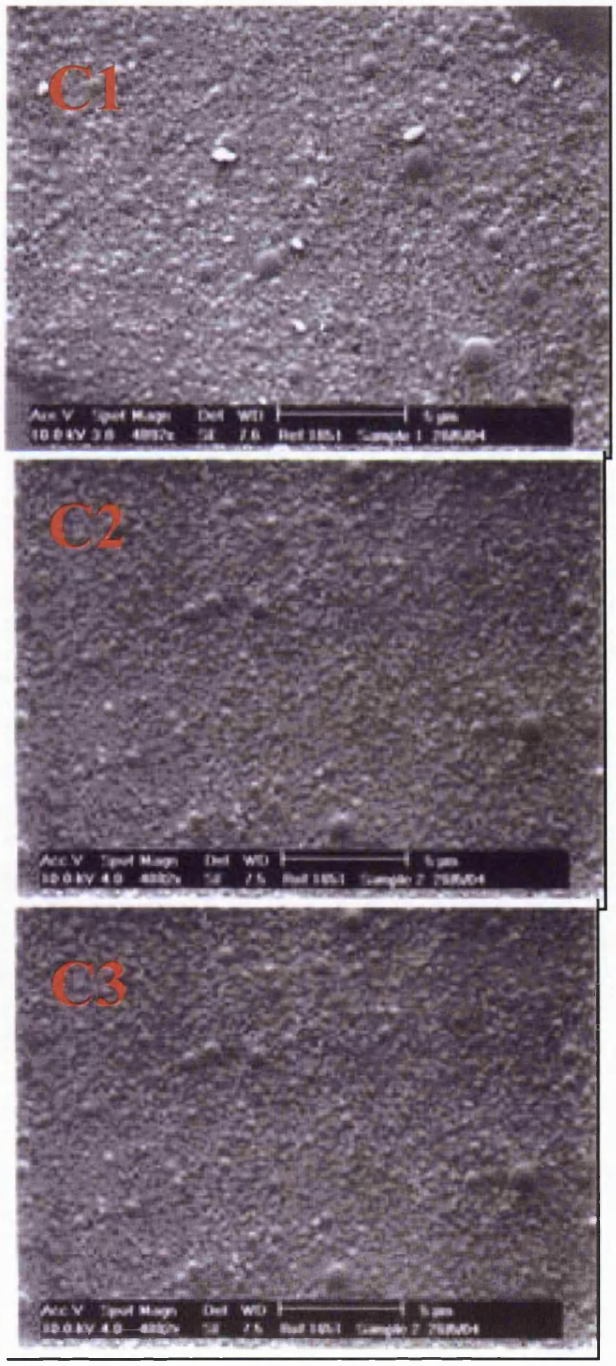

Figure 3.8: Scanning electron micrograph of 1% m/m HBsAg loaded PLA plus TPGS nanoparticles. TPGS was used either in the organic phase dissolved with PLA or as an internal phase stabilizing agent. The external phase stabilizing agent was 30ml of 2.5% w/v PVA. (C1) HBsAg loaded from PLA nanoparticles – 10% m/m PVA as internal phase stabilizer; (C2) HBsAg loaded PLA nanoparticles – 10% m/m TPGS dissolved in organic phase and 10% m/m PVA as internal phase stabilizer; (C3) HBsAg loaded PLA nanoparticles – 10% m/m TPGS dissolved in organic phase and 10% m/m TPGS as internal phase stabilizer.

According to the SEM observation, there was no difference in particle sizes between different formulations as indicated by the laser diffraction. The anamoly in the particle sizing by laser diffraction can be attributed to the aggregation of the particles during the freeze drying process.

The structural integrity of the extracted and released HBsAg was analysed using SDS-PAGE. The retention of structural integrity of the antigen is a prerequisite for the antigen to generate a suitable immune response, denaturation or inactivation of the protein during processing causes alteration of the native structure of the protein, indicated by a shift in band formation higher or lower than the standard. This was not seen for any of the samples (Figure 3.9) and therefore the structural integrity of HBsAg in the microparticles was not affected by process parameters and storage. We also can observe that the TPGS emulsified particles (C3) have a higher loading efficiency as shown by thick bands (higher intensity bands) in comparison to the other formulations.

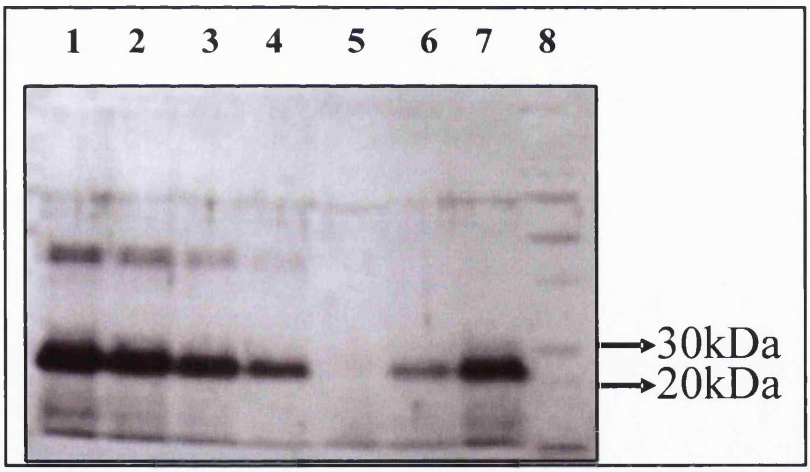

Figure 3.9: SDS–PAGE of HBsAg extracted by digesting the nanoparticles in PBS and NaOH overnight. Lanes are as follows: (1) Molecular Weight Marker; (2) 400 µg/ml of free HBsAg; (3) 300 µg/ml of free HBsAg (4) 200 µg/ml of free HBsAg; (5) 100 µg/ml of free HBsAg; (6) HBsAg extracted from PLA nanoparticles – 10% m/m PVA as internal phase stabilizer; (7) HBsAg extracted from PLA nanoparticles – 10% m/m TPGS dissolved in organic phase and 10% m/m PVA as internal phase stabilizer; (8) HBsAg extracted from PLA nanoparticles – 10% m/m TPGS dissolved in organic phase and 10% m/m TPGS as internal phase stabilizer; (9) Molecular Weight Marker.

3.3.1.4. HBsAg 1% m/m loaded PLA plus TA or TN nanoparticles

PLA plus tocopherol acetate (TA) or tocopherol nicotinate (TN) were dissolved in the organic phase of the emulsification process to prepare HBsAg loaded nanoparticles by w/o/w double emulsification solvent evaporation method. The results are summarized in Table 3.17.

Table 3.17: Effect of TA or TN concentration when used as a matrix on HBsAg loaded PLA nanoparticles. Particles were characterized for size, loading and zeta potential of nanoparticles. Particles were prepared by solvent evaporation method; the internal and external phase stabilizing agent was 1 ml of 10% m/m of PVA and 30 ml of 1.25% m/v PVA. (n=1, analysis was done 3 times on same formulation, mean ±s.d).

Code	% mass of TA or TN in organic phase	Yield % ±s.d	Z average particle size (nm) ±s.d	Poly-dispersity ±s.d	Zeta potential, mV ±s.d	Actual protein content (µg/mg) ±s.d	Encapsulation efficiency % ±s.d
I1	-	63.4 ±1.45	337.9 ±3	0.448 ±0.019	-6.34 ±2.2	3.91 ±0.06	24.79 ±2.3
I2	TA 20%	65.3 ±1.54	319.7 ±2	0.274 ±0.024	-6.54 ±1.4	13.2 ±0.11	86.2 ±0.4
I3	TN 20%	67.3 ±1.34	340.6 ±5	0.225 ±0.014	-7.65 ±1.6	8.73 ±0.12	58.72 ±0.3

The size of the particles before the freeze drying was found to be around 250 to 300 nm for the formulations. The particle size has slightly increased to 320-340 nm after freeze drying which is not unexpected as freeze drying tends to aggregate the nanoparticles. The PLA-TA particles showed an increase in encapsulation efficiency up to 86% compared with 25% for plain PLA particles, whereas the PLA-TN particles had encapsulation efficiency of 59%. This may have occurred due to the amphiphillic properties of tocopherol in the formulations. The structural integrity of the encapsulated antigen in the nanoparticles was determined by the SDS-PAGE. The gels show that the HBsAg was not degraded. The structural integrity of HBsAg remained following exposure to DCM, homogenization and lyophilisation. This was observed for the all the nanoparticle formulations.

3.3.1.4.1. MTT assay of the formulations to assess cytotoxicity

Based on the satisfactory encapsulation efficiency it was decided that particles containing 20% of either TPGS or TA or TN will be used in the *in vivo* animal studies. Although the literature suggests that the tocopherol derivatives are safe for human as well as animal use, it is imperative to assess the safety of these molecules in conjunction with the polymeric particles. Therefore the nanoparticles containing TPGS or TA or TN were assessed for the cytotoxicity in the cell lines by the MTT assay.

The MTT assay is a colorimetric assay system that measures the reduction of a tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into an insoluble formazan product by the mitochondria of viable cells. *In vitro* toxicity

of nanoparticles was evaluated by MTT assay in CHO-K1 cells using increasing concentration of nanoparticles (Figure 3.10). The nanoparticles assessed prepared by the solvent evaporation method, PLA only particles; PLA (20% m/m TPGS in organic phase); PLA (20% m/m TA in organic phase); PLA (20% m/m TN in organic phase) particles. Poly ethyleneimine (PEI) was used as a positive control. PEI is positively charged polyplex which interacts with the blood components activating the complement system and thereby is highly toxic at the cellular levels (Merdan *et al.*, 2002). Due to this known toxic properties of PEI, it was used as the positive control. The nanoparticle formulations were found to be non-toxic at the majority of concentrations studied. There was no significant difference ($p > 0.05$) in the toxicity of different formulations at any of the concentrations used. There was a slight reduction in cell viabilities at higher concentrations of 5 mg/ml of the particles.

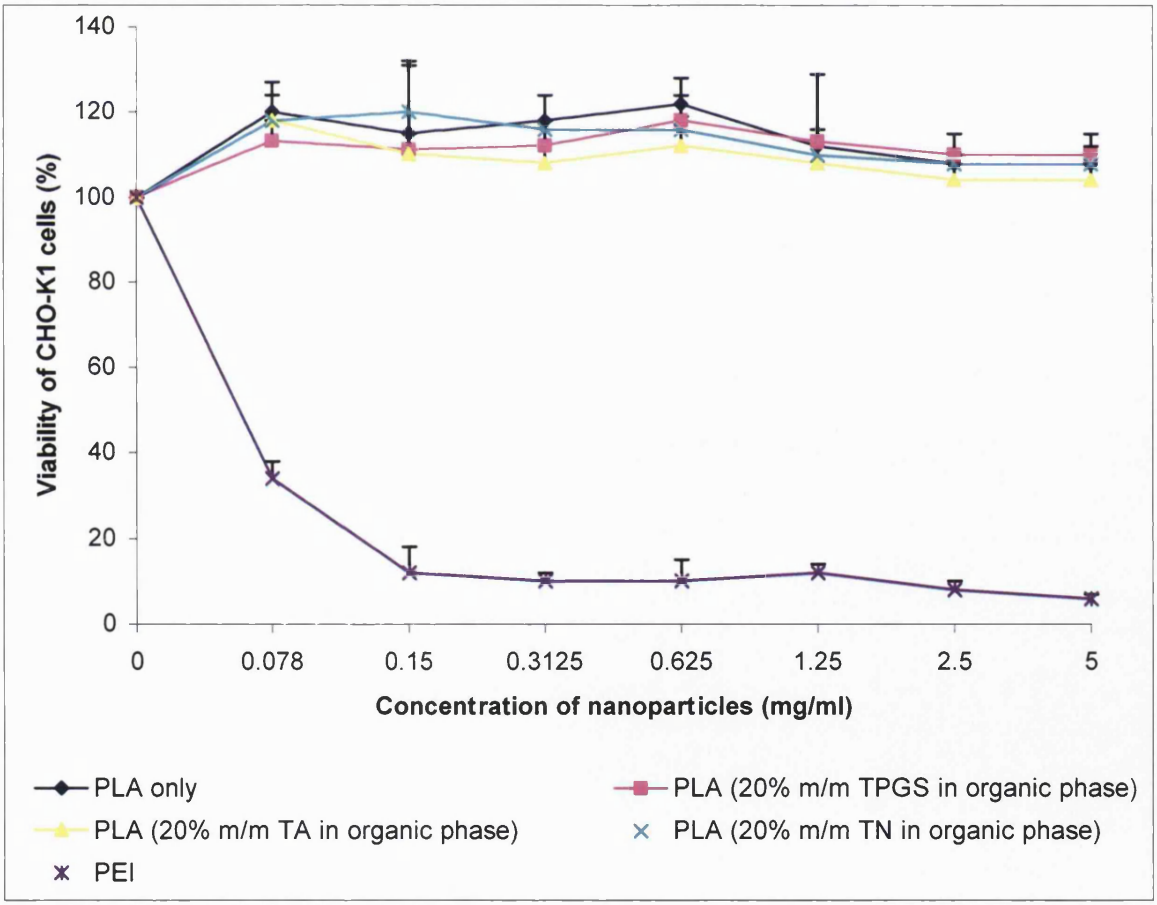

Figure 3.10: Cytotoxicity of HBsAg loaded PLA plus tocopherol derivative nanoparticles analyzed by MTT assay in CHO-K1 cells. The particles were prepared by double emulsion solvent evaporation with either TPGS or TA or TN mixed in the organic phase of the formulation. PEI was used as a positive control. Cells incubated in cell culture medium were used as a control to assay 100% viability. Viability of cells exposed to the formulations was expressed as percentage of the viability seen in control untreated cells ($n = 3$, mean ±s.d).

The average cell viabilities were between 90 to 125% of control (PEI) at the concentrations studied. TPGS, TA and TN are considered to be safe excipients (Eastman Inc datasheet; Hidiroglou and Charmley, 1990; 1991; Toutain *et al.*, 1995). Results of one-year chronic oral toxicity studies in rats and dogs conducted by the National Cancer Institute indicated that no safety concerns exist for either species regarding oral use of the excipient at the highest levels tested (1000 mg/kg/day). These reports substantiate the safety data available from Eastman toxicology studies. A monograph for TPGS was approved by the United States Pharmacopoeia (p4712 supplement no 9, 1998). The US FDA recently approved Agenerase® (active ingredient is Amprenavir from GlaxoSmithKline), a protease inhibitor for the treatment of HIV positive patients containing D-α-tocopheryl PEG 1000 succinate as an absorption enhancer in the dosage form. It has been shown that TPGS can be used as a stabilizer, an emulsifier, an absorption enhancer, a vehicle for lipid-based drug delivery formulation and a water soluble source of vitamin E (Eastman Chemical Co, datasheet). Tocopherol derivatives like TA and TN are also non-toxic in animal feeding studies, although very high doses (2 g/kg/day) have caused haemorrhages. TA and TN have also exhibited antimutagenic activity consistent with their antioxidant properties. Tocopherol was found not be carcinogenic. Some studies showing tumour promotion activity in mice have been found to be non-reproducible and inconsistent with the data suggesting that the antioxidant properties of these agents protect against tumour induction.

3.3.1.5. DT 2% m/m loaded PLA microparticles– TPGS as matrix with PLA or as stabilizing agent in the internal or external phase emulsion

In the present study TPGS was used as a matrix as well as an emulsifier in DT loaded microparticles and the effect of TPGS on the physicochemical characteristics (size, zeta potential and loading efficiency) of the microparticles was investigated (Table 3.18). The zeta potential slightly decreases when TPGS is used as a matrix, as in the preceding study. When TPGS is used as an emulsifier, the zeta potential becomes significantly more negative (<-20mV). This can be explained by the chemical nature of this molecule (especially by the presence of the alcoholic group -OH of the PEG) and by the presence of high concentrations of TPGS on the surface of the microspheres. This assumption is based on a previous study, wherein low concentrations of TPGS (0.015% m/v) were used as emulsifier and the atomic force microscopy (AFM) studies revealed the absence of TPGS on the surface (Mu and Feng, 2003).

Table 3.18: Characterisation of size, loading and zeta potential for 2% m/m DT loaded PLA microparticles. TPGS was used as matrix with PLA or as stabilizing agent in the internal or external phase emulsion (n=3, mean ±s.d).

Code	% mass of TPGS in organic phase	Internal phase stabilizing agent	External phase stabilizing agent	Yield % ±s.d	Mean volume diameter (μm) ±s.d	Span	Zeta potential, mV ±s.d	Actual protein content (μg/mg) ±s.d	Encapsulation efficiency % ±s.d
D1	-	PVA	PVA 2.5%	66.4 ±3.2	3.5 ±0.35	2.4	-4.3 ±0.2	3.72 ±0.18	49.4 ±2.4
D2	10	"	"	68.5 ±2.4	19.4 ±5.07	3.7	-5.3 ±0.3	2.78 ±0.25	38.1 ±3.4
D3	25	"	"	73.2 ±3.4	13.2 ±3.02	5.2	-5.7 ±0.4	1.81 ±0.33	26.5 ±4.8
D4	5	"	"	68.4 ±2.6	22.8 ±1.81	3.3	-4.4 ±0.1	3.08 ±0.25	42.2 ±3.4
D5	-	TPGS	"	59.3 ±2.6	5.7 ±1.94	3.2	-22.6 ±0.6	6.53 ±0.21	77.4 ±2.5
D6	-	"	TPGS 10%	54.6 ±2.3	190.6 ±48.2	1.8	-22.0 ±0.8	1.87 ±0.27	20.4 ±2.9

Laser diffraction (Malvern MasterSizer®) reveals a larger size for the microspheres prepared using TPGS as matrix (D2-D4) as well as an internal phase emulsifier (D5). This is probably due to the aggregation of the particles during freeze-drying. The microparticles of the formulations D1 to D5 have similar shape and size, contrary to the results obtained with the laser diffraction. The SEM pictures show smooth particles of homogeneous size. The microparticles were not formed when TPGS was used as internal and external phase emulsifier therefore there is no SEM picture of D6 (Figure 3.11). The microparticles containing TPGS were not easy to disperse in water.

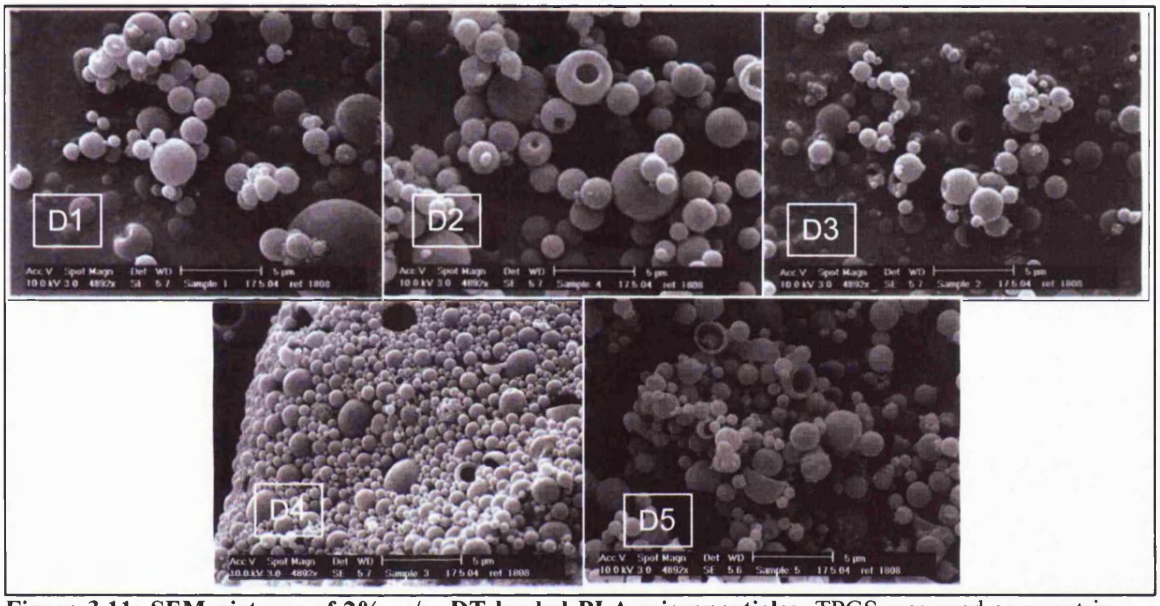

Figure 3.11: SEM pictures of 2% m/m DT loaded PLA microparticles. TPGS was used as a matrix with PLA or as stabilizing agent in the internal or external phase emulsion

From the SDS-PAGE (Figure 3.12), it can be observed that the formulation (D5), where TPGS is used as internal emulsifier, has the highest loading. For the particles containing TPGS as a matrix (D2-D4) the loading efficiency is lower, compared to the control formulation D1, prepared without using TPGS. However, for all the formulations it was difficult to observe the bands owing to incomplete digestion/extraction. The alternative method to observe these bands can be staining with silver stain which is more sensitive than commaissne blue staining. Overall, it can be said that the integrity of the encapsulated DT in these microparticles is inconclusive.

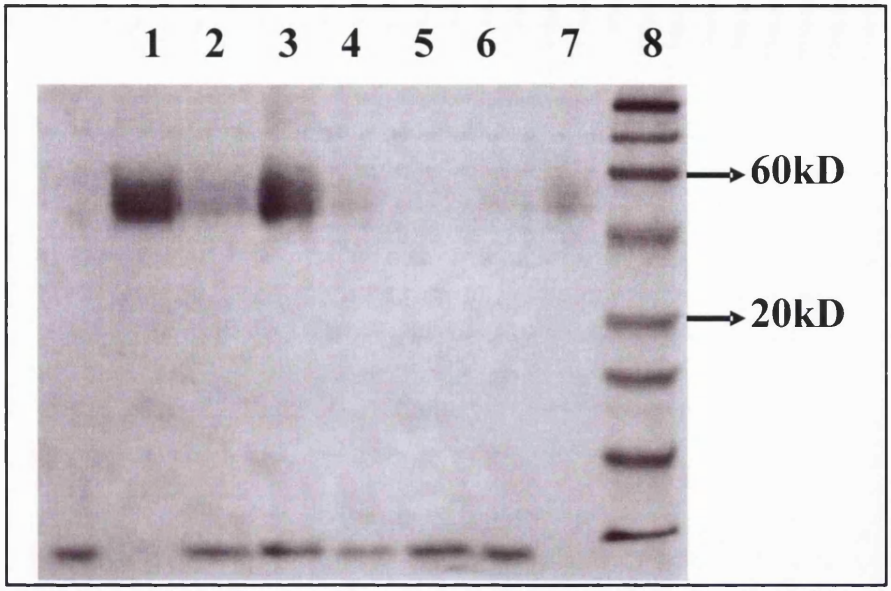

Figure 3.12: SDS–PAGE of DT in the PLA microparticles. Samples were extracted by digesting the PLA microparticles in PBS and NaOH overnight. Lanes are as follows: (1) 500 µg/ml of free DT; (2) DT extracted from PLA microparticles – 10% m/m TPGS as internal and external phase stabilizer; (3) DT extracted from PLA microparticles – 10% m/m TPGS as internal and 2.5% PVA as external phase stabilizer; (4) DT extracted from PLA microparticles – 10% m/m PVA as internal and 2.5% PVA as external phase stabilizer; (5) DT extracted from PLA microparticles – 25% m/m TPGS in organic phase – 10% m/m PVA as internal and 2.5% PVA as external phase stabilizer; (6) DT extracted from PLA microparticles – 10% m/m TPGS in organic phase – 10% m/m PVA as internal and 2.5% PVA as external phase stabilizer; (7) DT extracted from PLA microparticles –10% m/m PVA as internal and 2.5% PVA as external phase stabilizer; (8) Molecular Weight Marker.

3.3.1.6. DT 2% m/m loaded PLA microparticles with varying concentration of TPGS as internal phase stabilizing agent

In the present study four different concentrations (varying from 2.5% to 20% m/v) of TPGS as internal phase emulsifier were used for the preparation of the DT loaded PLA microparticles by the w/o/w double emulsion solvent evaporation method. The particles were characterized according to the size, surface charge and encapsulation efficiency (Table 3.19).

Table 3.19: Effect of varying concentration of TPGS used in the internal phase stabilizer on DT loaded PLA nanoparticles. Particles were characterized for size, loading and zeta potential of nanoparticles. (n=3, mean ±s.d).

Code	Internal phase stabilizer	Yield % ±s.d	Mean Volume Diameter (µm) ±s.d	Span	Zeta potential, mV ±s.d	Actual protein content (µg/mg) ±s.d	Encapsulation efficiency % ±s.d
E1	PVA 10%	64.4 ±4.2	1.9 ±0.2	1.7	-5.1 ±0.6	16.02 ±0.75	51.6 ±2.4
E2	TPGS 20%	68.9 ±5.6	8.3 ±1.9	3.7	-23 ±0.2	21.60 ±0.81	74.4 ±2.8
E3	TPGS 10%	66.7 ±6.4	5.4 ±0.1	6.1	-22.6 ±0.3	24.29 ±1.08	81 ±3.6
E4	TPGS 5%	68.6 ±2.9	3.8 ±1.3	4.3	-22.9 ±1.0	25.22 ±0.85	86.5 ±2.9
E5	TPGS 2.5%	62.1 ±4.8	6.5 ±1.2	3.4	-22.7 ±0.2	27.41 ±1.35	85.1 ±4.2

The product yield for all the formulations was 62-69%. The size of the particles prepared using TPGS as emulsifier (E2-E5) was comparatively larger than the particles prepared using PVA as stabilizing agent (E1). However, for all the formulations the size is < 10 μm. For concentrations higher than 2.5% TPGS, the size of the microparticles increases (from 3.8 to 8.3 μm), this is probably due to the relative increase in viscosity of the stabilizing agent. For all the TPGS emulsified particles (E2-E4) the surface charge decreased (-20 mV) compared to the PVA emulsified microparticles (E1), which had the surface charge of -5.1 mV. The loading efficiency is 25 to 30% higher for TPGS emulsified microparticles when compared with particles made using PVA as stabilizing agent (E1). Furthermore, for 5% TPGS emulsified particles (E4) to 20% TPGS emulsified ones (E2) the encapsulation efficiency decreased, whereas E4 (5%) and E5 (2.5%) show a similar encapsulation efficiency.

All the microparticles had smooth surface and there was no aggregation as observed by SEM (Figure 3.13). The particles prepared using the 20% TPGS (E2) had larger size than particles without TPGS (E1), thus confirming the results obtained by the laser diffraction.

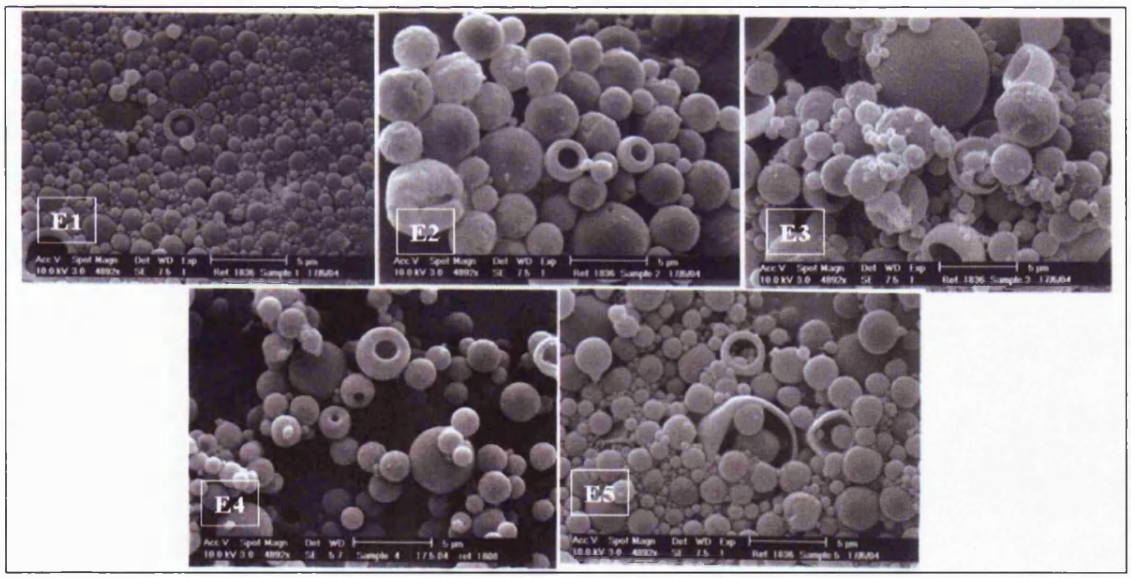

Figure 3.13: SEM pictures of 2% m/m DT loaded PLA microparticles. Particles were prepared with varying concentration of TPGS as internal phase stabilizing agent.

SDS-PAGE showed that there was no deterioration of DT when encapsulated in the microparticles (Figure 3.14). For TPGS emulsified microparticles, intense bands are revealed confirming higher encapsulation efficiency of these particles. It was not possible to observe the band of lane 6 which was containing the DT extracted/digested

from the PLA only microparticles. This can be due to incomplete extraction of the antigen from the microparticles.

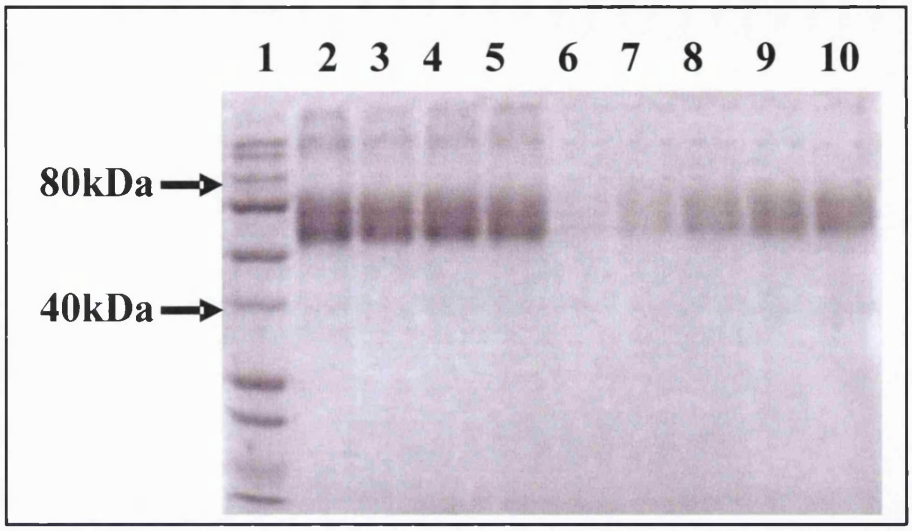

Figure 3.14: SDS–PAGE of DT extracted from PLA microparticles prepared using PVA and TPGS as stabilizing agent. Samples were obtained after extraction of antigen from PLA microparticles by digesting in PBS and NaOH. Lanes are as follows: (1) Molecular Weight Marker; (2) DT extracted from PLA microparticles – 2.5% m/m TPGS as internal and 2.5% m/m PVA as external phase stabilizer; (3) DT extracted from PLA microparticles – 5% m/m TPGS as internal and 2.5% m/m PVA as external phase stabilizer; (4) DT extracted from PLA microparticles – 10% m/m TPGS as internal and 2.5% m/m PVA as external phase stabilizer; (5) DT extracted from PLA microparticles – 20% m/m TPGS as internal and 2.5% m/m PVA as external phase stabilizer; (6) DT extracted from PLA microparticles – 10% m/m PVA as internal and 2.5% m/m PVA as external phase stabilizer; (7) 100 µg/ml of free DT; (8) 150 µg/ml of free DT; (9) 200 µg/ml of free DT; (10) 500 µg/ml of free DT.

3.3.1.7. DT loaded PCL microparticles with varying concentration of TPGS in the organic phase prepared by spray drying technique

DT loaded PCL microparticles were prepared by w/o/w double emulsification followed by spray drying of the emulsion. Compared to other biodegradable polyesters like polyglycolide, polylactide and their copolymers, PCL is less expensive and offers many advantages. It is non-toxic, non-mutagenic and shows minimal or insignificant tissue reaction. Moreover, its low glass transition temperature (Tg) of -60°C and melting point of about 60°C makes it more suitable for the spray drying technique (Cha and Pitt, 1990; Lin *et al,* 1999; Lin and Lu, 2002).

The particles were characterized with respect to the size, surface charge and encapsulation efficiency of the antigen (Table 3.20).

Table 3.20: Characterisation of size, loading and zeta potential for 2% m/m DT loaded PCL microparticles prepared by spray drying method. Particles were prepared with varying concentration of TPGS in the organic phase. (n=3 Mean ±s.d).

Code	% mass of TPGS dissolved in the organic phase with PCL	Internal phase stabilizer	External phase stabilizer	Yield % ±s.d	Mean volume diameter (µm) ±s.d	Span	Zeta potential, mV ±s.d	Actual protein content (µg/mg) ±s.d	Encapsulation efficiency % ±s.d
F1	-	7.5% PVA	2.5% PVA	32 ±3	2.33 ±1.28	1.8	-2.3 ±0.2	38.36 ±0.53	61.38 ±0.85
F2	10	"	"	39 ±2	2.11 ±1.09	2.4	-5.4 ±0.1	30.31 ±0.26	59.1 ±0.51
F3	20	"	"	42 ±4	2.43 ±1.36	2.1	-6.4 ±0.2	24.25 ±0.27	50.93 ±0.57
F4	40	"	"	45 ±3	2.59 ±1.65	1.8	-6.8 ±0.3	27.12 ±0.38	61.03 ±0.86
F5	-	7.5% Kollicoat®	2.5% Kollicoat®	51 ±2	2.66 ±1.72	1.4	-3.6 ±0.2	12.42 ±0.24	31.67 ±0.60
F6	-	"	5% Kollicoat®	88 ±4	2.23 ±1.32	2.4	-3.5 ±0.1	2.55 ±0.01	11.23 ±0.05
F7	-	15% Kollicoat®	10% Kollicoat®	121 ±5	4.89 ±4.72	2.6	-3.2 ±0.1	2.73 ±0.05	16.5 ±0.32
F8	-	"	20% Kollicoat®	148 ±8	7.86 ±6.86	1.6	-3.1 ±0.2	1.52 ±0.01	11.26 ±0.03

Generally the yield obtained from spray drying process is low. A 32% yield was obtained for diphtheria polycaprolactone microspheres (F1). When TPGS+PCL blends were used the yield gradually increased with the increase in concentration of TPGS. For 10, 20, 40% m/v of TPGS, the yield was 39, 42 and 45% respectively. A maximum yield of 45% was obtained for the 40% TPGS-PCL blend (formulation F4), which is significantly higher than the formulation F1 (only PCL). Although these results are contradicting to the work done by Mu and Feng (2003), where the yield was decreased with polymer-TPGS blends, the reasons can be attributed to different processing parameters such as preparation method (the solvent evaporation method was used) and emulsifying agent (no emulsifier was used). In that particular study paclitaxel loaded PLGA nanoparticles were made using a blend of TPGS and PLGA as matrix material.

In the formulations F5-F8 Kollicoat® was used instead of PVA as emulsifying agent. The yield for formulation F5 (2.5% Kollicoat®) was 51%, which is in fact higher than that obtained for 40% TPGS-PCL blend (F4). The yield increased with increase in concentration of Kollicoat® as shown in the Figure 3.2. For 5, 10, 20% m/v Kollicoat® the yield obtained was 88, 121, 148% respectively. This yield is surprisingly very high. The increase in yield can be because of encapsulation of the stabilizing agent (Kollicoat®) along with the polymer. The particle size of formulations from F1-F4 was in the size range of 2-5 μm.The distribution modal size of the particles prepared with only PCL and PCL+10% TPGS was more than that of with 20 and 30% TPGS. The zeta potential of the particles decreased for the formulations of TPGS-PCL blends (F2-F4) when compared with PCL formulation (F1). Among all the formulations, the lowest zeta potential was for the formulation of 40% TPGS-PCL blend (-6.4mV). Although there is a decrease in the zeta potential, the difference is not statistically significant.

For the formulations made with Kollicoat® as the stabilizing agent, there was a minimal increase of zeta potential with increase in concentration of the stabilizing agent when compared with the formulation made 2.5% Kollicoat® (F5) which had zeta potential of -3.6mV. The zeta potential of the formulation with 20% Kollicoat® has increased to -3.1mV, which again is statistically insignificant. The loading efficiency of the formulations made with PVA as emulsifier (F1-F4) had a satisfactory loading of about 50-60%. Among all the formulations, the 20% TPGS-PCL blend had the lowest loading efficiency of 50%. These results show that the TPGS-PCL blend had made no

significant difference in the loading efficiency of the diphtheria toxoid. On the other hand the formulations prepared with Kollicoat® as the stabilizing agent (F5-F8) demonstrated a very drastic difference in loading efficiency. Of the four formulations, a maximum loading efficiency of 31% was achieved for the formulation with 2.5% Kollicoat® used in the secondary emulsion (F5). For the remaining formulations (F6-F8) the loading decreased to 11-16%.This large difference in loading can be related to the high yield obtained from these formulations which subsequently decreased the loading efficiency. Therefore, there can be a significant error in the calculation of the loading efficiency because of the encapsulated PEG-PVA. The burst release study was performed for the formulations made with PVA as stabilizing agent (F1-F4). The formulation made with PCL alone (F1) has shown the highest release of ~44%; whereas, formulations made with TPGS-PCL blends have shown to have reduced release. The release decreased with increase in concentration of TPGS. This decrease in release is not very significant. Under SEM observation (Figure 3.15), all the microparticles had a fine spherical shape and appear to have smooth surface without any deformities.

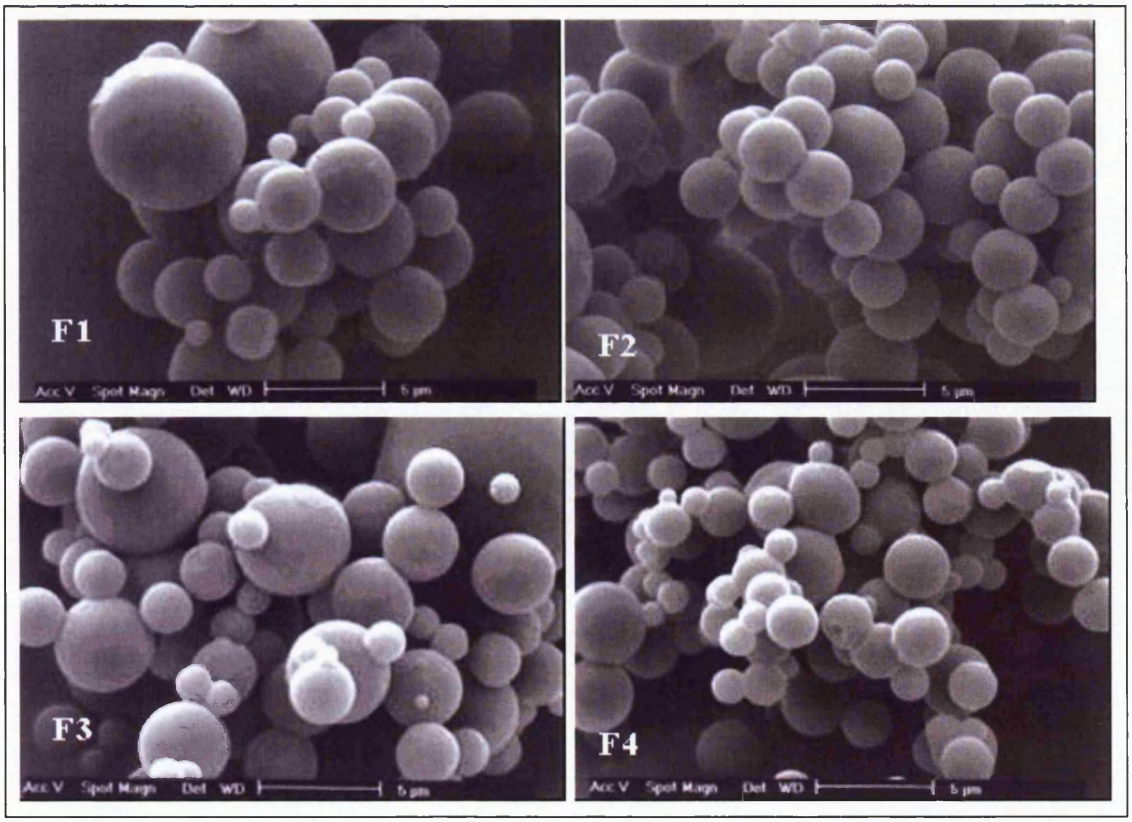

Figure 3.15: SEMs of DT encapsulating PCL plus microparticles by spray drying technique with varying concentration of TPGS in the organic phase (PVA as stabilizing agent).

The particles without TPGS (F1) are bigger than the particles with TPGS (F2-F4). The highest concentration of 40% m/m TPGS particles (F4) were smaller than the others, however the laser diffraction analysis did not show any prominent difference in the particle size. This decrease in particle size suggests that TPGS has affected the physical properties of the particles.

Although PVA plus PEG has been used previously in stabilising emulsions, Kollicoat® comprises of 75% PVA and 25% PEG graft copolymer and is mainly applied in instant-release coatings of tablets and pellets as highly flexible film-former with low viscosity. It also contains approximately 0.3% colloidal silica to improve its flow properties. The molecular weight is approximately 45K. The viscosity of aqueous solutions of Kollicoat® is much lower than that of equivalent solutions for instance, cellulose derivatives (www.basf.com). To the best of my knowledge, there is no literature showing use of Kollicoat® as a stabilizing agent for emulsions so far. In this work, Kollicoat® IR was used as a stabilizing agent instead of PVA in some of the formulations to prepare DT loaded PCL microparticles. Although use of high concentrations of a stabilizing agent is not advisable, a very high concentration of Kollicoat® was used to evaluate the physicochemical characteristics of the microparticles. As observed in the SEM (Figure 3.16), the particle size was not affected by using 2.5% Kollicoat® (F5), it was comparatively similar to the formulation made with 2.5% PVA (F1). As the concentration of Kollicoat® increased, the particle size increased. The formulation F8, where 20% Kollicoat® was used, the particle size was in the range of 7-10 μm. Increase in the Kollicoat® concentration has also appeared to contribute to the distortion of the particle shape as seen in the SEM.

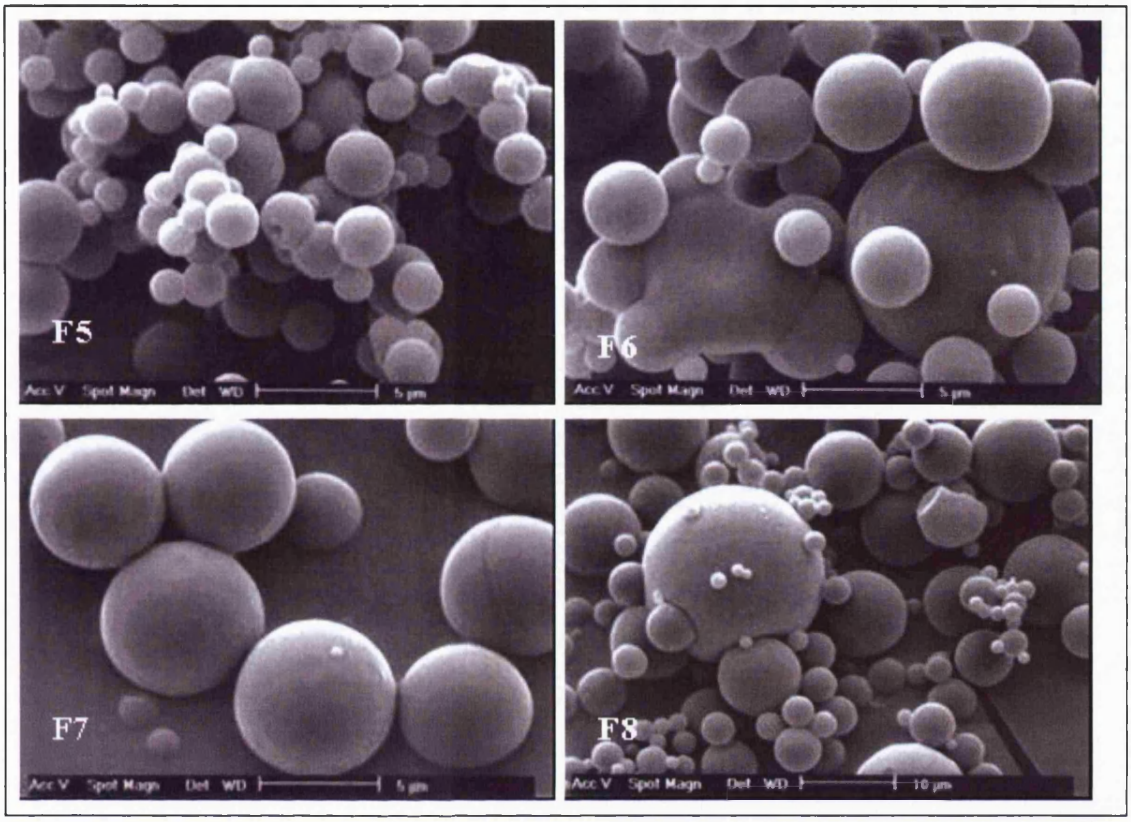

Figure 3.16: SEMs of DT encapsulating PCL plus microparticles by spray drying technique with varying concentration of TPGS in the organic phase (Kollicoat® as stabilizing agent).

3.3.2. *In vivo* studies in mice with the HBsAg loaded PLA nanoparticles

3.3.2.1. Immune response following subcutaneous administration of HBsAg loaded PLA nanoparticles with TPGS in organic phase or aqueous phase

In the present study when HBsAg encapsulated in PLA and TPGS blend nanoparticles were delivered subcutaneously in mice, the TPGS-containing formulations have enhanced the specific serum IgG reciprocal endpoint titres in comparison to free antigen or free antigen plus alum or antigen encapsulated in the PLA only nanoparticles (Figure 3.17). In addition to the magnitude of the observed systemic humoral immune response, the duration of the response was shown to be maintained for an excess of 400 days. On day 15 the HBsAg-specific IgG antibody titres obtained were significantly higher ($P < 0.05$) for the formulations containing TPGS compared to the free antigen and PLA only nanoparticles. The antibody responses for free antigen were higher than the PLA only

nanoparticles at day 15, however the PLA only nanoparticles gave better immune response than free antigen over the time course of the experiment. This was expected as the polymer used was high molecular weigh PLA (147 K), the release of the antigen will be in a gradual and controlled manner over longer time period.

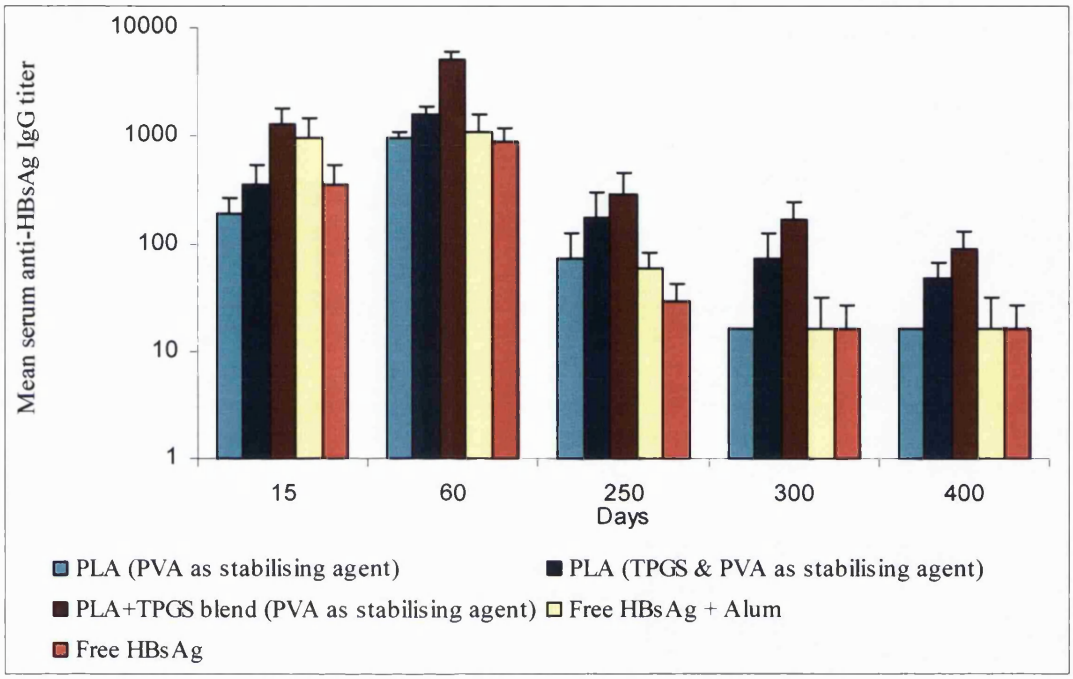

Figure 3.17: Mean serum antibody response to HBsAg loaded PLA nanoparticles. Mice were administered subcutaneously with PLA nanoparticles prepared by prepared by w/o/w solvent evaporation method with TPGS in organic phase or aqueous phase or free antigen or free antigen plus alum. The priming dose was 1 µg HBsAg containing formulations on day 1 followed by a booster dose of 0.5 µg of HBsAg formulations on day 21 ($n = 4$ ±s.d)

After 60 and 250 days, formulations containing TPGS (PLA plus TPGS blend nanoparticles and PLA particles prepared with TPGS as internal phase stabilizing agent) displayed enhanced antibody titres, approximately 5-6 times ($P < 0.024$) higher than PLA only nanoparticles. After 300 and 400 days, the free antigen, free antigen plus alum and the PLA only nanoparticles had similar antibody responses. Although it was expected that the antibody responses of the free antigen and free antigen plus alum would be reduced in the long term, the surprising aspect was reduced antibody responses of PLA only nanoparticles. This can be attributed to the physical characteristics of the PLA only particles. Owing to the low encapsulation efficiency of PLA only nanoparticles more particles were injected compared to the TPGS containing particles so as to correspond the required dose. As mentioned in the characterization section earlier the PLA only particles aggregated after freeze drying, therefore when the particles were injected as a suspension, the aggregated particles might have blocked in

the hypodermic syringe resulting in under dosing of the antigen. Whereas, the PLA plus TPGS blend particles and PLA with TPGS as the stabilising agent gave 6 and 10 fold higher antibody titres respectively ($P < 0.037$). This clearly emphasises the potential of TPGS as an adjuvant.

TPGS has been used in our laboratory as a mucosal adjuvant in the past (Alpar *et al.*, 2001). In these studies a solution of TPGS was co-administered with TT/DT solutions on day 1 (5 LF) and 49 (2.5 LF) intranasally in the concentrations of 0.5% m/v, 5% m/v, 10% m/v and 20% m/v. As compared with solutions of TT/DT in PBS, TPGS evoked many folds higher specific serum IgG reciprocal endpoint titres. The higher concentration of TPGS resulted in higher antigen specific immune responses, to co-delivered TT/DT. Although 0.5% m/v TPGS potentiated a lower immune response to antigen; it is still significantly increased in comparison to the free toxoid. The mechanism of action of TPGS as a mucosal adjuvant is not well understood. The antioxidant properties of TPGS could explain or be responsible for its adjuvant effect. The enhancement of immune responses may not only be due to the innate nature of TPGS which a known immunostimulant protects rapidly proliferating cells of the immune system from oxidation damage (Tengerdy and Lacetera, 1991).

For the analysis of HBsAg specific cytokines, splenocytes were stimulated with 5 μg/ml of HBsAg after 400 days of the experiment and naïve mice were used as controls to correct the background. Th1 cells secrete IFN-γ along with IL-2 and tumour necrosis factor-beta increases gene expression in APC and activate B cells to differentiate and secrete IgG2a, while Th2 produces IL-4, -5, -6, -10 and -13, IgG1 being the major isotype elicited from B cells following activation of Th2 cells. Therefore, Th1 cells are involved in the cell mediated immunity, while Th2 cells are related with humoral immunological response (Johansen *et al.*, 2000). However, due to logistical reasons, only IL-4, IL-6 and secretions were evaluated during these experiments, with IFN-γ giving the Th1 orientation and IL-4 and IL-6 giving the Th2 orientation.

Stimulation with HBsAg successfully elicited IL-4, IL-6 and IFN-γ secretion. Cytokine production was higher for the nanoparticle formulations when compared with the free antigen and free antigen plus alum (Figure 3.18).

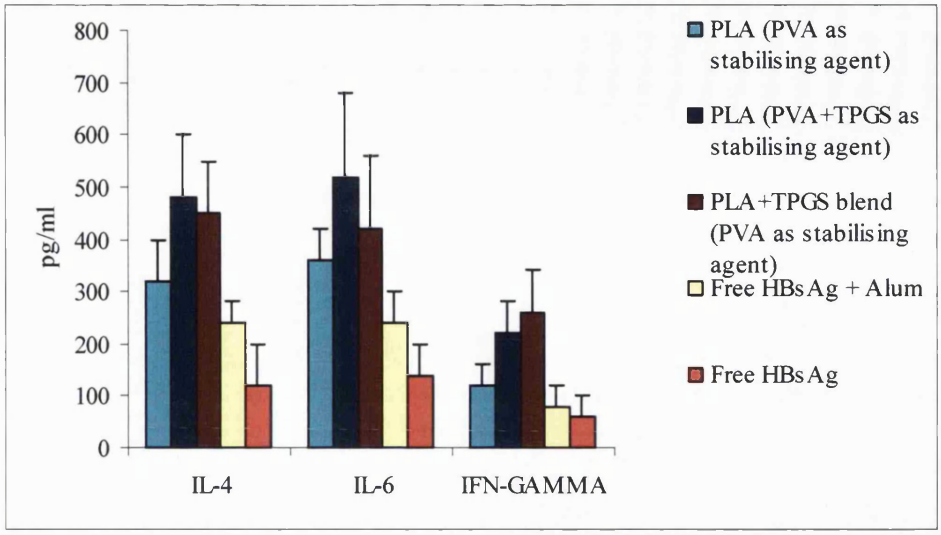

Figure 3.18: Interleukin 4 (IL-4), Interleukin 6 (IL-6) and Interferon gamma (IFN-γ) production by pooled spleens cells after culturing with 5 μg/ml concentrations of soluble HBsAg after 400 days. Mice were administered subcutaneously with PLA nanoparticles prepared by prepared by w/o/w solvent evaporation method with TPGS in organic phase or aqueous phase or free antigen or free antigen plus alum. The priming dose was 1 μg HBsAg on day 1 followed by a booster dose of 0.5 μg of HBsAg on day 21 ($n = 4$, mean ±s.d)

The nanoparticle formulation primed spleen cells induced the highest IL-4 and IL-6 production, significantly higher than the free HBsAg and alum plus free HBsAg primed spleen cells. The incorporation of TPGS either as polymer blend or internal phase stabilizer significantly increased IL-4 and IL-6 production by spleen cells ($P < 0.05$). The PLA particles prepared with TPGS as an internal phase stabilizer had higher IL-4 and IL-6 levels when compared with the PLA plus TPGS blended nanoparticles. The IFN-γ production pattern was different than the IL-4 and IL-6 production. The presence of TPGS in the formulations has shown a significant increase in the IFN-γ production by spleen cells when compared with the free antigen ($P < 0.018$), free antigen plus alum ($P < 0.038$) and PLA nanoparticles ($P < 0.05$) prepared with PVA as stabilizer in both internal and external phases.

3.3.2.2. Immune response following intramuscular and intranasal administration of HBsAg loaded PLA nanoparticles TA or TN in the organic phase

To the best of my knowledge, there is no literature stating the use of TA and TN as adjuvants. Based on the results obtained with TPGS, these studies were carried out to investigate the potential of TA and TN as adjuvants as the main reason for the adjuvancy of TPGS is to believed to be the presence of tocopherol. Although the immune response obtained with these molecules was lower when compared with TPGS, nevertheless PLA-TA and PLA-TN when administered intramuscularly or intranasally have been shown to act as better adjuvants to some extent when compared with PLA only nanoparticles and to a large extent when compared with the free antigen (Figure 3.19 and 3.20).

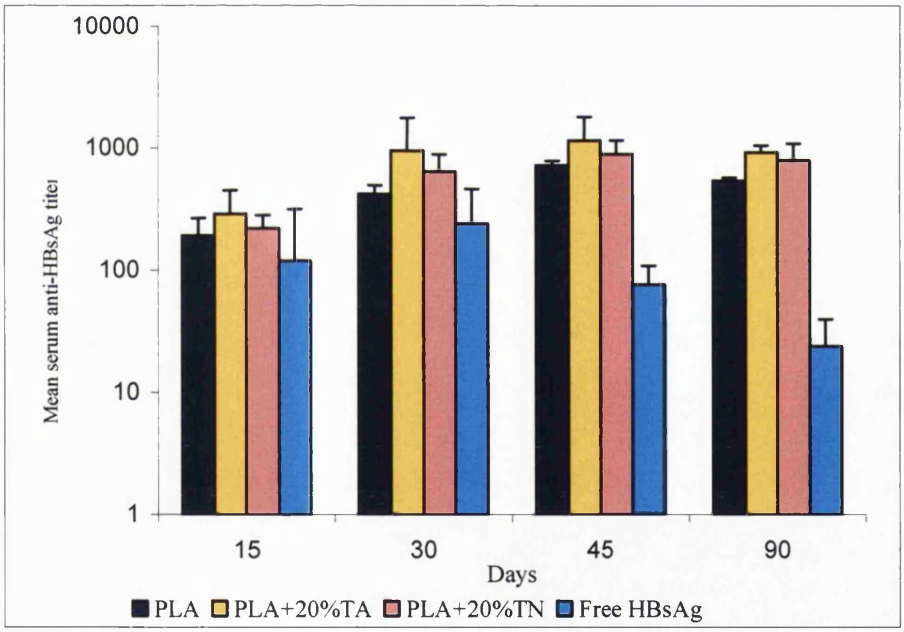

Figure 3.19: Mean serum antibody response to intramuscularly administered HBsAg loaded PLA nanoparticles with or without TA or TN. Mice were administered intramuscularly with PLA nanoparticles prepared by w/o/w solvent evaporation method with or without TA or TN in organic phase or free antigen. The priming dose was 1 µg HBsAg on day 1 followed by a booster dose of 0.5 µg of HBsAg on day 21 (n = 4, mean ±s.d)

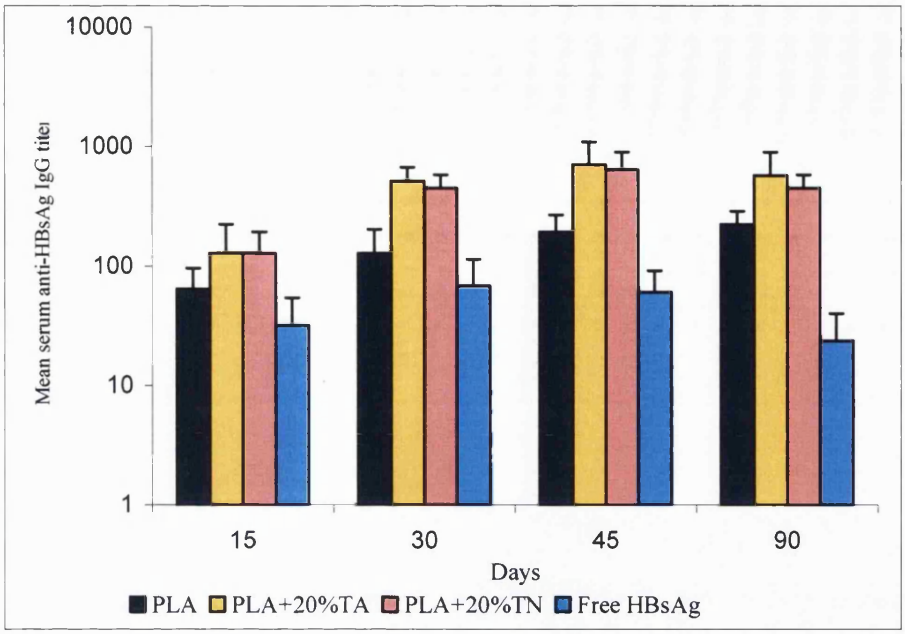

Figure 3.20: Mean serum antibody response to intranasally administered HBsAg loaded PLA nanoparticles with or without TA or TN. Mice were administered intranasally with PLA nanoparticles prepared by w/o/w solvent evaporation method with or without TA or TN in organic phase or free antigen. The priming dose was 1 μg HBsAg on day 1 followed by a booster dose of 0.5 μg of HBsAg on day 21 ($n = 4$, mean ±s.d)

After intramuscular delivery of HBsAg encapsulated PLA, PLA-TA and PLA-TN, the primary serum IgG response after 15 days was not significantly different when compared with the free antigen ($P < 0.18$). The serum IgG antibody responses gradually increased for the formulations post boosting dose and at the end of the study (90 days). PLA-TA and PLA-TN containing nanoparticles induced higher serum IgG immune responses than PLA only nanoparticles. The highest serum IgG antibody responses were observed with PLA-TN nanoparticles. This can be due to the fact that TA has better bioavailability compared to TN which might have influenced the uptake of the particles (Hidiroglou and Charmley, 1990; 1991). At day 90, PLA-TA nanoparticles particles gave ~38 times higher serum IgG antibody responses when compared with the free antigen ($P < 0.005$). The PLA-TN nanoparticles particles gave ~22 times higher serum IgG antibody responses when compared with the free antigen after 90 days ($P < 0.008$). However, the serum antibody responses were not significantly different between PLA-TA and PLA-TN ($P < 0.12$).

For the nasally immunised mice the same trend was observed but the intensity of the antibodies produced was much lower when compared with the intramuscularly

immunised mice. At day 90, PLA-TA nanoparticles particles gave ~23 times higher serum IgG antibody responses when compared with the free antigen ($P < 0.037$). The PLA-TN nanoparticles particles gave ~18 times higher serum IgG antibody responses when compared with the free antigen after 90 days. Although the serum antibody titres of PLA-TA and PLA-TN particles were significantly higher than the free antigen ($P < 0.037$ and $P < 0.05$ respectively), this difference was not as pronounced when compared to the PLA only particles ($P < 0.17$ and $P < 0.23$).

3.3.3. *In vivo* studies in mice with the DT loaded PLA nanoparticles

3.3.3.1. Immune response following intramuscular or intranasal administration of DT loaded PLA microparticles with TPGS in organic phase or aqueous phase

The intramuscularly immunized groups gave significantly higher immune responses for the TPGS containing formulations (Figure 3.21). After 30 days, the PLA-TPGS formulations gave ~7 and ~10 times higher antibody responses than the plain PLA particles and free antigen respectively. At the end of the study, the TPGS containing formulations gave ~4 times higher response than the PLA only particles; however this was not statistically significant ($P < 0.06$). Although the TPGS containing formulations gave ~20 times higher serum antibody levels than the free antigen and this was statistically significant ($P < 0.03$). When the intramuscular immune responses of the TPGS containing PLA particles were compared with the intranasally administered TPGS containing particles, the intramuscular responses are significantly higher ($P < 0.017$).

After 21 days of intranasal delivery of the formulations and free antigen the antibody responses among the groups were not significant to each other, however, comparatively the microparticles gave a better response than the free antigen (Figure 3.22). The immune responses were significantly higher after the booster dose, 30 days after immunization the TPGS containing formulations gave ~10 fold higher response than the plain PLA particles and ~20 times higher than the free antigen. This trend continued at 45 and 90 days as well. After 90 days, the antibody responses were higher for the PLA-TPGS formulations were not statistically significant than the plain PLA particles ($P < 0.06$). A significant higher response of almost ~20 times higher responses were observed for the TPGS containing PLA particles when compared with the free antigen.

This clearly shows that the TPGS containing PLA nanoparticles can elicit better immune responses when compared with the free antigen. The potential of the nasal route as a mucosal site for drug and vaccine delivery has now been firmly established (Eyles *et al.*, 1999; Jabbal-Gill *et al.*, 2001; Klinguer *et al.*, 2001; Olszewska and Steward, 2001; Scheerlinck *et al.*, 2006; Amidi *et al.*, 2007; Illum, 2007). The nasal mucosa possesses many advantages for drug and vaccine delivery, including a highly vascularised epithelium of considerable surface area. Conditions in the nasal cavity may be less harsh than those present in the gastrointestinal tract. This is due to the lower enzymatic activity and the reduced exposure to low pH. The existence of nasal-associated lymphoid tissue, which has a role that is analogous to that of the GALT, is thought to be of particular importance for the uptake of particulate carriers for the purposes of immunization. Several studies have demonstrated the potential of PLGA microparticles as vaccine carriers for intranasal delivery. The intranasal immunization of microencapsulated *Bordetella pertussis* antigens has been shown to give protective immunity in mice (Shahin *et al.*, 1995). Intranasal immunization with microparticles has also induced protection in mice against aerosol challenge with ricin toxin (Yan *et al.*, 1996). In our laboratories, the intranasal delivery of TT in microparticles has been shown to induce markedly higher systemic and local immune responses than the free toxoid (Almeida *et al.*, 1993; Eyles *et al.*, 1999).

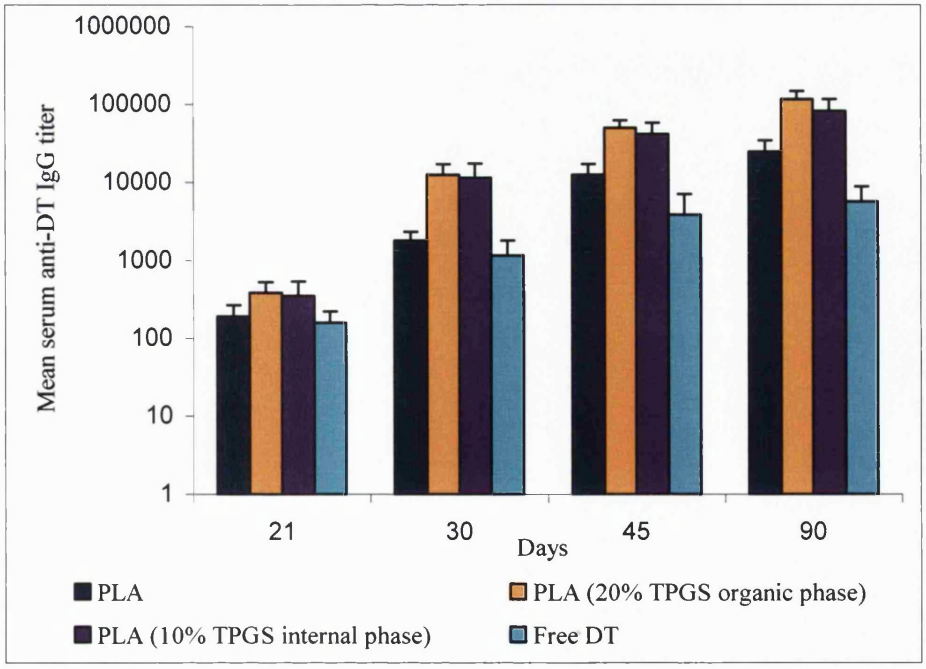

Figure 3.21: Mean serum antibody response to DT loaded PLA microparticles. Mice were administered intramuscularly with PLA microparticles prepared by w/o/w solvent evaporation method with TPGS in organic phase or aqueous phase or free antigen. The priming dose was 10 µg DT on day 1 followed by a booster dose of 5 µg of DT on day 21 ($n = 4$, mean ±s.d)

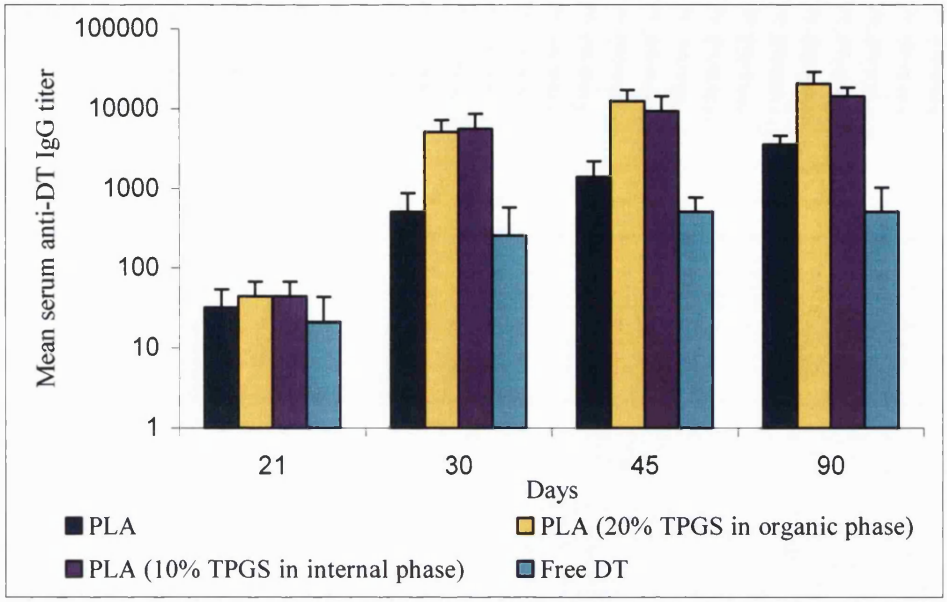

Figure 3.22: Mean serum antibody response to DT loaded PLA microparticles. Mice were administered intranasally with PLA microparticles prepared by w/o/w solvent evaporation method with TPGS in organic phase or aqueous phase or free antigen. The priming dose was 10 μg DT on day 1 followed by a booster dose of 5 μg of DT on day 21 (n = 4, mean ±s.d)

Secretory immunoglobulin A (sIgA) is an important component of mucosal immunity which induces systemic antibody and cell mediated immunity. After the intranasal vaccination the systemic and local immune responses are induced depending upon the nature of antigen, adjuvant, dose volume and frequency of dose. If a large volume of dose is administered in the nose, it can be swallowed leading to oral or lung delivery. It has been previously shown 40% of the administered dose of labelled microparticles reached the lung when 50 μl volume of dose was administered. Whereas most of the particles were retained in the nose when 10 μl volume of dose was administered (Eyles *et al.*, 1998). Moreover, soluble antigens need a carrier which can act as an adjuvant when nasally administered so that they can cross the nasal epithelium, interact with dendritic cells, macrophages and lymphocytes, and then be transported to posterior lymph nodes. This will result in elicitation of local and distant mucosal immune responses due to maturation of the precursors of IgA mucosal cells in regional lymph nodes or, after being transported, in the lamina propria of distant mucosal sites of the CMIS (Davis, 2001).

In the present study, a 50 μl volume of the formulations were administered in mice. Therefore it was anticipated that the administered dose might have reached the lung and gut cavities resulting in the induction of mucosal IgG and IgA antibodies. At the end of the study the mice were sacrificed and the lung and gut washes were analysed for

mucosal IgG and IgA antibody levels (Figures 3.23, 3.24, 3.25). The lung washes from mice dosed with DT-loaded PLA-TPGS blend microparticles showed significantly higher IgA titres ($P < 0.05$) compared to lung washes of control mice (which received free DT), in which IgA responses were almost undetectable. The gut washes of mice dosed with DT loaded PLA microparticles containing TPGS exhibited high IgA and IgG titres compared to lung washes of control mice, where antigen specific IgA responses were low. However, the gut IgA and IgG responses were not significant ($P < 0.13$ and $P < 0.08$ respectively).

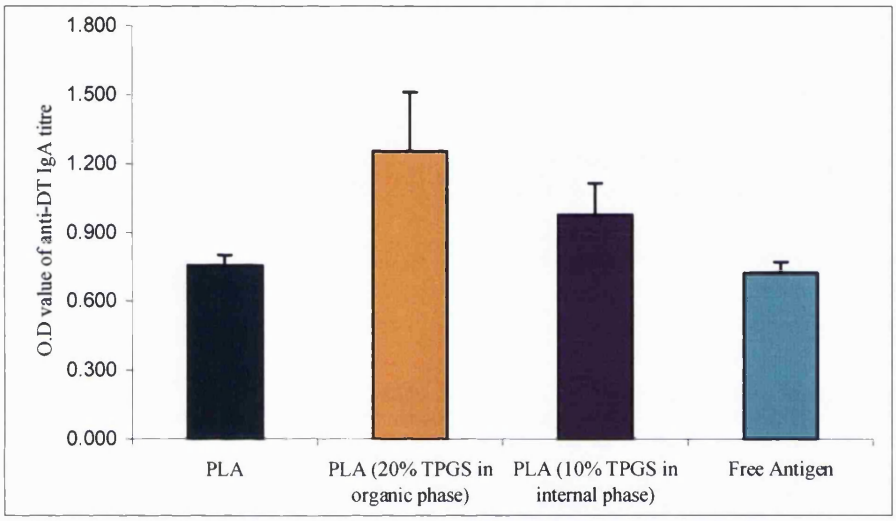

Figure 3.23: Mucosal immune responses in lung washes (IgA) to DT loaded PLA microparticles. Mice were administered intranasally with PLA microparticles prepared by w/o/w solvent evaporation method with TPGS in organic phase or aqueous phase or free antigen. The priming dose was 10 μg DT on day 1 followed by a booster dose of 5 μg of DT on day 21. Mice were sacrificed on day 90 ($n = 4$, mean ±s.d)

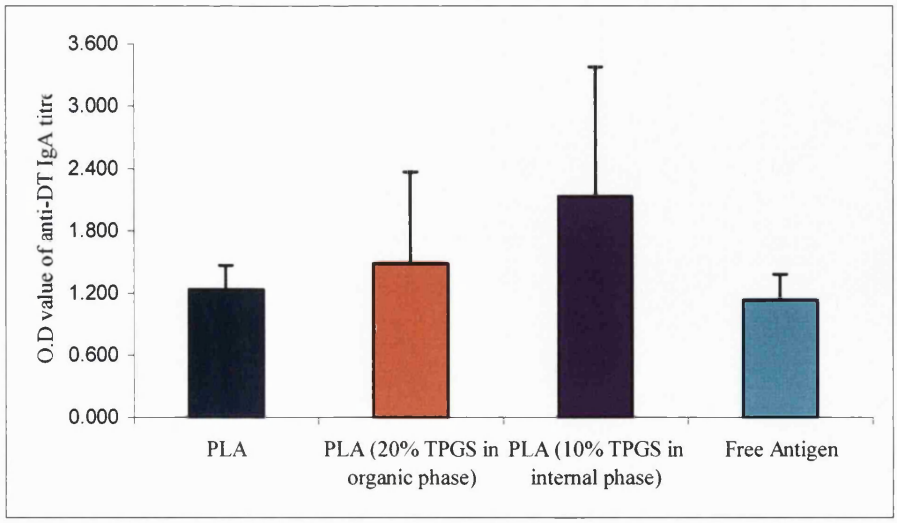

Figure 3.24: Mucosal immune responses in gut washes (IgA) to DT loaded PLA microparticles. Mice were administered intranasally with PLA microparticles prepared by w/o/w solvent evaporation method with TPGS in organic phase or aqueous phase or free antigen. The priming dose was 10 μg DT on day 1 followed by a booster dose of 5 μg of DT on day 21. Mice were sacrificed on day 90 ($n = 4$, mean ±s.d)

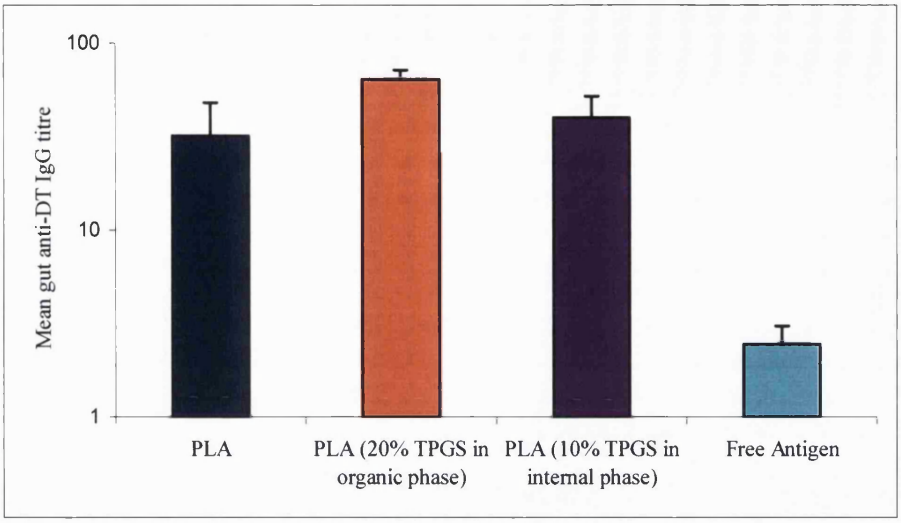

Figure 3.25: Mucosal immune responses in gut washes (IgG) to DT loaded PLA microparticles. Mice were administered intranasally with PLA microparticles prepared by w/o/w solvent evaporation method with TPGS in organic phase or aqueous phase or free antigen. The priming dose was 10 μg DT on day 1 followed by a booster dose of 5 μg of DT on day 21. Mice were sacrificed on day 90 (n = 4, mean ±s.d)

For the analysis of DT specific cytokines, splenocytes were stimulated with 5 μg/ml of DT, which successfully elicited IL-6 and IFN-γ secretion (Figure 3.26). The IL-6 responses for the nasally delivered particles were higher when compared with the intramuscularly delivered particles. The IL-6 responses were particularly higher for the PLA-TPGS blend particles when compared with the other formulations and the free antigen. There were no significant differences with the IFN-γ responses for both intramuscular and intranasal formulations among all the groups ($P < 0.08$). The PLA only microparticle formulation primed spleen cells induced the highest elevated IL-6 productions significantly higher than the free DT primed spleen cells ($P < 0.05$). However, incorporation of TPGS either as a polymer blend or internal phase stabilizer significantly increased IL-6 production by spleen cells when compared with the group of animals dosed with the free antigen ($P < 0.021$ and $P < 0.04$). When TPGS was used as internal phase stabilizer, the formulations have shown a significant increase in the IFN-γ production by spleen cells when compared with the free antigen ($P < 0.05$). However this was not significant ($P < 0.08$) when compared with the PLA particles prepared with PVA as stabilizer in both internal and external phases. The TPGS blend particles showed significantly higher IFN-γ production when compared with both free antigen as well as the PLA only particles ($P < 0.032$ and $P < 0.05$ respectively).

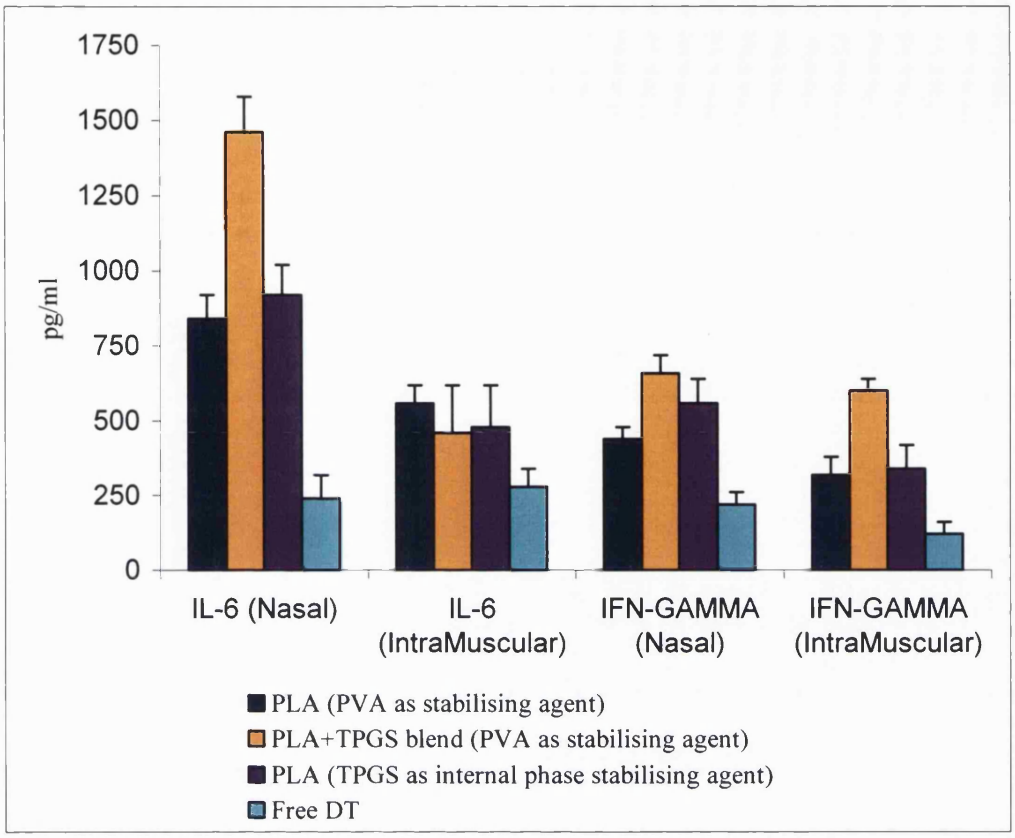

Figure 3.26: Interleukin 6 (IL-6) and Interferon gamma (IFN-γ) production by pooled spleens cells after culturing with 10 µg/ml concentrations of soluble DT after 90 days. Mice were administered either intranasally or intramuscularly with PLA microparticles prepared by w/o/w solvent evaporation method with TPGS in organic phase or aqueous phase or free antigen. The priming dose was 10 µg DT on day 1 followed by a booster dose of 5 µg of DT on day 21 (n = 4, mean ±s.d)

3.3.3.2. Immune response following intranasal administration of DT loaded PCL microparticles prepared by spray drying method with varying concentration of TPGS in the organic phase

The primary serum IgG response after 15 days was not significant ($P < 0.12$) for all the formulations when compared with the free antigen (Figure 3.27). However, after the boosting dose on day 21, the antibody responses increased significantly for all the formulations when compared with the free antigen. At day 45, PCL-10% TPGS and PCL-20% TPGS microparticles gave ~ 28 and ~40 times higher antibody responses than free antigen ($P < 0.05$ and $P < 0.031$ respectively). The PCL-10% TPGS and PCL-20% microparticles gave a 5 and 13 fold higher serum specific antibody responses compared to PCL only microparticles, however this was not statistically significant ($P < 0.21$). Saying this, these results are after 45 days of dosing and there may well be a better response after a longer period of time.

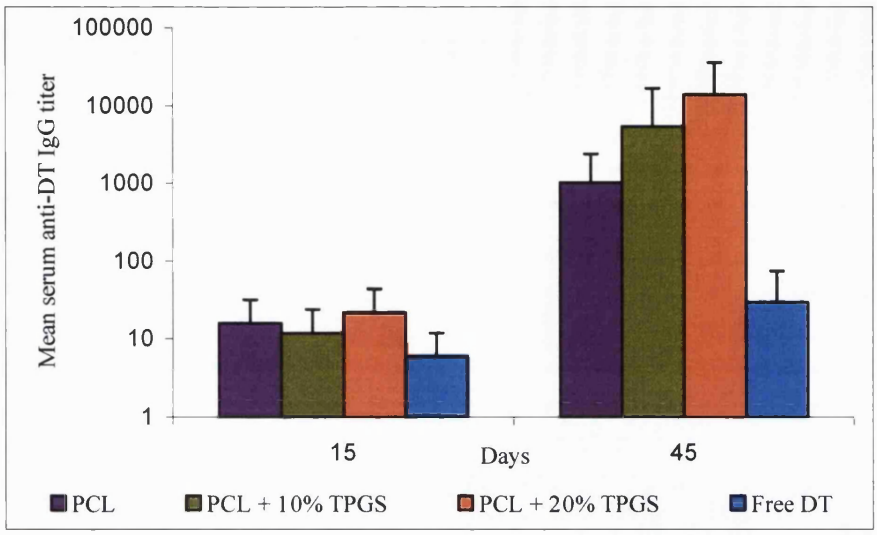

Figure 3.27: Mean serum antibody response to DT loaded PCL microparticles. Mice were administered intranasally with PCL microparticles prepared by spray drying method with varying concentration of TPGS in the organic phase or free antigen. The priming dose was 10 μg DT on day 1 followed by a booster dose of 5 μg of DT on day 21 (n = 4, mean ±s.d)

Alpar *et al* (2001) investigated the potential utility of penetration enhancers (dimethyl β-cyclodextrin, deoxycholic acid, poly-ornithine and TPGS) as adjuvants for nasally applied DT in BALB/c mice. Co-administration of the enhancer solutions markedly improved the humoral responses evoking specific serum IgG. They also produced positively charged PLA microspheres containing DT using a modified double emulsion solvent process in which chitosan was present in both the internal and external phases. They tested the hypothesis that positively charged PLA microspheres might be better mucosal adjuvants than the conventional PLA microspheres, due to the mucoadhesive and biocompatible properties of chitosan. Mice were nasally immunized with DT encapsulated in positively charged PLA microspheres, another group with DT encapsulated in "conventional" PLA microspheres and the last received antigen as a solution (free). Animals immunized with cationic microspheres had significantly elevated secondary serum anti-DT IgG titres relative to the ones treated with the other DT formulations. TPGS being an amphiphilic molecule can function as a safe mucosal adjuvant. An important consideration for its use as a mucosal adjuvant is the safety of TPGS, in comparison with alternative absorption enhancers. Such as, TPGS has been used as a vitamin E supplement for treatment of vitamin E deficiency in children. The acute oral LD50 of vitamin E TPGS is over 7,000 mg/kg for adult rats. In clinical studies, TPGS is used at 2256 mg twice daily. Thus the concentrations in our studies were low and should not cause any tissue damage. The mechanism of action of this

molecule is not known, therefore it needs to be explored more thoroughly because compared to known mucosal adjuvants TPGS has been shown to be extremely safe molecule.

There have been a number of studies showing the potential of the polymeric carriers for the delivery of the antigens. Singh *et al* (1991) microencapsulated DT using PLA (49 K), by the in-water drying technique. They obtained microcapsules with a mean size in the range of 30-100 μm. *In vitro*, they studied the erosion kinetics of the polymer, leading to a controlled release of the antigen (initially pulse then continuous release) and 88% of encapsulated DT was detected *in vitro* release media. Antibody titres in immunized BALB/c mice on day 75 were equal to those in the group receiving conventional three-dose injection of DT with calcium phosphate as an adjuvant (DT-$CaPO_4$ dosed on days 0, 30 and 60). Schröder and Svenson (1999) showed that mono-olein/oleic acid (monoglyceride/fatty acid suspension system) vesicles (~200nm) enhance the immunogenicity of admixed DT in mice to the same level as Alum adsorbed (or Freund's complete adjuvant) when administered parentally or nasally. This lipid matrix acted as a penetration enhancing molecule and as an immunological adjuvant due to its amphiphilic character. Furthermore, organic solvents are not needed in the preparation procedure and low cost endogenous lipid based raw materials can be employed. Johansen *et al* (1999) examined the immunogenicity of DT encapsulated in different types of PLA and PLGA microparticles prepared by the methods of spray-drying and coacervation. They used PLGA 50:50 of low Mw (14 K), PLA of high Mw (130 K) and non commercial 10 and 18 K PLA with stearyl end-groups. They utilized BSA and D (+)-trehalose dihydrate as additives. The co-encapsulated trehalose lowered the DT entrapment efficiency in the spray-dried particles from 75% to 56%, whereas albumin alone had no effect in the spray-drying, but improved the encapsulation in the coacervation process. DT microspheres made with the relatively hydrophilic PLGA 50:50 and administered to guinea pigs subcutaneously exhibited specific and sustained antibody responses over 40 weeks, comparable to the responses to alum-adjuvanted toxoid. In contrast, undetectable or very low antibody responses were determined after immunization with microspheres made with hydrophobic polymers. Peyre *et al* (2004) reported the uptake and biodistribution of fluorescent labelled PLGA microspheres loaded with DT and administered subcutaneously to BALB/c mice. They were prepared by spray-drying a mixture of antigen, fluorescein and rhodamine and their size range was between 0.5 and 10 μm diameter. Fluorescent particles were detected inside cells of

the peritoneal flush as early as 10 minutes post-inoculation, predominantly in cells of macrophage morphology. In the first week after injection, microspheres were uptaken and digested by dendritic cells, thereby triggering the immune response against DT. Fluorescent PLGA microspheres were also observed in cells of lymphoid tissues such as the mesenteric lymph-nodes and spleen but in these sites they decreased rapidly as they were degraded inside the cells. As a result, they enable the presentation of the antigen to specific cells of the immune system. After two weeks from the first injection, anti-DT specific antibodies were detected in the blood serum.

3.4. Conclusions

In this study OVA or DT or HBsAg loaded PLA or PCL micro/nanoparticles were prepared by the w/o/w double emulsification solvent evaporation or spray drying method. The effect of TPGS, TA and TN on the physicochemical characteristics of these particles was investigated. The particles were characterized with respect to size, surface charge and encapsulation efficiency. The antigen loaded particles were tested *in vivo* for their immunogenicity.

The particles prepared with w/o/w double emulsification solvent evaporation, the concentration of TPGS in the organic phase did not affect the particle size and the encapsulation efficiency of OVA loaded PLA nanoparticles. However, the presence of TPGS in the organic phase or when used as an internal phase stabilizing agent markedly improved the encapsulation efficiency of HBsAg in the PLA nanoparticles. Pluronic F-127 also improved the encapsulation efficiency of HBsAg to some extent. TA and TN also improved the encapsulation efficiency of HBsAg in the PLA nanoparticles. TPGS when used as internal phase stabilizing agent increased the loading efficiency of DT in the PLA microparticles. When DT loaded PCL microparticles were prepared with varying concentration of TPGS in the organic phase by the spray drying method, there was no significant differences between the particle size and encapsulation efficiency.

The integrity of the encapsulated antigen in the polymeric particles was found to be inconclusive owing to the improper extraction technique (digestions with NaOH and PBS). As a future study, the integrity of the protein can be evaluated using size exclusion chromatography or mass spectroscopy.

The MTT assay confirmed that the particles containing TPGS or TA or TN are not cytotoxic. There was no significant difference in the toxicity of different formulations at any of the concentrations used. The average cell viabilities were between 90 to 125% of control (PEI) at the concentrations studied.

HBsAg-loaded PLA nanoparticles containing TPGS were administered subcutaneously in mice to evaluate the serum antibody responses. All the particles irrespective of the presence of TPGS induced an HBsAg specific serum IgG antibody response higher than free HBsAg and the duration of the response was shown to be maintained for in excess of 400 days. After 60 and 250 days the PLA only particles gave similar antibody responses when compared with alum plus free antigen. However during this period, the particles containing TPGS gave better serum specific antibody responses when compared with alum plus free antigen. The immune responses were higher for the particles containing TPGS in the organic phase when compared with particles made using TPGS in the internal phase stabilizing agent. The nanoparticles eliciting the highest IgG antibody responses elicited the highest levels of cytokine IL-6 and IFN-γ. The presence of TPGS in the formulations has shown a significant increase in the IFN-γ production by spleen cells when compared with the free antigen, free antigen plus alum and PLA nanoparticles prepared with PVA as stabilizer in both internal and external phases.

The potential of TA and TN as adjuvants was investigated by administering HBsAg loaded PLA nanoparticles containing TA and TN in mice intramuscularly and intranasally. Although the immune response obtained with these molecules was lower when compared with TPGS, nevertheless PLA-TA and PLA-TN have been shown to act as better adjuvants when compared with PLA only nanoparticles and the free antigen. After the intramuscular administration, PLA plus TA and PLA plus TN gave ~ 22 and ~38 times higher serum IgG antibody responses when compared with the free antigen after 90 days. Intranasally, PLA plus TA and PLA plus TN gave ~23 and ~18 times higher serum IgG antibody responses when compared with the free antigen after 90 days.

DT encapsulated PLA microparticles containing TPGS were also investigated for their immunogenicity. Both intramuscular and intranasal administered mice showed

significantly higher immune responses for formulations containing TPGS compared to the free antigen. The gut washes of mice dosed with DT loaded PLA microparticles containing TPGS exhibited high IgA and IgG titres compared to lung washes of control mice, where antigen specific IgA responses were very low. The cytokine analysis showed that the IL-6 responses were particularly higher for the PLA-TPGS blend particles when compared with the other formulations and the free antigen. There were not much significant differences with the IFN-γ responses for both intramuscular and intranasal formulations.

DT-loaded PCL microparticles prepared by spray drying method with varying concentrations of TPGS were administered intranasally in mice. After 45 days PCL-10% TPGS and PCL-20% microparticles gave a 5 and 13 fold higher serum specific antibody responses compared to PCL only microparticles.

This study has shown the potential of TPGS, TA and TN as co-adjuvants together with PLA or PCL micro/nanoparticles to induce serum IgG antibody responses higher than PLA or PCL only micro/nanoparticles and free antigen.

3.5. Key observations

- Formulations containing TPGS were found to be the best among all the tested formulations with respect to improving the adjuvancy of the antigens.

- The unknown adjuvants, TA or TN were also better than plain micro/nanoparticles as well as free antigen.

- The MTT assay confirmed that the particles containing TPGS or TA or TN are not cytotoxic therefore these formulations can be assumed to be safe.

- The mechanism of adjuvant action of TPGS, TA and TN needs to be further explored.

Chapter 4

Co-encapsulation of mineral compounds with HBsAg

4. Co-encapsulation of mineral compounds with HBsAg in micro/nanoparticles

4.1. Co-encapsulation of mineral compounds

In the earlier chapter (chapter 3), previously untried adjuvants were co-encapsulated along with the antigens in the polymeric nano/microparticles. The results have shown that co-encapsulating the adjuvants with the antigen can improve the adjuvancy of these carriers. It will be interesting to investigate if the adjuvancy of known adjuvants can also be improved if co-encapsulated. The focus of this study is to co-encapsulate the mineral compounds (aluminium hydroxide, zinc sulfate and zinc oxide) in micro/nanoparticles to improve the adjuvancy of HBsAg. Alum is the most widely used adjuvant in the marketed vaccines. However, very little has been reported on the effect of co-encapsulation of alum and antigen on the physicochemical properties of the micro/nanoparticles and on immune responses. Zinc is also a mineral compound which is known to have beneficial effects on the immune system. Therefore, this chapter explores the effects of co-encapsulating these mineral compounds in the polymeric carriers. Moreover, modification of the surface charge may profoundly affect the efficiency with which the microparticles target the delivery of bioactive agents to mucosally associated lymphoid tissues and to antigen presenting cells. Therefore chitosan has been used to modify the surface charge of the microparticles and its effect on the immune response. Three types of polymers, PCL (80 K), PLA (80 K) and PLA (147 K) were used in this study. These high molecular weight polymers were chosen as they have a long degradation time and therefore would help to ascertain the long term immune responses of these carriers.

4.1.1. Alum

Aluminium compounds include aluminium hydroxyphosphate commonly called aluminium phosphate, aluminium oxyhydroxide or hydroxide and alum-precipitated vaccines. The term alum is often used to refer to all these substances. However, the general term alum is not correct if used for all aluminium adjuvants, because aluminium hydroxide and aluminium phosphate have different physical characteristics and adjuvants properties. For example, as Gupta *et al.* (1995) had reported, aluminium hydroxide (Alhydrogel®) was found to be a more potent adjuvant in comparison with

aluminium phosphate. Aluminium compounds cannot be frozen or lyophilized because the gel would collapse causing aggregation and precipitation and during the lyophilisation process, stabilizers and cryopreservatives are often used because they desorb antigens from aluminium adjuvants on reconstitution of lyophilized vaccines. Alum is known to work chiefly though a depot effect. Small antigens, injected into the bloodstream, can quickly become degraded in the liver or filtered by the kidneys. Alum, by precipitating soluble antigens, forces them to linger longer at the site of injection, where antigen presenting cells (APCs), mainly macrophages and dendritic cells, can ingest and process them, thus ensuring a greater immunological response. Although considered safe Alum can occasionally produce subcutaneous nodules, granuloma and abscesses (Saraf *et al*, 2006).

Aluminium hydroxide has been widely used as adjuvant and is in use currently an adjuvant in many human vaccine formulations (Iyer *et al.* 2004; Moschos *et al.* 2006). The mechanism of enhancement of immune responses by alum is due to the triggering of humoral immunity by selective stimulation of type 2 T-helper (Th2) responses (Cox and Coulter 1997). The effect of incorporation of alum in TT encapsulated PLA (45 K) microspheres were studied by Raghuvanshi *et al* (2002). Results of this study indicated that alum-adsorbed TT microspheres elicited significantly higher anti-TT antibody titres, as compared to plain TT microspheres. In case of the HBsAg, there are substantial published data that demonstrate that there is considerable potential for the development of single dose HBsAg vaccines using controlled release biodegradable polymeric microspheres, and there is extensive information about the release kinetics of the encapsulation (Singh *et al.* 1997; Nellore *et al.* 1992; Shi *et al.* 2002). The incorporation of adjuvants to enhance immune responses has been investigated, and a number of immunostimulatory adjuvants evaluated (O'Hagan *et al.* 1993; Diwan *et al.* 2001; Raghuvanshi *et al.* 2002; Bramwell *et al.* 2003; Moschos *et al.* 2006). Therefore, incorporation of adjuvants with the polymeric carriers is considered to be beneficial.

4.1.2. Zinc

The supplementation of zinc prior to immunization has been shown to stimulate the antigen specific responses in a number of studies. Moreover, zinc plays an important role in the immune functions in humans. The deficiency of zinc is associated with

impaired immunity and increased susceptibility to infectious diseases (Golden *et al.*, 1978; Duchateau *et al.*, 1981; Shankar and Prasad, 1998).

Zinc is a nutritionally valuable element which plays an important role in the immune system and zinc-deficient subjects are susceptible to a variety of pathogens. Moreover, zinc deficiency causes an imbalance between Th1 and Th2 functions which adversely affect the cell mediated immunity (CMI) and prevents regeneration of new CD4+ T-lymphocytes. There is also evidence suggesting the use of zinc in HIV-positive patients. Such patients require higher dietary levels of the element owing to malabsorption and repeated infection resulting from the syndrome. By avoiding deficient states of the element it is believed that normal or improved immune function can be maintained in such patients and may inhibit apoptosis of T-lymphocytes in individuals who are HIV positive (Black and Miguel., 2002). Ozgenc *et al.*, (2006) carried out a study, wherein they investigated the effect of zinc deficiency on the antibody responses of HBsAg on rats. Wistar-Albino rats in two groups were fed with constant diet with a zinc supplementation of 10 mg/kg in group one and 30 mg/kg in group two. Hepatitis B vaccine (Engerix B, 4 μg) was administered intramuscularly after 8 weeks on feeding and a booster dose was applied 4 weeks after the first injection. It was found that zinc-deficient rats can have ~8 fold lower anti-HBsAg antibody titres and the study concluded that marginal zinc deficiency might influence the efficacy of hepatitis B vaccination in humans. In a randomized clinical trail study, administration of zinc over a month has shown increased number of circulating T lymphocytes, increased delayed type hypersensitivity (DTH) response and increased antibody response to tetanus vaccine (Duchateau *et al.*, 1981). Zinc has been shown to induce the production of IL-1β, tumour necrosis factor (TNF)-alpha and the mRNA corresponding to human peripheral blood mononuclear cells (PBMC) (Wellinghausen *et al.*, 1997). These observations have led to the hypothesis that zinc supplementation can improve the antigen-specific responses; therefore in the present study zinc was co-encapsulated with HBsAg in the polymeric particles to increase the adjuvanticity of the antigen.

4.1.3. Aims and objectives

The development of a delivery system for the hepatitis B vaccine with an alternative adjuvant which could induce the desired antibody response from a reduced number of injections would be of enormous benefit. In this regard this present study was

undertaken to combine the known beneficial properties of nanoparticles with alum and zinc. The formulations were prepared to investigate the following parameters:

- Effect of molecular weight of the polymer on OVA loaded PCL nanoparticles
- Effect of zinc sulfate on the physicochemical characteristics of OVA loaded PCL and PLA nanoparticles
- Effect of zinc oxide on the physicochemical characteristics of OVA loaded PCL and PLA nanoparticles
- Formulation of FITC-BSA loaded nanoparticles to evaluate the cellular uptake of the particles
- Effect of alum or zinc sulfate or zinc oxide on the physicochemical characteristics of HBsAg loaded PCL and PLA nanoparticles
- Effect of chitosan on the physicochemical properties of the HBsAg loaded PLA microparticles

These formulations were characterized with respect to size, yield, surface charge, loading efficiency and uptake by cell lines. The best formulations were chosen for *in vivo* animal studies (in the mouse model) and the antibody responses were measured together with the antigen specific cytokine responses. The important aspect of the *in vivo* studies was to recognize the best formulation which can improve the adjuvancy of the antigens for which the following parameters were studied:

- Effect of PCL *versus* PLA nanoparticles on immune responses in mice
- Effect of alum *versus* zinc sulfate on HBsAg immunogenicity
- Comparison of intranasal *versus* intramuscular administration route
- Long term immune responses of subcutaneously administered PLA microparticles – comparison of chitosan *versus* alum

4.2. Materials and Methods

4.2.1. Materials

Hepatitis B surface antigen (HBsAg) at a concentration of 1.68 mg/ml was provided as a gift by Shantha Biotechnics, India. Poly (L-lactic acid) (LACTEL®, 80 and 147 K) was obtained from Birmingham polymers Inc, USA. Albumin Chicken Egg (OVA) Grade V, polyvinyl alcohol (13,000-23,000 and 87-89% hydrolyzed), Polycaprolactone (PCL) low Mw (10 K), PCL medium Mw (40 K), PCL high Mw (80 K), zinc sulfate 0.3 N solution, zinc oxide, L-glutamine and penicillin/streptomycin, QuantiPro® Bicinchoninic acid assay kit was from Sigma Aldrich, UK. Alhydrogel® 2% (Aluminium Hydroxide Gel Adjuvant) was from Superfos Biosector, Denmark. RPMI and fetal bovine serum were from Gibco, UK. Horseradish peroxidase conjugated Goat anti-mouse isotype specific immunoglobulin from Serotec, UK. DuoSet® sandwich ELISA kits was from R&D Systems, UK. Dichloromethane (DCM) was from BDH laboratory suppliers, UK. All other reagents used were of analytical grade.

4.2.2. Methods

4.2.2.1. Formulation of OVA loaded PCL nanoparticles

OVA 2% m/m loaded PCL nanoparticles were prepared using PVA as a stabilizing agent by w/o/w emulsification solvent evaporation method. Approximately 100 mg of PCL (low, medium or high molecular weight) was dissolved in 6 ml of dichloromethane (DCM) to make the organic phase (Table 4.1). The primary emulsion (w/o) was formed by dispersing 1 ml of 10% m/v PVA along with the protein solution (internal phase) in the organic phase using high shear homogenizer (Ultra-turrax) at 24,000 rpm for 2 minutes. This w/o emulsion was added drop wise to 30 ml of 2.5% m/v PVA solution and homogenized for 6 minutes at 10,000 rpm using the Silverson® homogenizer (Silverson, model L4RT). The formed w/o/w emulsion was left stirring for 4 hours using a magnetic stirrer to evaporate the organic solvent. 1 ml of 10% m/v trehalose was added in each formulation prior to centrifugation and the emulsion was centrifuged (20,000 rpm, 45 minutes, 4 °C) to obtain a pellet of nanoparticles. This pellet was resuspended in a mixture of 4 ml of 2.5% m/v trehalose solution and freeze-dried (Virtis, England) to obtain a fine powder of nanoparticles. Trehalose is a disaccharide

with high thermal stability and a high pH-stability range and one of the most stable saccharides. As it is a non-reducing sugar, it does not show Maillard reaction with amino compounds such as amino acids or proteins. It has good stabilizing functions such as preventing starch retrogradation, protein denaturation and lipid degradation, so it can be good stabilizer for the antigen encapsulated into nanoparticles.

Table 4.1: Formulations of 2% m/m OVA loaded PCL nanoparticles. The internal and external phase stabilizing agents were 1ml of 10% m/v PVA together with OVA and 30 ml of 2.5% m/v PVA respectively

Code	Type of PCL in the organic phase
A 1	Low Mw (10 K)
A 2	Medium Mw (40 K)
A 3	High Mw (80 K)

4.2.2.2. Formulation of OVA plus zinc sulfate co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles

OVA plus zinc sulfate co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles were prepared by the w/o/w emulsification solvent evaporation method. As the effect of PCL and PLA was evaluated on the physicochemical characteristics of the formulation, same molecular weights of the polymers (PCL and PLA) were used in this study. Zinc sulfate (solution containing 0.3N zinc sulfate) was mixed in 1 ml of 10% m/v PVA. The composition of the internal phase and the formulation details are in Table 4.2. The preparation method of the nanoparticles is described in the previous section (4.2.2.1.1).

Table 4.2: The internal phase composition of the formulations of OVA plus zinc sulfate co encapsulated PCL (80 K) or PLA (80 K) nanoparticles. The external phase stabilizing agent was 30 ml of 1.25% m/v PVA.

Code	Polymer dissolved in the organic phase	Composition of OVA or zinc sulfate solution in the internal phase stabilizing agent with 1 ml 10% m/v PVA (corresponding zinc sulfate in weight)
B1	PCL	-
B2	"	200 µl zinc sulfate (4.84mg)
B3	"	2% OVA m/m + 100 µl zinc sulfate (2.42mg)
B4	"	2% OVA m/m + 200 µl zinc sulfate (4.84mg)
B5	"	2% OVA m/m + 300 µl zinc sulfate (7.26mg)
B6	"	2% OVA m/m + 400 µl zinc sulfate (9.68mg)
B7	PLA	-
B8	"	200 µl zinc sulfate (4.84mg)
B9	"	2% OVA m/m + 100 µl zinc sulfate (2.42mg)
B10	"	2% OVA m/m + 200 µl zinc sulfate (4.84mg)
B11	"	2% OVA m/m + 300 µl zinc sulfate (7.26mg)
B12	"	2% OVA m/m + 400 µl zinc sulfate (9.68mg)

4.2.2.3. Formulation of OVA plus zinc oxide co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles

OVA plus zinc oxide co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles were prepared by the w/o/w emulsification solvent evaporation method. Zinc oxide was mixed in 1 ml of 10% m/v PVA. Zinc oxide is not water soluble therefore the internal phase in these formulations contained zinc oxide as a suspension form. The composition of the internal phase and the formulation details are in Table 4.3. The preparation method of the nanoparticles is described in the previous section (4.2.2.1.1).

Table 4.3: The internal phase composition of the formulations of OVA plus zinc oxide co encapsulated PCL (80 K) or PLA (80 K) nanoparticles. The external phase stabilizing agent was 30 ml of 1.25% m/v PVA.

Code	Polymer dissolved in the organic phase	Composition of OVA or zinc oxide in the internal phase stabilizing agent with 1 ml 10% m/v PVA
C1	PCL	-
C2	"	10 mg zinc oxide
C3	"	2% OVA m/m
C4	"	2% OVA m/m + 5 mg zinc oxide
C5	"	2% OVA m/m + 10 mg zinc oxide
C6	"	2% OVA m/m + 20 mg zinc oxide
C7	PLA	-
C8	"	10 mg zinc oxide
C9	"	2% OVA m/m
C10	"	2% OVA m/m + 5 mg zinc oxide
C11	"	2% OVA m/m + 10 mg zinc oxide
C12	"	2% OVA m/m + 20 mg zinc oxide

4.2.2.4. Formulation of FITC-BSA plus zinc sulfate or zinc oxide co-encapsulated PCL (80 K) nanoparticles

To assess the uptake of the particles by the macrophage cells J774A.1, FITC-BSA plus zinc sulfate or zinc oxide co-encapsulated PCL (80 K) nanoparticles were prepared by the w/o/w emulsification solvent evaporation method. FITC-BSA was commercially sourced for this study, although a FITC labelled HBsAg would give a better indication of the uptake of these particles. Zinc sulfate or zinc oxide was mixed in 1 ml of 10% m/v PVA. The composition of the internal phase and the formulation details are in Table 4.4. The preparation method of the nanoparticles is described in the previous section (4.2.2.1.1).

Table 4.4: Formulation of FITC-BSA plus zinc sulfate or zinc oxide co-encapsulated PCL (80 K) nanoparticles. The external phase stabilizing agent was 30 ml of 1.25% m/v PVA.

Code	Composition of FITC-BSA and zinc sulfate or zinc oxide in the internal phase stabilizing agent with 1 ml 10% m/v PVA
D1	-
D1	2% FITC-BSA m/m + 20 mg zinc oxide
D3	2% FITC-BSA m/m + 40 mg zinc oxide
D4	2% FITC-BSA m/m + 200 µl zinc sulfate (4.84mg)
D5	2% FITC-BSA m/m + 400 µl zinc sulfate (9.68mg)

4.2.2.5. Formulation of HBsAg plus zinc sulfate or zinc oxide or alum co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles

HBsAg 1% m/m plus zinc sulfate or zinc oxide or alum co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles were prepared the by w/o/w emulsification solvent evaporation method. Zinc sulfate or zinc oxide or alum (in the form of 3 %, m/v aluminium hydroxide gel) was mixed in 1 ml of 10% m/v PVA. The concentration of the HBsAg was 1.68 mg/ml therefore 540 µl of HBsAg plus the relevant co-adjuvant together with PVA was used as the internal phase. The composition of the internal phase and the formulation details are in Table 4.5. The preparation method of the nanoparticles is described in the previous section (4.2.2.1.1).

Table 4.5: Formulation of HBsAg plus zinc sulfate or zinc oxide or alum co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles. The external phase stabilizing agent used for all the formulations was 30 ml of 1.25% m/v PVA.

Code	Polymer dissolved in the organic phase	Composition of HBsAg or zinc sulfate or zinc oxide or alum in the internal phase stabilizing agent with 1 ml 10% m/v PVA
E1	PCL	1% HBsAg m/m
E2	"	1% HBsAg m/m + 20 mg zinc oxide
E3	"	1% HBsAg m/m + 400 µl zinc sulfate (9.68mg)
E4	"	1% HBsAg m/m + 100 µl alum
E5	PLA	1% HBsAg m/m
E6	"	1% HBsAg m/m + 400 µl zinc sulfate (9.68mg)
E7	"	1% HBsAg m/m + 100 µl alum

4.2.2.6. Formulation of HBsAg loaded PLA (147 K) microparticles

The theoretical loading of HBsAg for all formulations was 1 % m/m of the polymer (Table 4.6). Briefly, PLA was dissolved in 4 ml dichloromethane. HBsAg was mixed with 0.5 ml of aqueous solution containing 10 % m/v PVA. In case of the alum containing formulation, the antigen was initially adsorbed to aluminium hydroxide gel

(3 % m/v) and then mixed with 0.5 ml of 10 % m/v PVA (Table 6.1). The primary emulsion was formed by dispersing the two immiscible solutions using an ultra-turrax (T25 Janke & Kunkel, IKA-Labortechnik, Germany) for 2 minutes at 24,000 rpm. This emulsion was added drop wise to 30 ml of 1.25 % m/v PVA solution in deionised water or 0.75 % m/v chitosan solution in 1 % v/v acetic acid and homogenized for 6 minutes at 10,000 rpm with a high shear homogenizer (Silverson® L4RT, Silverson Machines, UK). The resultant w/o/w emulsion was magnetically stirred at room temperature overnight to evaporate the organic solvent. The microparticles were collected by centrifugation (Beckman J2-21, USA) after 15 minutes at 20,000 rpm and washed once with deionised water. The resultant suspension was freeze-dried (Virtis, UK) to obtain a free flowing powder.

Table 4.6: Formulation details of the alum or chitosan incorporated HBsAg-loaded PLA microparticles.

Code	Antigen	Volume of oil phase (ml)	External aqueous phase 30ml (%)
F1	HBsAg	5	PVA (2.5)
F2	HBsAg adsorbed alum	5	PVA (2.5)
F3	HBsAg	5	Chitosan (0.75)
F4	HBsAg	10	PVA (2.5)
F5	-	5	PVA (2.5)
F6	-	5	PVA (2.5)
F7	-	5	Chitosan (0.75)
F8	-	10	PVA (2.5)

4.2.2.7. Characterization of micro/nanoparticles

The micro/nanoparticles obtained were characterized with respect to their size distribution, surface charge and encapsulation efficiency as described in sections 2.2.2.1, 2.2.2.2, 2.2.2.3, 2.2.2.5. Integrity of the protein or antigen was determined using SDS-PAGE as described in section 2.2.2.6. The morphology of the micro/nanoparticles was studied using SEM as described in section 2.2.2.4. The MTT assay for PCL or PLA plus zinc sulfate nanoparticles encapsulated with HBsAg was carried out as described in section 2.2.3.4. The culturing of J774A.1 cells (mouse BALB/c monocyte macrophage) and the uptake studies of nanoparticles by macrophage cells were carried out as described in sections 2.2.3.1 and 2.2.3.2. Statistical analysis was preformed using an analysis of variance (ANOVA) general linear model with SPSS software (Version 17, Microsoft) assuming P values of $P \leq 0.05$ as significant.

4.2.2.8. *In vivo* studies

4.2.2.8.1. Immunization schedule used for PCL (80 K) or PLA (80 K) nanoparticles

A total of 16 groups (n = 4) of mice were used for this study (Table 4.7). Eight groups (n = 4) of female BALB/c mice (25g, 4-6 weeks old) were anaesthetized with an inhaled gaseous mixture of 3% v/v halothane (RMB Animal Health, UK) in oxygen (300 cm^3 min^{-1}) and nitrous oxide (100 cm^3 min^{-1}) for intranasal dosing procedures. The particles were administered intranasally to the mice using a micropipette. Eight groups (n = 4) of female BALB/c mice (25g, 4-6 weeks old) were intramuscularly (at the hind-legs) administered with the antigen formulations using a hypodermic syringe.

Table 4.7: Intramuscular *versus* intranasal immunization schedule of HBsAg loaded PCL and PLA nanoparticles containing zinc sulfate or zinc oxide or alum. The priming dose was 1 µg of HBsAg *per* mice on day 1 followed by one booster dose of 0.5 µg of HBsAg *per* mice on day 21.

No	Formulations	Route
1	HBsAg loaded PCL nanoparticles	Intramuscular
2	HBsAg loaded PLA nanoparticles	
3	HBsAg plus zinc sulfate co encapsulated PCL nanoparticles	
4	HBsAg plus zinc sulfate co encapsulated PLA nanoparticles	
5	HBsAg plus alum co encapsulated PCL nanoparticles	
6	HBsAg plus alum co encapsulated PLA nanoparticles	
7	Alum plus free HBsAg	
8	Free HBsAg	
9	HBsAg loaded PCL nanoparticles	Intranasal
10	HBsAg loaded PLA nanoparticles	
11	HBsAg plus zinc sulfate co encapsulated PCL nanoparticles	
12	HBsAg plus zinc sulfate co encapsulated PLA nanoparticles	
13	HBsAg plus alum co encapsulated PCL nanoparticles	
14	HBsAg plus alum co encapsulated PLA nanoparticles	
15	CpG plus free HBsAg	
16	Free HBsAg	

Each mouse received a volume of 50 µl antigen loaded particle suspension dispersed in PBS or antigen alone as appropriate. Tail-vein blood samples were taken on days 15, 30, 45 and 90, to assess the serum specific antibody responses by ELISA as described in section 2.2.4.5.

4.2.2.8.2. Immunization schedule used for PLA (147 K) microparticles

Six groups (n = 4) of female BALB/c mice (25g, 4-6 weeks old) were subcutaneously administered with the formulations using a hypodermic syringe (Table 4.8). Each mouse received a volume of 50 µl antigen loaded particle suspension dispersed in PBS or antigen alone as appropriate. Tail vein blood samples were taken on days 15, 60, 250,

300 and 400, to assess the serum-specific antibody responses by ELISA as described in section 2.2.4.5.

Table 4.8: Immunization schedule of HBsAg loaded PLA (147 K) microparticles by subcutaneous route. The priming dose was 1 μg of HBsAg *per* mice on day 1 followed by one booster dose of 0.5 μg of HBsAg *per* mice on day 21.

No	Formulations
1	HBsAg loaded PLA microparticles with PVA as external phase stabilizing agent
2	Alum plus HBsAg co encapsulated PLA microparticles with PVA as external phase stabilizing agent
3	HBsAg loaded PLA microparticles with chitosan as external phase stabilizing agent
4	Free HBsAg plus alum
5	Free HBsAg plus chitosan
6	Free HBsAg

4.2.2.8.3. Enzyme linked immunosorbent assay (ELISA) for the determination of IgG antibodies and cytokine analysis

Antibody responses in serum of the immunized animals were monitored using a microplate ELISA as described in chapter 2 (section 2.2.4.5). The cytokine analysis was carried on as described in chapter 2 (section 2.2.4.6) for the determination of endogenous cytokines levels, interleukin-4 (IL-4), interleukin-6 (IL-6) and interferon-γ (IFN-γ).

4.3. Results and discussion

4.3.1. Characterization of nanoparticles

4.3.1.1. Effect of molecular weight of the polymer on OVA loaded PCL nanoparticles

In the present work the effect of PCL molecular weight on the particle size, zeta potential and loading of OVA in the nanoparticles were investigated; the results are described in the Table 4.9. The z-average diameter of the particles for the low molecular weight nanoparticles was found to be around 291 nm (A1) and the size of the particles increased to 349 nm (A3) with the high molecular weight polymer. From these results it can be seen that the size decreases as the molecular weight of the polymer decreases. This can be attributed to the increase in the viscosity of the polymer solution with increase in the molecular weight of the polymer.

Table 4.9: Effect of molecular weight of the polymer on OVA loaded PCL nanoparticles. Particles were characterized with respect to size, loading and zeta potential. The internal and external phase stabilizing agent was 1 ml of 10% m/v PVA and 30 ml of 1.25% m/v PVA respectively (n=3, mean ±s.d).

Code	Type of PCL in the organic phase	Yield % ±s.d	Z average particle size (nm) ±s.d	Poly-dispersity index ±s.d	Zeta potential, mV ±s.d	Actual protein content (µg/mg) ±s.d	Encapsulation efficiency % ±s.d
A1	Low Mw (10 K)	66.3 ±0.84	291.3 ±5	0.283 ±0.016	-4.86 ±1.9	3.12 ±0.18	20.7 ±1.2
A2	Medium Mw (40 K)	65.4 ±2.23	320.6 ±4	0.262 ±0.018	-5.61 ±1.8	4.40 ±0.35	28.8 ±2.3
A3	High Mw (80 K)	65.8 ±1.95	349.8 ±4	0.368 ±0.028	-5.89 ±2.1	4.82 ±0.36	31.7 ±2.4

A viscous solution needs a high shear stress to be dispersed. If the shear stress remains constant (homogenization) the less viscous solution can be dispersed more easily and this will result in smaller particles. Benoit *et al* (1999) found that an increase in the concentration (weight) of polymer (PCL) in a fixed volume of organic solvent has increased the mean particle size by at least 2.7 times. Jeffery *et al.* (1993) also found similar increase of particle size with increase in the polymer viscosity and suggested that the higher concentration of polymer in the sample might have led to an increased frequency of collisions creating a fusion of semi-formed particles resulting in the increased particle size. The zeta potential measured of the formulations was found to be between –5.8 and –4.8 mV. The molecular weight of the polymer did not influence the surface charge of the particles.

The encapsulation efficiency of the nanoparticles increased with increase in the molecular weight of the polymer. The low molecular weight PCL nanoparticles had the encapsulation efficiency of 20.7% (A1, actual protein content 6.24 µg/mg of nanoparticles), whereas the encapsulation efficiency increased to 28.8% (A2, actual protein content 8.8 µg/mg of nanoparticles) and 31.7% (A3, actual protein content 9.6 µg/mg of nanoparticles) for the medium and high molecular weight polymer respectively. Mehta *et al* (1996) reported that the loading of the polymeric particles increases as the polymer molecular weight increases; this can be attributed to the viscosity of the polymer wherein the protein cannot escape from the interface easily when the viscosity of the polymer is higher during the double emulsion solvent evaporation method. It can also be argued that the higher encapsulation efficiency of the high molecular weight PCL nanoparticles is due to its high crystallinity as the crystalline phase is generally impermeable to water; the antigen is likely to be entrapped in the amorphous region (Conway *et al.*, 1997).

The scanning electron micrographs showed that the particles are spherical surface (Figure 4.1). There was not much difference between the morphology of the nanoparticles made with different molecular weights of polymer.

Figure 4.1: (A) Scanning electron micrograph (SEM) of OVA loaded low Mw PCL nanoparticles prepared by a w/o/w solvent evaporation method (B) SEM of OVA loaded medium Mw PCL nanoparticles prepared by a w/o/w solvent evaporation method (C) SEM of OVA loaded high Mw PCL nanoparticles prepared by a w/o/w solvent evaporation method

The factors such as the nature of polymer, concentration of the stabilizer and the preparation parameters affects the surface morphology of the particles (Rafati *et al.*, 1997). The morphology and size of the microspheres is an important determinant of the physical stability of the nanoparticles and their ability to induce an immune response. For example, smooth and spherical surface morphologies of the particles are indicators of physical stability and increase the likelihood of good release profiles for the particles, while presence of pores on the surface of the particles increases the possibility of leakage of the antigen and rough surfaces may lead to aggregation of the particles (Igartua *et al.*, 1998).

4.3.1.2. Formulation of OVA plus zinc sulfate co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles

OVA plus zinc sulfate co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles were prepared by w/o/w double emulsification solvent evaporation method. The particles were characterized with respect to size, zeta potential and encapsulation efficiency. The results are described in the Table 4.10.

The size of the particles before the freeze drying was found to be around 250 nm for the PCL formulations and around 270 to 300 nm for the PLA formulations. The particle size increased to 300-370 nm for PCL formulations and 360-410 nm for the PLA formulations after freeze drying, which is not unexpected as freeze drying tends to aggregate the nanoparticles. PCL-zinc sulfate particles showed an increase in encapsulation efficiency of 43% compared with 30.6% for plain PCL particles. Similarly, the PLA-zinc sulfate particles showed an increase in encapsulation efficiency of 56.7% compared with 40.3% for plain PLA particles. This may have occurred for a variety of reasons. The first reason is based on studies performed on w/o/w emulsions by Wen and Papadopoulos (2000, 2001). In these studies it was found that when a 5M solution of sodium chloride was used as the external aqueous phase, water was transported from the internal aqueous phase outwards, however no transfer of the salt was observed. The authors proposed that this was a consequence of osmotic pressure. They found that in w/o/w emulsions where no contact between the internal and external aqueous phase was present, water transport occurred via spontaneously emulsified droplets and reverse micelles.

Table 4.10: Effect of mineral compounds on the physical characteristics of the OVA loaded PCL nanoparticles. Particles were characterized for the yield, size, loading and zeta potential (n=3, mean ±s.d).

Code	Polymer dissolved in the organic phase	Composition of OVA or zinc sulfate solution in the internal phase stabilizing agent with 1 ml 10% m/v PVA	Yield % ±s.d	Z average particle size (nm)				Zeta potential, mV ±s.d	Actual protein content (µg/mg) ±s.d	Percentage encapsulation efficiency ±s.d
				Before Freeze drying ±s.d	Poly-dispersity index ±s.d	After Freeze drying ±s.d	Poly-dispersity index ±s.d			
B1	PCL	-	60.8 ±1.58	247.8 ±4	0.212 ±0.014	303.3 ±8	0.221 ±0.021	-4.73 ±2.2	n/a	n/a
B2	"	200 µl zinc sulfate	62.5 ±1.47	252.3 ±4	0.139 ±0.021	334.8 ±9	0.312 ±0.026	-5.21 ±2.3	n/a	n/a
B3	"	2% OVA m/m + 100 µl zinc sulfate	63.4 ±1.85	256.6 ±3	0.154 ±0.013	332.3 ±7	0.291 ±0.032	-5.73 ±1.8	4.83 ±0.35	30.6 ±2.2
B4	"	2% OVA m/m + 200 µl zinc sulfate	62.4 ±2.45	260.1 ±3	0.150 ±0.015	343.0 ±7	0.306 ±0.038	-5.74 ±1.7	4.92 ±0.43	30.7 ±2.7
B5	"	2% OVA m/m + 300 µl zinc sulfate	65.5 ±3.45	253.2 ±4	0.168 ±0.022	311.4 ±8	0.269 ±0.029	-6.43 ±2.4	5.47 ±0.29	35.8 ±1.9
B6	"	2% OVA m/m + 400 µl zinc sulfate	64.8 ±2.48	254.4 ±5	0.154 ±0.026	370.8 ±11	0.382 ±0.036	-6.54 ±2.3	6.64 ±0.35	43.0 ±2.3
B7	PLA	-	62.3 ±1.84	270.8 ±6	0.286 ±0.018	360.2 ±3	0.243 ±0.034	-5.73 ±1.2	n/a	n/a
B8	"	200 µl zinc sulfate	62.4 ±1.57	294.6 ±5	0.156 ±0.028	367.4 ±4	0.254 ±0.033	-5.84 ±2.4	n/a	n/a
B9	"	2% OVA m/m + 100 µl zinc sulfate	64.2 ±1.25	267.3 ±4	0.254 ±0.023	389.4 ±5	0.239 ±0.039	-5.86 ±2.5	6.28 ±0.37	40.3 ±2.4
B10	"	2% OVA m/m + 200 µl zinc sulfate	62.5 ±1.92	268.2 ±6	0.286 ±0.029	386.4 ±3	0.275 ±0.042	-6.96 ±2.6	6.62 ±0.19	41.4 ±1.2
B11	"	2% OVA m/m + 300 µl zinc sulfate	63.8 ±1.84	296.2 ±4	0.231 ±0.024	384.3 ±4	0.213 ±0.038	-8.67 ±2.8	7.57 ±0.42	48.3 ±2.7
B12	"	2% OVA m/m + 400 µl zinc sulfate	64.2 ±1.75	297.4 ±4	0.324 ±0.022	410.4 ±6	0.354 ±0.046	-8.83 ±3.6	8.83 ±0.44	56.7 ±2.8

Bearing this in mind it is possible that during the preparation of PCL-zinc or PLA-zinc sulfate nanoparticles water is transported from the external phase in the direction of the internal phase. In the double emulsion process one of the reasons that antigen is lost is mixing between the internal aqueous phase which contains the antigen and the external phase. Based on the work discussed by Wen and Papadopoulos (2000, 2001) it is possible that during the production of zinc sulfate co-encapsulated particles the osmotic pressure created by the presence of zinc sulfate in the internal aqueous phase results in the transport of water from the external phase inwards. This would create an opposing force to the transfer of the internal aqueous phase into the external.

The structural integrity of the protein encapsulated in the nanoparticles was determined by SDS-PAGE (Figures 4.2 and 4.3). The gels show that OVA had not deterioted. The structural integrity of OVA remained following exposure to DCM, homogenization and lyophilisation. This was observed for the all the nanoparticle formulations.

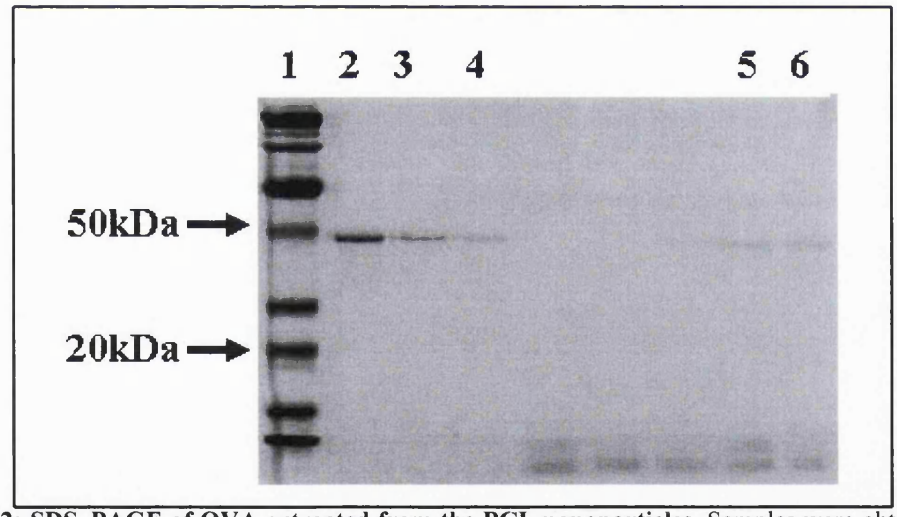

Figure 4.2: SDS–PAGE of OVA extracted from the PCL nanoparticles. Samples were obtained after digestion of PCL nanoparticles with PBS and NaOH. Lanes are as follows: (1) molecular weight marker; (2) 100 μg/ml of free OVA; (3) OVA extracted from PCL nanoparticles - 100 μl zinc sulfate in the internal phase); (4) OVA extracted from PCL nanoparticles-200 μl zinc sulfate in the internal phase (5) OVA extracted from PCL nanoparticles-300 μl zinc sulfate in the internal phase; (6) OVA extracted from PCL nanoparticles- 400 μl zinc sulfate in the internal phase.

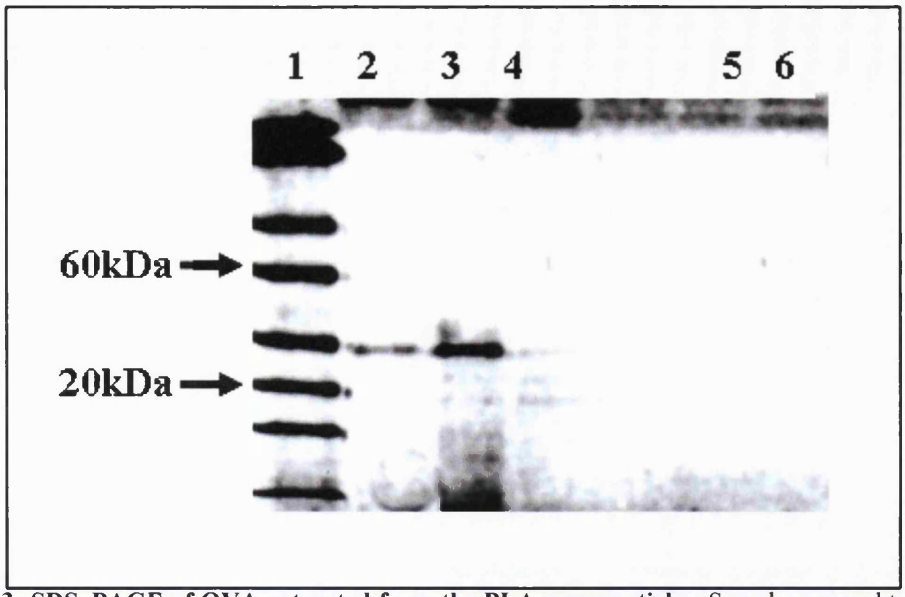

Figure 4.3: SDS–PAGE of OVA extracted from the PLA nanoparticles. Samples were obtained after digestion of PLA nanoparticles with PBS and NaOH. Lanes are as follows: (1) molecular weight marker; (2) 100 µg/ml of free OVA; (3) OVA extracted from PLA nanoparticles - 100 µl zinc sulfate in the internal phase); (4) OVA extracted from PLA nanoparticles-200 µl zinc sulfate in the internal phase (5) OVA extracted from PLA nanoparticles-300 µl zinc sulfate in the internal phase; (6) OVA extracted from PLA nanoparticles- 400 µl zinc sulfate in the internal phase.

4.3.1.3. Formulation of OVA plus zinc oxide co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles by w/o/w double emulsification solvent evaporation method

OVA plus zinc oxide co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles were prepared by w/o/w double emulsification solvent evaporation method. The particles were characterized with respect to size, zeta potential and encapsulation efficiency. The results are described in the Table 4.11. The results for the zinc oxide plus OVA co-encapsulated PCL or PLA nanoparticles are similar to that obtained for zinc sulfate. Interestingly, the particle size was found to be in the submicron range, although zinc oxide is a powder that is micron in size. This can be explained by the hypothesis that zinc oxide might have not been encapsulated inside the particles. The size of the particles before the freeze drying was found to be around 250 to 270 nm for all the formulations. The particle size increased to 280-370 nm after freeze drying which is not unexpected as freeze drying tends to aggregate the nanoparticles.

Table 4.11: Characterisation of size, loading and zeta potential for OVA plus zinc sulfate co-encapsulated PCL nanoparticles prepared by solvent evaporation method (n=3, mean ±s.d).

Code	Polymer dissolved in the organic phase	Composition of OVA or zinc sulfate solution in the internal phase stabilizing agent with 1 ml 10% m/v PVA	Yield % ±s.d	Z average particle size (nm)				Zeta potential, mV ±s.d	Actual protein content (µg/mg) ±s.d	Percentage encapsulation efficiency ±s.d
				Before Freeze drying	Poly-dispersity index ±s.d	After Freeze drying	Poly-dispersity index ±s.d			
C1	PCL	-	59.3 ±2.48	255.0 ±4	0.153 ±0.024	343.9 ±8	0.193 ±0.038	-6.48 ±2.3	n/a	n/a
C2	"	10 mg zinc oxide	61.2 ±2.36	261.0 ±6	0.203 ±0.026	290.8 ±5	0.225 ±0.036	-6.24 ±2.6	n/a	n/a
C3	"	2% OVA m/m	62.5 ±1.89	243.3 ±3	0.148 ±0.034	323.6 ±8	0.258 ±0.046	-6.64 ±1.8	4.82 ±0.43	30.14 ±2.7
C4	"	2% OVA m/m + 5 mg zinc oxide	64.2 ±2.45	252.1 ±7	0.292 ±0.027	288.7 ±6	0.306 ±0.056	-10.64 ±2.4	4.89 ±0.30	31.4 ±1.9
C5	"	2% OVA m/m + 10 mg zinc oxide	64.5 ±2.65	243.9 ±4	0.173 ±0.026	307.6 ±9	0.378 ±0.054	-10.65 ±3.1	5.76 ±0.36	37.16 ±2.3
C6	"	2% OVA m/m + 20 mg zinc oxide	63.2 ±2.26	249.0 ±4	0.179 ±0.031	299.8 ±5	0.398 ±0.056	-10.34 ±2.8	6.65 ±0.49	42.02 ±3.1
C7	PLA	-	62.5 ±2.38	247.8 ±2	0.165 ±0.018	315.8 ±3	0.237 ±0.029	-4.92 ±1.8	n/a	n/a
C8	"	10 mg zinc oxide	64.5 ±3.21	257.1 ±2	0.202 ±0.026	287.4 ±4	0.245 ±0.034	-5.56 ±1.9	n/a	n/a
C9	"	2% OVA m/m	66.8 ±1.92	261.2 ±4	0.215 ±0.022	297.6 ±6	0.284 ±0.034	-5.68 ±2.2	4.88 ±0.21	32.6 ±1.4
C10	"	2% OVA m/m + 5 mg zinc oxide	62.6 ±2.48	271.3 ±3	0.317 ±0.029	287.4 ±6	0.228 ±0.039	-6.24 ±2.5	5.86 ±0.26	36.7 ±1.6
C11	"	2% OVA m/m + 10 mg zinc oxide	64.9 ±2.34	283.2 ±4	0.274 ±0.031	316.8 ±8	0.364 ±0.046	-6.48 ±1.9	6.52 ±0.43	42.3 ±2.8
C12	"	2% OVA m/m + 20 mg zinc oxide	64.2 ±1.98	268.5 ±5	0.264 ±0.024	322.8 ±9	0.378 ±0.043	-6.89 ±2.6	6.95 ±0.37	44.6 ±2.4

PCL-zinc oxide particles showed an increase in encapsulation efficiency of 42 % compared with 30 % for plain PCL particles, whereas PLA-zinc oxide particles showed an increase of 44.6% compared to 32.6% for plain PLA particles. This increase in loading can be attributed to the possibility osmotic pressure created by the presence of zinc oxide similar to the zinc sulfate.

It can also be observed that the intensity of the bands was not similar for all the formulations. This is can be due to the extraction/digestions technique used in this study. It might be possible that the nanoparticles were not completely digested when PBS and NaOH were used. However, the integrity of the protein was inconclusive with the presence of a thin band appearing at the 30KDa range. The alternative method to examine the protein integrity is either size exclusion chromatography or mass spectroscopy, although this was not done during this project (Figures 4.4 and 4.5). After these preliminary studies, the uptake of the particles by the macrophages was also explored, the results of which are described in the next section.

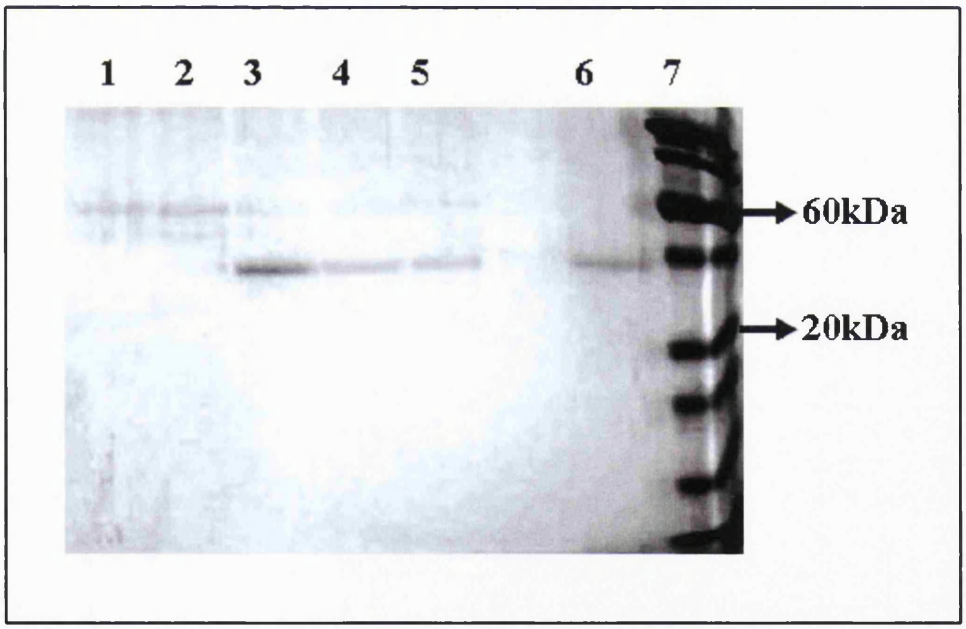

Figure 4.4: SDS–PAGE of OVA extracted from the PCL nanoparticles containing zinc oxide. Samples were obtained after digestion of PCL nanoparticles with PBS and NaOH. Lanes are as follows: (1) Placebo PCL nanoparticles – 20 mg zinc oxide in the internal phase; (2) Placebo PCL nanoparticles; (3) OVA extracted from PCL nanoparticles – 5 mg zinc oxide in the internal phase; (4) OVA extracted from PCL nanoparticles – 10 mg zinc oxide in the internal phase (5) OVA extracted from PCL nanoparticles – 20 mg zinc oxide in the internal phase; (6) 100 µg/ml of free OVA; (7) molecular weight marker.

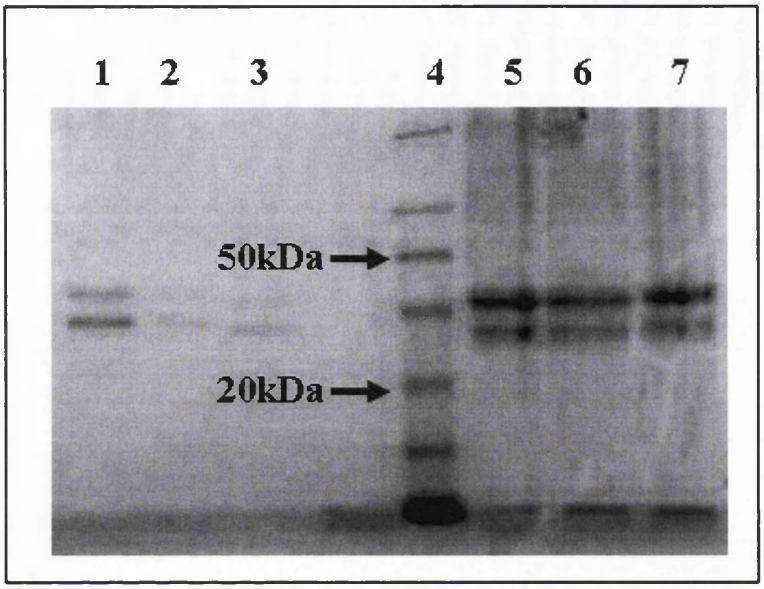

Figure 4.5: SDS–PAGE of OVA extracted from the PCL nanoparticles containing zinc oxide. Samples were obtained after digestion of PCL nanoparticles with PBS and NaOH. Lanes are as follows: (1) OVA extracted from PLA nanoparticles – 5 mg zinc oxide in the internal phase; (2) OVA extracted from PLA nanoparticles – 10 mg zinc oxide in the internal phase (3) OVA extracted from PLA nanoparticles – 20 mg zinc oxide in the internal phase (4) molecular weight marker; (5) 100 µg/ml of free OVA; (6) 50 µg/ml of free OVA; (7) 25 µg/ml of free OVA.

4.3.1.4. Formulation of FITC-BSA plus zinc sulfate or zinc oxide co-encapsulated PCL (high molecular weight) nanoparticles

To assess the uptake of the particles by the macrophage cells J774A.1, FITC-BSA plus zinc oxide co encapsulated PCL (80 K) nanoparticles were prepared by w/o/w emulsification solvent evaporation method. The particles were characterized with respect to size and zeta potential. The results are described in the Table 4.12.

Table 4.12: The particle size of FITC-BSA plus zinc sulfate or zinc oxide co encapsulated PCL nanoparticles (n=3, mean ±s.d).

Code	Composition of FITC-BSA and zinc sulfate or zinc oxide in the internal phase stabilizing agent with 1 ml 10% m/v PVA	Z average particle size (nm) ±s.d			
		Before Freeze drying	Poly dispersity	After Freeze drying	Poly dispersity
D1	-	249.2 ±3	0.162 ±0.011	331.1 ±4	0.395 ±0.024
D1	2% FITC-BSA m/m + 20 mg zinc oxide	303.9 ±4	0.254 ±0.024	346.0 ±8	0.565 ±0.083
D3	2% FITC-BSA m/m + 40 mg zinc oxide	280.3 ±2	0.142 ±0.029	244.3 ±7	0.614 ±0.086
D4	2% FITC-BSA m/m + 200 µl zinc sulfate (4.84mg)	311.6 ±4	0.187 ±0.019	304.0 ±3	0.111 ±0.022
D5	2% FITC-BSA m/m + 400 µl zinc sulfate (9.68mg)	274.3 ±2	0.219 ±0.021	257.8 ±3	0.098 ±0.024

Although there was no appreciable difference in the particle size before and after freeze drying irrespective of the type of zinc used, we decided to use the zinc sulfate plus FITC-BSA co-encapsulated nanoparticles for the uptake studies, because of the better solubility of the zinc sulfate compared to zinc oxide. Although, zinc is considered safe and is taken as part of nutrition, the uptake of nanoparticles by macrophage cells was investigated to assess if zinc sulfate or zinc oxide has any untoward effects on the uptake of nanoparticles. The structural integrity of the FITC-BSA was intact as shown by the SDS-PAGE (Figure 4.6).

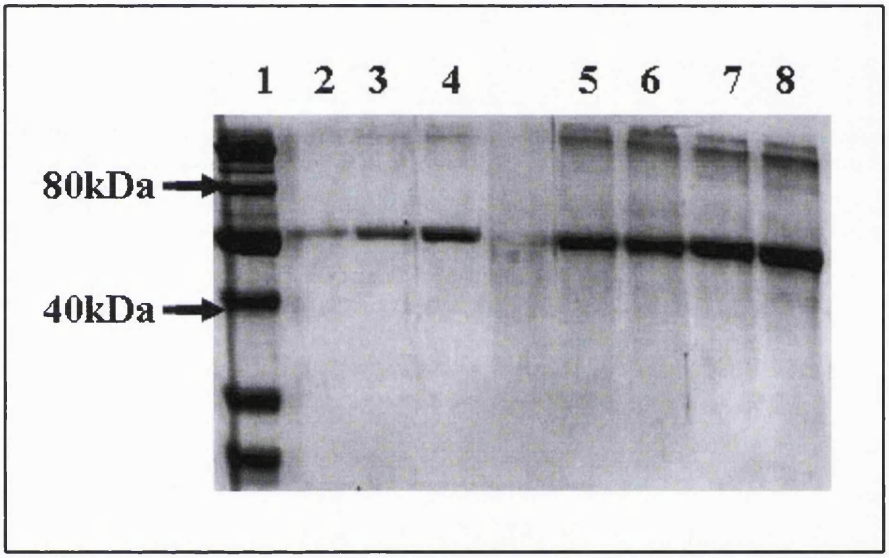

Figure 4.6: SDS–PAGE of FITC-BSA extracted from the PCL nanoparticles containing zinc sulfate or zinc oxide. Lanes are as follows: (1) molecular weight marker; (2) 25 µg/ml of free FITC-BSA; (3) 50 µg/ml of free FITC-BSA; (4) 25 µg/ml of free FITC-BSA; (5) FITC-BSA extracted from PCL nanoparticles with 10 mg zinc oxide in the internal phase; (6) FITC-BSA extracted from PCL nanoparticles with 20 mg zinc oxide in the internal phase; (7) FITC-BSA extracted from PCL nanoparticles with 200 µl zinc sulfate in the internal phase; (8) FITC-BSA extracted from PCL nanoparticles with 200 µl zinc sulfate in the internal phase.

In this study, the particle size and the charge of the nanoparticles was similar, moreover the hydrophobicity is similar as the polymer used was same for all the formulations. Size of the nanoparticles is an important criterion in the induction of an antibody response, and as mentioned above, in terms of phagocytosis by antigen presenting cells such as macrophages. All these nanoparticles are in the size range of 200-400 µm and therefore in a range suitable for phagocytosis and uptake (Morris *et al.*, 1994). It was observed by confocal microscopy that the particles are taken up by the by macrophages (Figure 4.7). As seen in Figure. 4.7 (A), cells tested do not have background in the fluorescence emission range of FTIC, whereas after 90 minutes they have a fluorescence background (Figure. 4.7 – F). The nanoparticles containing zinc sulfate were readily taken up by the macrophages in a similar manner as nanoparticles without

zinc sulfate as shown by the confocal microscopy. These studies show that zinc sulfate has no untoward effect on the uptake of the nanoparticles.

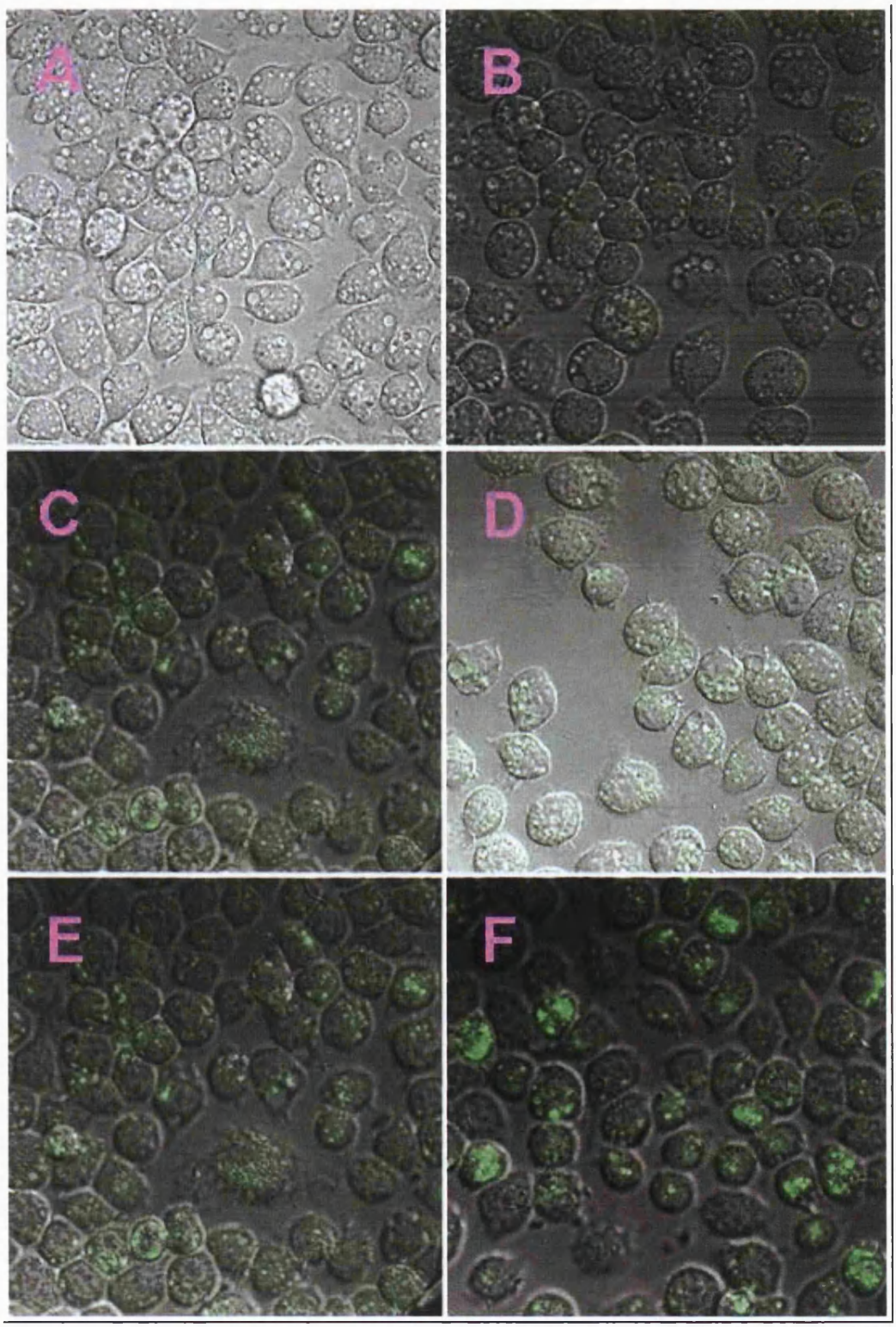

Figure 4.7: Uptake of FITC-BSA loaded PCL nanoparticles containing zinc sulfate by J774A.1 macrophage cells (A) 0 mins (B) 15 mins (C) 30 mins (D) 45 mins (E) 60 mins (F) 90 mins.

However, this is a qualitative assessment only and the full understanding of the uptake can be done quantitatively by using radio labelled particles. Moreover, it would be

clearer if the nucleus of the cell is focussed rather than the whole cell, which was not done during this study.

4.3.1.5. MTT assay of the formulations to assess cytotoxicity

Based on the satisfactory encapsulation efficiency it was decided that particles containing 400 zinc sulfate or alum will be used in the *in vivo* animal studies. As the co-encapsulation of zinc oxide lowered the encapsulation efficiency of the antigen in the particles they were excluded from the animal studies. It is essential to check the toxicity of these particles as they were being used for *in vivo* studies, therefore *In vitro* toxicity of nanoparticles was evaluated by MTT assay in CHO-K1 cells using increasing concentration of nanoparticles (Figure 4.8). The MTT assay is a colorimetric assay system that measures the reduction of a tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into an insoluble formazan product by the mitochondria of viable cells. The nanoparticles assessed prepared by the solvent evaporation method, PCL only particles; PCL with zinc sulfate; PCL with alum particles. Poly ethyleneimine (PEI) was used as a positive control. PEI is positively charged polyplex which interacts with the blood components activating the complement system and thereby is highly toxic at the cellular levels (Merdan *et al.*, 2002). Due to this known toxic properties of PEI, it was used as the positive control.

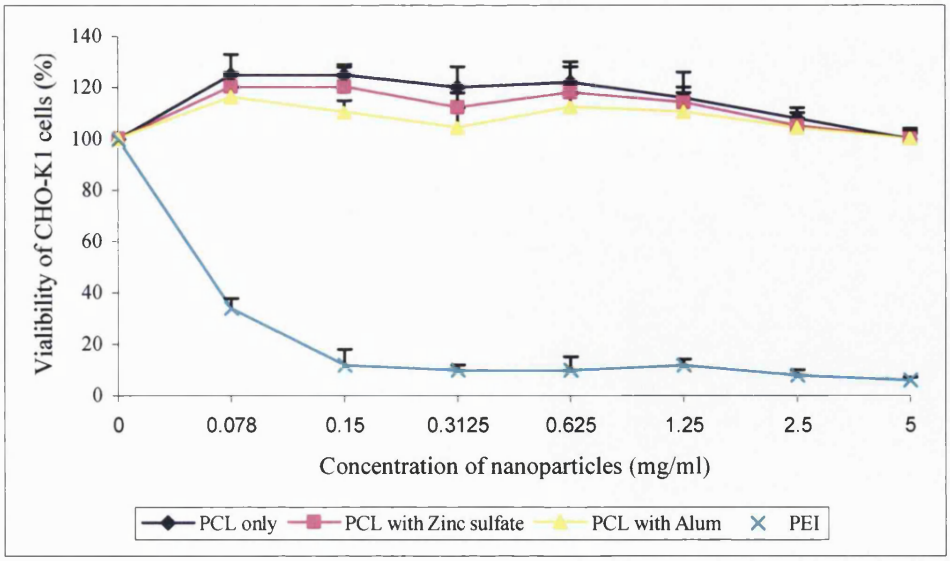

Figure 4.8: Cytotoxicity of PCL nanoparticles co-encapsulating HBsAg and zinc sulfate or alum analyzed by MTT assay in CHO-K1 cells. The particles were prepared by double emulsion solvent evaporation. PEI was used as a positive control. Cells incubated in cell culture medium were used as a control to assay 100% viability. Viability of cells exposed to the formulations was expressed as percentage of the viability seen in control untreated cells ($n = 3$).

The nanoparticle formulations were found to be non-toxic at the majority of concentrations studied. There was no significant difference ($P > 0.06$) in the toxicity of different formulations at any of the concentrations used. There was a slight reduction in cell viabilities at higher concentrations of 5 mg/ml of the particles. The average cell viabilities were between 90 to 125% of control (PEI) at the concentrations studied. Based on these MTT assay results, these formulations were assumed to be safe to be used in the *in vivo* studies.

4.3.1.6. Formulation of HBsAg plus zinc sulfate or zinc oxide or alum co-encapsulated PCL (80 K) or PLA (80 K) nanoparticles

In a study conducted by Raghuvanshi *et al* (2002) alum was co-encapsulated with TT in PLA microparticles and the results showed that alum-adsorbed TT microparticles elicited significantly higher anti-TT antibody titres, as compared to plain TT microspheres. In case of the HBsAg, there are substantial published data that demonstrates that there is considerable potential for the development of single dose HBsAg vaccines using controlled release biodegradable polymeric microspheres, and there is extensive information about the release kinetics of the encapsulation (Singh *et al.*, 1997; Nellore *et al.*, 1992; Shi *et al.*, 2002). However, there are no published data on the effect of co-encapsulation of zinc sulfate and antigen on the physiochemical properties of the polymeric nanoparticles and immune responses. The fundamental requirements for the success of the use of biodegradable polymers for delivery of vaccine antigens are that the particles should efficiently entrap the antigen; they should be in the suitable size range and surface properties to interact with the cell membrane and be taken up by antigen presenting cells. The particles co-encapsulating HBsAg plus mineral compounds in PCL or PLA were characterized and the results are summarized in the Table 4.13.

Table 4.13: Characterization of size, zeta potential and encapsulation efficiency of HBsAg plus zinc sulfate or zinc oxide or alum co encapsulated PCL or PLA nanoparticles (n=1, analysis was done 3 times on same formulation, mean ±s.d).

Code	Polymer dissolved in the organic phase	Composition of HBsAg or zinc sulfate or zinc oxide or alum in the internal phase stabilizing agent with 1 ml 10% m/v PVA	Yield % ±s.d	Z average particle size (nm)				Zeta potential, mV ±s.d	Actual protein content (µg/mg) ±s.d	Percentage encapsulation efficiency ±s.d
				Before Freeze drying	Polydispersity index ±s.d	After Freeze drying	Polydispersity index ±s.d			
E1	PCL	1% HBsAg m/m	64.2 ±1.82	262.6 ±5	0.108 ±0.024	315.8 ±4	0.237 ±0.031	-5.42 ±2.2	10.36 ±0.19	66.5 ±1.2
E2	"	1% HBsAg m/m + 20 mg zinc oxide	66.5 ±3.24	320.4 ±7	0.157 ±0.034	384.2 ±8	0.345 ±0.043	-7.48 ±2.5	2.38 ±0.09	15.8 ±0.6
E3	"	1% HBsAg m/m + 400 µl zinc sulfate (9.68mg)	64.25 ±2.46	282.8 ±5	0.217 ±0.024	320.6 ±5	0.284 ±0.021	-6.64 ±1.8	13.82 ±0.09	88.8 ±0.6
E4	"	1% HBsAg m/m + 100 µl alum	63.8 ±2.72	426.1 ±6	0.668 ±0.058	564.2 ±7	0.648 0.048	-8.42 ±2.7	10.99 ±0.24	70.1 ±1.5
E5	PLA	1% HBsAg m/m	66.5 ±2.26	285.2 ±2	0.335 ±0.019	325.6 ±6	0.232 ±0.016	-4.89 ±1.8	6.44 ±0.35	42.85 ±2.3
E6	"	1% HBsAg m/m + 400 µl zinc sulfate (9.68mg)	62.4 ±1.89	319.6 ±4	0.463 ±0.014	423.6 ±5	0.564 ±0.026	-6.78 ±1.6	9.42 ±0.29	58.80 ±1.8
E7	"	1% HBsAg m/m + 100 µl alum	63.8 ±3.24	468.9 ±7	0.441 ±0.032	568.2 ±6	0.583 ±0.046	-8.42 ±0.4	8.32 ±0.38	53.05 ±2.4

The nanoparticles had particle size smaller than 500 nm and Z average particle size of the PCL nanoparticles ranged from 263 to 426 nm before freeze drying and from 316 to 564 nm after the freeze drying (Table 4.13). For the PLA particles, the size ranged from 285 to 469 nm PCL nanoparticles before freeze drying and from 326 to 568 nm after freeze drying (Table 4.11). Incorporation of zinc sulfate did not influence the particle size but presence of alum and zinc oxide increased the particle size. This can be attributed to the size of alum and zinc oxide. The size of the polymeric particles is considered to be one of the most important parameters in elucidating the immune responses. Although, there have been several studies which has investigated the effect of particle size on the nasal delivery of the encapsulated antigens, the results are not conclusive.

The particles used for immunization in this study had a size range from 250 nm to 500 nm. The particle size is considered to influence the immune responses; however this is debatable as a number of studies have shown contradictory results. In a study conducted by Gutierro *et al.*, (2002) the influence of the BSA encapsulated PLGA particles of different sizes (1,000 nm, 500 nm, 200 nm) on the immune response by different routes of administration was investigated. It was found that the particle size does not influence the type (IgG1 or IgG2a) of immune response suggesting that the antigen processing and presentation is similar in all cases irrespective of the particle size. However, the administration of 1,000 nm particles generally elicited a higher serum IgG response than that obtained with the administration of 500 or 200 nm sized nanoparticles, the immune response for 500 nm particles being similar than that obtained with 200 nm by the subcutaneous and the oral route, and higher by the intranasal route. Jung *et al*, (2001) studied the effect of particle size and route of administration of the sulfobutylated PVA-graft-PLGA particles adsorbed with TT. Here, particles of approximately 100 nm, 500 nm and 1.5 μm diameter were dosed to BALB/c mice orally (28.9 μg of TT in 200 μl of saline) and nasally (2.9 μg of TT in 20 μl of saline). The authors reported a size-dependant response for serum IgG and IgA titres for orally dosed animals and found a inverse relation of particles size to immune titres, i.e. immune titres in the following order 100nm>500nm>1.5 μm. The nasal group followed a similar trend, although no difference between the 100 nm and 500 nm group was observed. Almeida *et al.* (1993) delivered 500 nm polystyrene particles nasally to Wistar rats and found the particles in the systemic circulation giving the evidence of systemic absorption when particles are

delivered nasally. In the same study, TT adsorbed PLA particles of 830 nm were given to guinea pigs and analysed the serum IgG titres for up to 15 weeks following priming. It was found that there was a rapid increase in the serum IgG levels after the booster dose (one month after the priming dose), which indicates the serum IgG memory. Based on these results the authors concluded that particles entered the systemic circulation and may have translocated to other immune organs such as the spleen, leading to the responses observed.

These studies show that the different particle sizes show contradicting immune response, therefore based on these studies it is difficult to establish the lower size limit required for particle administration using the nasal route. Additionally, the above studies report the results in the context of antigen-specific IgG titres. However, these studies show that nanoparticles with sizes lower than 500 nm have potential for successful vaccine carriers.

The production yield was between 63.8 to 66.5 % for PCL particles and 62.4 to 66.5 % for PLA particles. The co-encapsulation of zinc sulfate or zinc oxide or alum did not affect the yield of nanoparticles. The actual HBsAg content of nanoparticles, expressed as a percentage of the amount of antigen entrapped in the nanoparticles, varied between 2.38-13.82 μg/mg for PCL nanoparticles and 2.38-9.42 μg/mg for PLA nanoparticles (Table 4.13). It was found that the antigen amount of PCL nanoparticles prepared with zinc sulfate (E3) (13.82 ± 0.55 μg/mg) and PLA nanoparticles prepared with zinc sulfate (E6) (9.42 ± 0.64 μg/mg) were significantly higher than the nanoparticles containing antigen alone or with zinc oxide or alum. When zinc sulfate plus HBsAg were co-encapsulated in the PCL or PLA nanoparticles the loading efficiency of the antigen increased. The actual content and encapsulation efficiency can be enhanced by the use of excipients like salts or mineral compounds. Iyer *et al* (2004) have shown that HBsAg adsorbs strongly onto aluminium hydroxide adjuvant by means of ligand exchange between the HBsAg phospholipids and surface hydroxyls of the adjuvant. The increase in loading efficiency of the antigen by the use of zinc sulfate is advantageous. This is because for the antigen carrying nanoparticles, to function as controlled release systems for the generation of long lasting immune responses, it is necessary that they should contain high levels of antigen. However, the effects observed on encapsulation efficiency are in contrast to those reported in literature. Pistel and Kissel (2000) investigated the effect of the addition of sodium chloride to the internal aqueous phase

on the characteristics of microspheres. They found that addition of sodium chloride led to an increasingly porous structure with an irregular shape. The particles produced were reported to be of a larger size, lower encapsulation efficiency and experienced a higher initial burst of antigen compared with those particles produced without sodium chloride in the internal aqueous phase. A similar effect was reported by Hermann and Bodmeier (1995) when calcium chloride and sodium chloride were added to the internal aqueous phase. This would indicate that the results found in the present study may be specific to zinc salts. Complexation between proteins and zinc has been documented (Costantino *et al.*, 2000). Therefore, HBsAg and zinc may produce complexes. Such a scenario would also explain the increased encapsulation efficiency achieved by the particles. This is because the loss of the complexes to the external phase may be reduced owing to the larger size of the complexes having a lower tendency to diffuse out of the internal aqueous phase as compared to free HBsAg. However, particles co-encapsulated with zinc oxide had lower encapsulation efficiency of the antigen this might be because of the solubility of zinc oxide when compared with zinc sulfate. Zinc oxide is not water soluble and might have released the antigen from the internal phase during the formulation of the second emulsion. However, these results are contrary to the ones obtained with OVA and zinc oxide co-encapsulation. This can be because of the different nature of HBsAg from OVA. The loss of antigen can occur during the several stages of the formulation process such as during emulsification as well as washings, during which a significant portion of the un-encapsulated antigen is lost.

The particles size and morphology as observed by the scanning electron microscopy (Figure 4.9) showed that particles were mainly monodisperse and spherical, regardless of the co-encapsulation of zinc sulfate or alum. However, it was not possible using the scanning electron to investigate the finer structure of the nanoparticles.

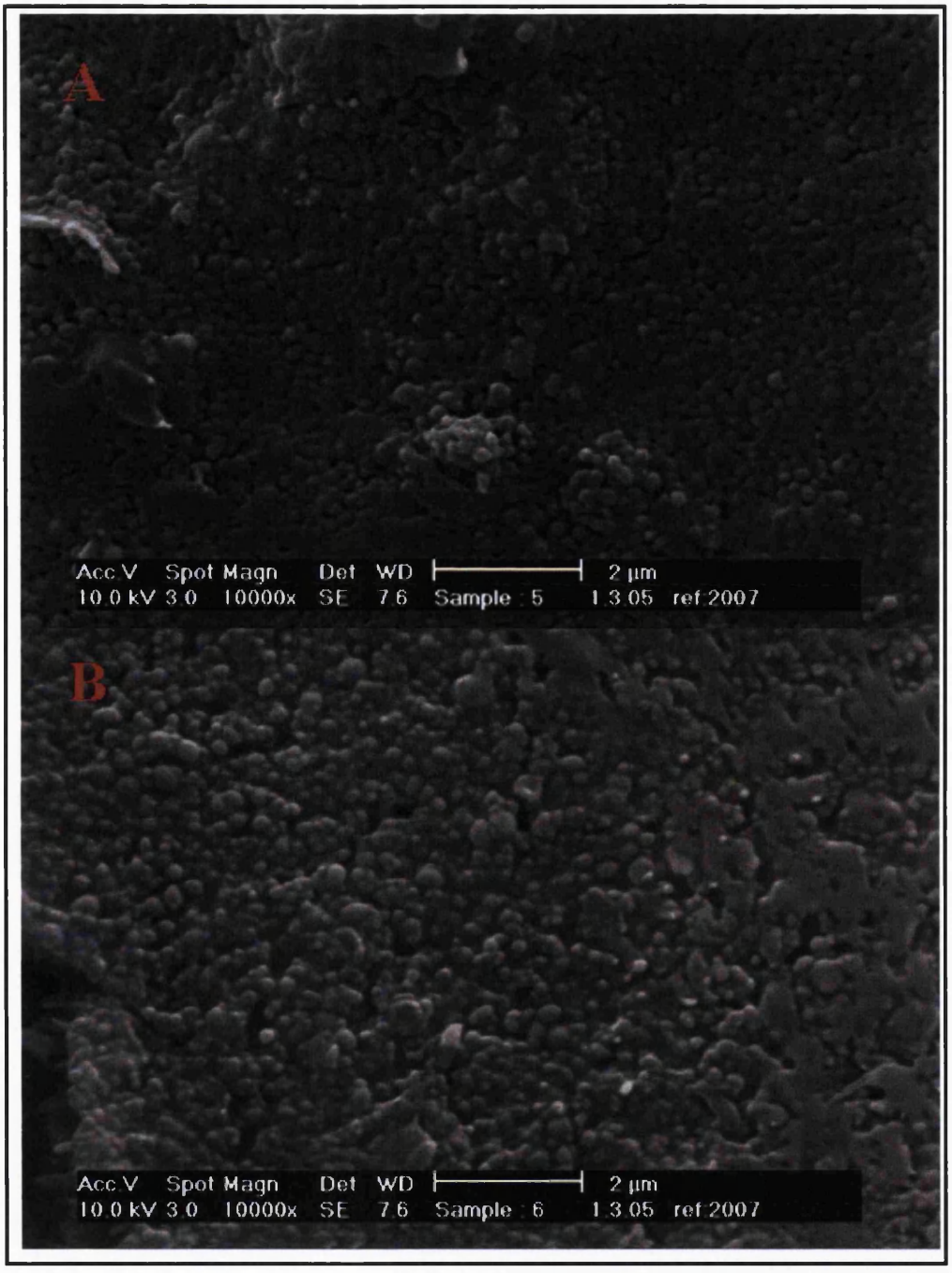

Figure 4.9: Scanning electron micrograph of HBsAg loaded PLA nanoparticles prepared by a w/o/w solvent evaporation method (A) HBsAg plus zinc sulfate co-encapsulated PLA nanoparticles (B) HBsAg plus alum co-encapsulated PLA nanoparticles

The retention of structural integrity of the antigen is a prerequisite for the antigen to generate a suitable immune response, denaturation or inactivation of the protein during processing causes alteration of the native structure of the protein, indicated by a shift in band formation higher or lower than the standard. This was not seen for any of the samples (Figure 4.10 and 4.11) and therefore the structural integrity of HBsAg in the

nanoparticles was not affected by either process parameters or by storage as shown by SDS-PAGE.

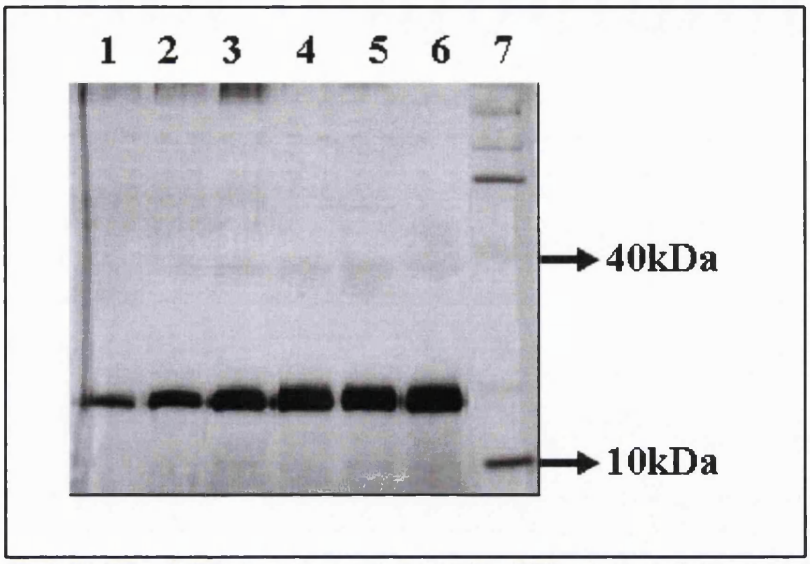

Figure 4.10: SDS–PAGE of HBsAg extracted from the PCL nanoparticles containing mineral compounds. Samples were obtained after digestion of PCL nanoparticles with PBS and NaOH. Lanes are as follows: (1) 12.5 µg/ml of free HBsAg; (2) 25 µg/ml of free HBsAg; (3) HBsAg extracted from PCL nanoparticles; (4) HBsAg extracted from PCL nanoparticles – 20 mg zinc oxide in the internal phase; (5) HBsAg extracted from PCL nanoparticles – 400 µl zinc sulfate in the internal phase; (6) HBsAg extracted from PCL nanoparticles – 100 µl alum in the internal phase; (7) molecular weight marker.

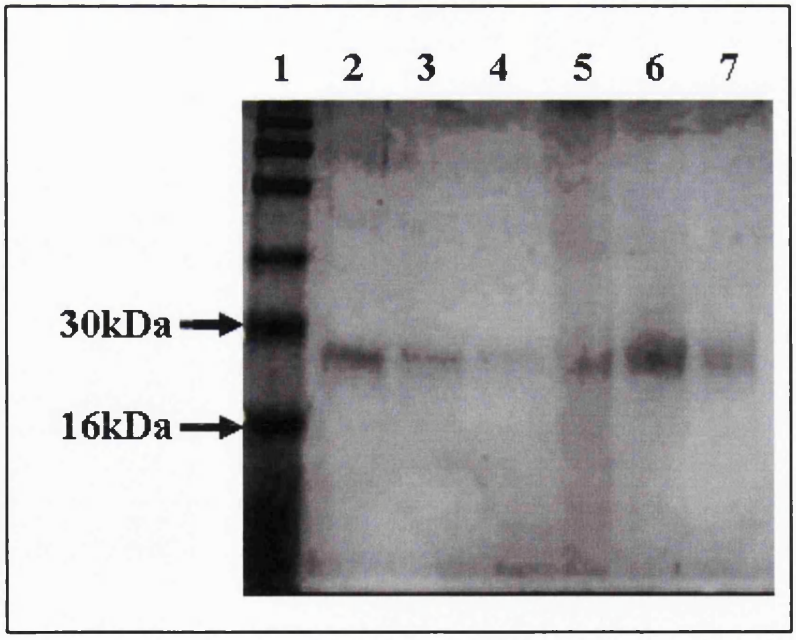

Figure 4.11: SDS–PAGE of HBsAg extracted from the PLA nanoparticles containing mineral compounds. Samples were obtained after digestion of PLA nanoparticles with PBS and NaOH. Lanes are as follows: (1) molecular weight marker; (2) 100 µg/ml of free HBsAg; (3) 50 µg/ml of free HBsAg; (4) 25 µg/ml of free HBsAg; (5) HBsAg extracted from PLA nanoparticles; (4) HBsAg extracted from PLA nanoparticles – 400 µl zinc sulfate in the internal phase; (7) HBsAg extracted from PLA nanoparticles – 100 µl alum in the internal phase.

Preservation of the integrity and stabilisation of proteins/antigens like DT has been shown in literature after formulation into polymeric particles using the ultra turrax to produce the w/o/w emulsion (Singh *et al.*, 1998; Johansen *et al.*, 1999). PVA acts as a protective polymer to the antigen by being adsorbed at the oil/water interface of droplets to produce a stearic barrier, which protects the antigen against the external organic solvent. Other proteins have also been reported to be successfully encapsulated into PCL nanoparticles (Lamprecht *et al.*, 2000) and PLGA nanoparticles (Tabata *et al.*, 1996) without losing their properties.

4.3.1.7. Formulation of HBsAg loaded PLA (147 K) nanoparticles

The characteristics of the HBsAg encapsulated microparticles fabricated here is summarised in Table 4.14.

4.14: Characterization of size, loading and zeta potential for 1% m/m HBsAg-loaded PLA microparticles. 1 ml of 10% m/v PVA used as primary stabilizing agent for all the formulations. The external phase stabilizing agent was either 30 ml of 2.5% m/v PVA or 0.75% m/v chitosan. (n=3, mean ±s.d).

Code	Antigen	Volume of oil phase (ml)	External aqueous phase (ml)	Yield % ±s.d	Mean volume diameter µm ±s.d	Zeta potential mV ±s.d	Actual antigen content (µg/mg) ±s.d	Encapsulación efficiency % ±s.d
F1	HBsAg	5	PVA (2.5%)	62.8 ±1.12	1.36 ±0.02	-23.9 ±1.0	1.97 ±0.11	12.34 ±1.02
F2	HBsAg adsorbed alum	5	PVA (2.5%)	67.2 ±1.34	1.56 ±0.18	-30.6 ±3.1	14.62 ±0.26	98.24 ±1.28
F3	HBsAg	5	Chitosan (0.75%)	68.8 ±2.13	2.12 ±0.16	21.5 ±2.2	12.55 ±0.21	86.36 ±2.14
F4	HBsAg	10	PVA (2.5%)	63.2 ±2.87	1.13 ±0.07	-24.8 ±1.5	7.11 ±0.22	44.96 ±2.09
F5	-	5	PVA (2.5%)	68.4 ±3.08	1.34 ±0.03	-11.5 ±2.0	n/a	n/a
F6	-	5	PVA (2.5%)	73.6 ±5.13	1.78 ±0.03	-19.5 ±0.9	n/a	n/a
F7	-	5	Chitosan (0.75%)	79.2 ±3.89	2.44 ±0.14	27.5 ±0.9	n/a	n/a
F8	-	10	PVA (2.5%)	60.8 ±2.75	1.33 ±0.02	-17.8 ±0.6	n/a	n/a

The size of the microparticles is an important criterion in the induction of an antibody response, as these microparticles are in the size range of 1-2 µm they are therefore in a range suitable for phagocytosis and uptake by the antigen presenting cells (Morris *et al.* 1994; Yamaguchi and Anderson 1993; Kanke *et al.* 1986). The surface morphology of

the particles depends upon factors such as the nature of the polymer, concentration of the stabiliser, and fabrication parameters (Rafati *et al.* 1997). All the microsphere preparations produced in this study are smooth and spherical, with no aggregation (Figure 4.12).

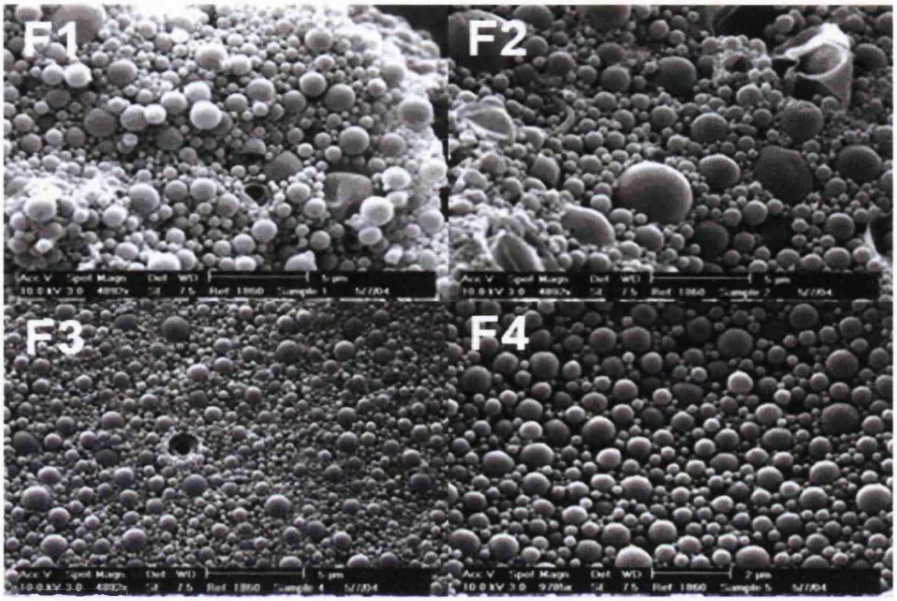

Figure 4.12: Scanning electron micrographs of HBsAg-loaded PLA microparticles made by w/o/w double emulsion technique (with 5 ml DCM in the organic phase unless specified). F1) HBsAg-loaded PLA microparticles prepared with PVA as external phase stabilizing agent; b) Alum adsorbed HBsAg-loaded PLA microparticles prepared with PVA as external phase stabilizing agent; c) HBsAg-loaded PLA microparticles prepared with chitosan as the external phase stabilizing agent and F4) HBsAg-loaded PLA microparticles prepared with PVA as external phase stabilizing agent (10 ml DCM in the organic phase).

Microparticles have a particle size smaller than 2 μm diameter and the mean particle size of microparticles ranged from 1.33 ± 0.02 to 1.93 ± 0.03 μm for empty microparticles (F5-F8) and from 1.13 ± 0.07 to 1.98 ± 0.16 μm for HBsAg encapsulated microparticles (F1-F4). Incorporation of the antigen or the inclusion of alum and chitosan did not change the particle size and distribution of microparticles (Table 4.14).

The surface charge in terms of the Zeta potential of the microparticles is an important determinant for the *in vivo* behaviour of microparticles and determines their degree of interaction with the cell surface and their uptake by phagocytotic cells. Surface charges of the microparticles (Table 4.14) were negative for all formulations except microsphere formulations containing chitosan (F3 and F7). Surface charge values between -19.5 and +27.5 mV for empty microparticles (F5-F8) and between -30.6 and +21.5 mV for antigen loaded microparticles (F1-F4) were measured. Incorporation of the antigen or

the inclusion of alum reduced the surface charge of microparticles. Alum adsorbed microparticles displayed a slightly higher negative charge than HBsAg loaded microparticles, suggesting that the adsorption of the antigen on alum has caused a shift in charge towards negative. Although using chitosan as a stabiliser in the external aqueous phase instead of negatively charged PVA increased the surface charge of microparticles when comparing these formulations (F3: +21.5 mV; F7: +27.5 mV). The zeta potential values measured, as expected, were positive (due to the amine groups of the chitosan molecule) for the formulations where chitosan was used as the external phase stabiliser. This indicates that the outer surface of F3 and F7 formulations consist largely of chitosan.

The production yield was between 60.8-79.2 % for empty particles and 62.8-68.8 % for antigen-loaded microspheres. Antigen loading didn't affect the yield of microparticles significantly ($p>0.05$). In order for the antigen encapsulated microparticles to function as controlled release delivery systems for the generation of long lasting immune responses, it is necessary that they should contain suitably high levels of the antigen and for this to take place, it is necessary to achieve sufficiently high protein loading efficiencies. The actual HBsAg content of microparticles, expressed as a percentage of the amount of antigen entrapped in the microparticles, varied between 1.97-14.62 μg/mg (Table 6.3). It was found that the antigen content of microparticles which were prepared with alum (F2) (14.62 ± 0.26 μg/mg) and chitosan (F3) (12.55 ± 0.21 μg/mg) were significantly higher than the microparticles containing antigen alone (F1) (1.97 ± 0.11 μg/mg) ($p<0.01$).

While the encapsulation efficiency of the F1 formulation was 12.34 ± 1.02 %, the use of alum (F2) and chitosan (F3) increased the encapsulation efficiency of formulations to 98.24 ± 1.02 % and 86.36 ± 2.14 %, respectively. Raghuvanshi *et al* (2002) have demonstrated that single doses of antigen encapsulated PLA particles exhibit markedly enhanced antibody titers when administered with alum. Alum is used as an adsorbent for antigen in the current HBsAg vaccine formulation, and adsorbing first might inhibit the loss of antigen that takes place during several stages of the formulation process such as during emulsification as well as washings, during which a significant portion of the unencapsulated antigen is lost. Iyer *et al* (2004) have shown that HBsAg adsorbs strongly onto aluminium hydroxide adjuvant by means of ligand exchange between the

HBsAg phospholipids and surface hydroxyls of the adjuvant. The adsorption of HBsAg onto alum adjuvant prior to microencapsulation therefore significantly enhanced the antigen loading efficiency of the PLA microparticles. In the case of chitosan, this is a positively charged polymer and protonated amine groups of chitosan are able to form complexes with negatively charged surface antigens. It is suggested that, as a result of complexation between chitosan and HBsAg, the encapsulation efficiency of the F3 formulation was significantly increased.

4.3.2. *In vivo* studies

4.3.2.1. Immune response for PCL or PLA nanoparticles co-encapsulated with zinc sulfate or zinc oxide or Alum and HBsAg

There have been previous attempts at encapsulating the HBsAg in PLA, PLGA, chitosan micro/nanoparticles, and HBsAg encapsulated in particles is known to elicit good immune responses in several investigations (Singh *et al.*, 1997; Lee *et al.*, 1997; Shi *et al.*, 2002; Jaganathan and Vyas, 2006; Borges *et al.*, 2007). In the present study, HBsAg co-encapsulated with zinc sulfate and alum in PCL or PLA nanoparticles and free HBsAg were administered intramuscularly or intranasally in mice to evaluate the immune responses. Alum plus free antigen was used as a positive control for the intramuscularly administered group of animals. For the intranasal group of animals, free antigen and CpG was used as a positive control. CpG is a well known mucosal adjuvant. CpG motifs are six deoxynucleotides-long DNA sequences with a central CpG dinucleotide which normally occur in bacterial DNA. The bacterial DNA has been shown to induce B-cell proliferation, antibody secretion and Th1-type responses dominated by the IL-12. This was attributed to specific single-stranded oligonucleotide sequences containing unmethylated CpG dinucleotides (CpG motifs). A similar immuno modulating effect can be seen with synthetic oligodeoxynucleotides (ODS) containing CpG motifs. Given these immunostimulatory properties, synthetic CpG ODNs have been extensively tested for therapeutic applications and for immunopotentiating activity to co-administered vaccine antigens (Partidos, 2000). McCluskie and Davis (1999) evaluated the potential of CpG as a mucosal adjuvant to HBsAg when administered alone or with cholera toxin (CT). CpG and CT both augmented systemic and mucosal immune responses against HBsAg. Antibody isotypes with CT alone were

predominantly Th2 whereas they were predominantly Th1 with CpG ODN alone or in combination with CT.

Both PCL and PLA nanoparticles containing zinc sulfate or alum when administered intramuscularly or intranasally have been shown to act as better adjuvants when compared with PCL or PLA only nanoparticles and the free antigen (Figures 4.13 and 4.14).

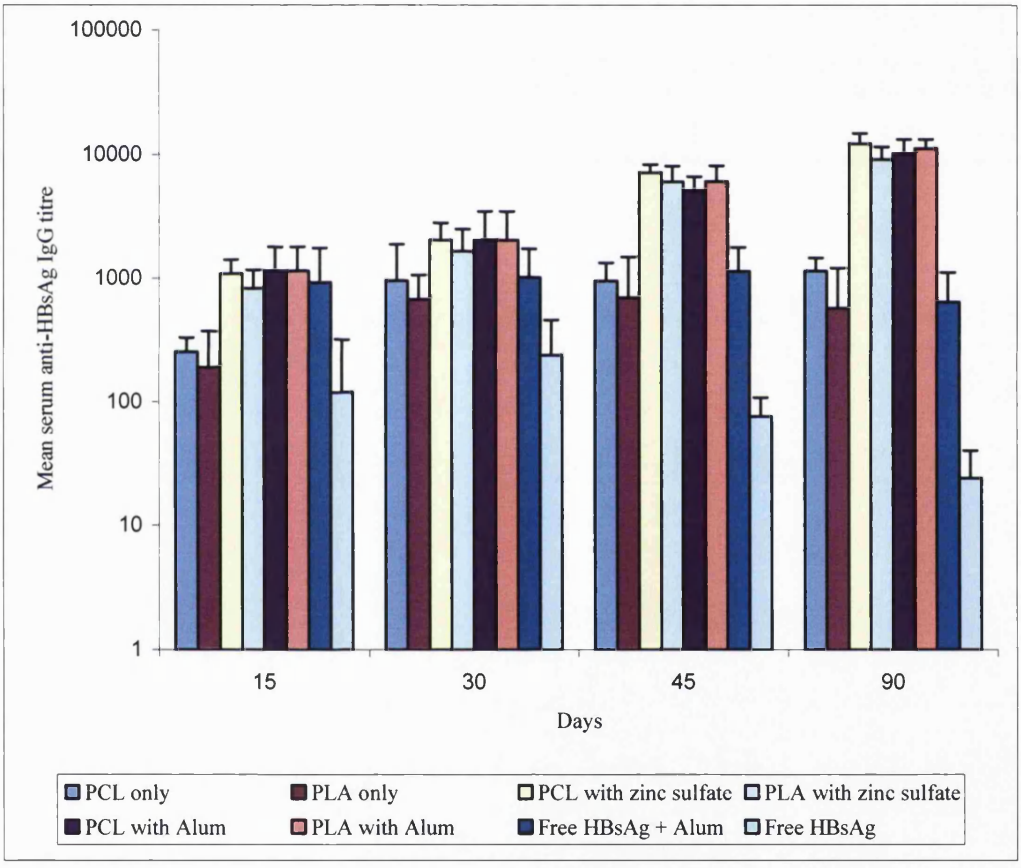

Figure 4.13: Mean serum antibody response to intramuscularly administered HBsAg loaded PCL or PLA nanoparticles with or without zinc sulfate or alum. Mice were also administered with free antigen or free antigen plus alum. The priming dose was 1 μg HBsAg on day 1 followed by a booster dose of 0.5 μg of HBsAg on day 21 ($n = 4$, mean ±s.d).

After intramuscular delivery of HBsAg plus zinc sulfate or alum co-encapsulated PCL or PLA nanoparticles, the primary serum IgG response after 15 days was ~6 to 10 times higher than the free antigen. The serum IgG antibody responses gradually increased for the formulations post-boosting (day 21) dose and at the end of the study (90 days) antibody responses were ~200 to 300 times higher than free antigen. This increase in the antibody responses were significantly higher ($P < 0.001$). The antibody titres for PLA

only and PCL only nanoparticles was lower than the alum plus free antigen until day 45 but at the end of the study the particles without zinc sulfate and alum gave a better response, however this was not significantly higher ($P < 0.08$). The IgG responses for the formulations with zinc sulfate or alum have given ~20 times higher response than the alum plus free antigen ($P < 0.024$ and $P < 0.03$ respectively).

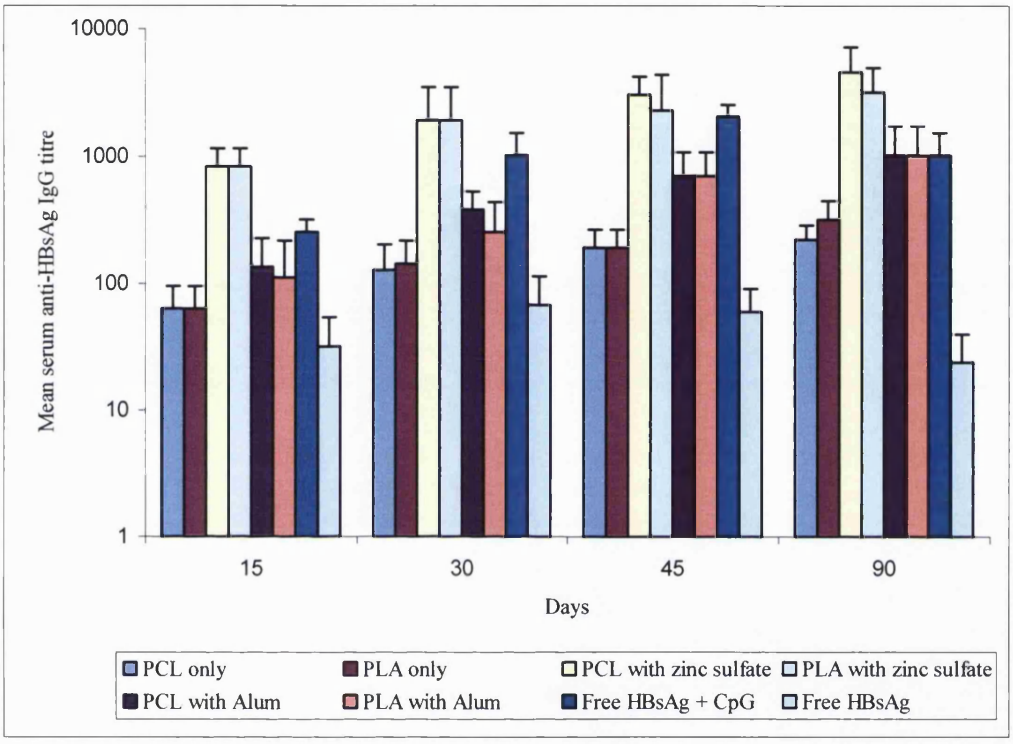

Figure 4.14: Mean serum antibody response to intranasally administered HBsAg loaded PCL or PLA nanoparticles with or without zinc sulfate or alum. Mice were also administered with free antigen or free antigen plus CpG. The priming dose was 1 μg HBsAg on day 1 followed by a booster dose of 0.5 μg of HBsAg on day 21 ($n = 4$, mean ±s.d).

After nasal delivery, high titres of anti-HBsAg antibodies were induced in all mice when HBsAg was either encapsulated or co-encapsulated with zinc sulfate or alum in the PCL or PLA nanoparticles compared with free antigen. The free antigen plus CpG gave a better immune response when compared with particles with alum and PCL only or PLA only nanoparticles. The particles containing zinc sulfate gave significantly higher antibody responses compared with all the other formulation, free antigen and free antigen plus CpG ($P < 0.05$). PCL-zinc sulfate nanoparticles gave a greater HBsAg specific IgG response than PLA-zinc sulfate nanoparticles, but this difference was not very significant ($P < 0.08$). After 90 days PCL-zinc sulfate nanoparticle formulation gave ~20 times higher IgG responses than PCL only nanoparticles ($P < 0.05$), whereas PLA-zinc sulfate particles gave ~10 times higher HBsAg specific IgG responses than PLA only nanoparticles ($P < 0.05$). This study shows that delivery of HBsAg with zinc

sulfate in the form of PCL or PLA nanoparticles enhances systemic immune response after nasal delivery.

In a recent clinical study involving 241 infants for immunization of pneumococcal conjugate vaccine, it was observed that supplementation of zinc from 4 to 33 weeks of age resulted in significantly higher antibody titres compared to titres in infants who had received a placebo. Zinc deficiency is associated with reduced T- and B-lymphocytes, impaired epithelial barrier functions, atrophy of lymphoid organs and decreased macrophage functions resulting in a depressed delayed hypersensitivity. Therefore supplementation of zinc can improve the antibody titres for the delivered antigen (Osendarp *et al.*, 2007). In another randomized clinical trial, the zinc and vitamin A supplementation in 118 HIV-positive injection drug users have shown to increase the antibody responses to pneumococcal conjugate vaccine; however it did not improve the immunogenicity of the vaccine (Deloria-Knoll *et al.*, 2006). Therefore, the presence of zinc in the nanoparticles with HBsAg might have improved the antibody levels in the mice.

The higher serum antibody responses for particles containing alum is contradictory with a previous study, where microencapsulation of alum-adsorbed antigen proved to be unsuccessful in achieving increased immunogenicity of the antigen, compared to formulations encapsulating the plain antigen (Esparza and Kissel, 1992). However, results from that study cannot be compared directly to those presented here as the antigen used was TT in that particular study. The efficacy of encapsulated vaccines is affected by many factors such as polymer size, composition, rate of release of antigen and its loading and the presence of stabilisers and co-adjuvants. It has been postulated that the lower antibody response produced by microencapsulated TT compared with the conventional vaccine may be due to several factors. These might be an altered immunological response as a result of its association with microspheres or biochemical changes in the toxoid originating from microsphere loading and subsequent degradation during storage or following administration (Alonso *et al.*, 1994).

After 90 days, the PCL only formulation produced 2 fold higher IgG titres than the PLA only particles. Studies with polymeric particles have shown that the immune response elicited is dependant on a variety of factors, including particle size, polymer molecular

weight and hydrophobicity (Johansen *et al.*, 2000). These properties are believed to influence the antigen presentation and hence influence the type of immune response elicited. In the present study PCL is relatively hydrophobic in comparison to PLA and hence might have been recognized by APCs to a higher extent, which explains the elevated serum IgG levels elicited by mice when compared with the PLA nanoparticles.

For the analysis of HBsAg-specific cytokines, splenocytes were stimulated with 2.5 µg/ml of HBsAg after 90 days of the experiment and naïve mice were used as controls to correct the background. Th1 cells secrete IFN-γ along with IL-2 and tumour necrosis factor-beta increases gene expression in APC and activate B cells to differentiate and secrete IgG2a, while Th2 produces IL-4, -5, -6, -10 and -13, IgG1 being the major isotype elicited from B cells following activation of Th2 cells. Therefore, Th1 cells are involved in the cell mediated immunity, while Th2 cells are related with humoral immunological response (Johansen *et al.*, 2000). However, due to logistical reasons, only IL-4, IL-6 and secretions were evaluated during these experiments, with IFN-γ giving the Th1 orientation and IL-4 and IL-6 giving the Th2 orientation.

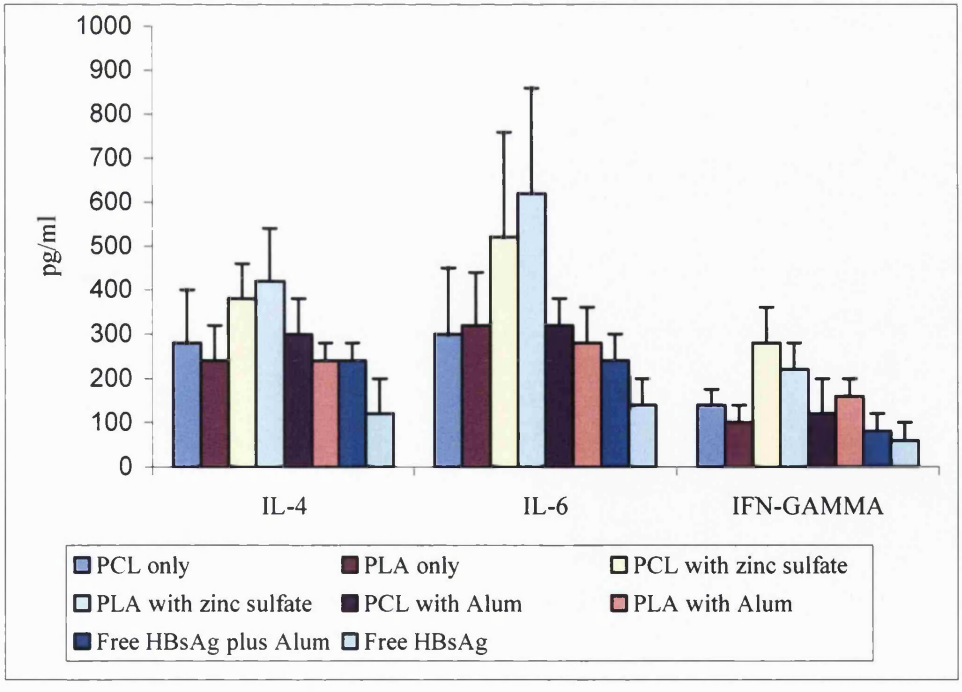

Figure 4.15: Interleukin 4 (IL-4), Interleukin 6 (IL-6) and Interferon gamma (IFN-γ) production by pooled spleens cells after culturing with 2.5 µg/ml concentrations of soluble HBsAg after 90 days. Mice were administered intramuscularly with PCL or PLA with zinc sulfate or alum nanoparticles or free antigen or free antigen plus alum. The priming dose was 1 µg HBsAg on day 1 followed by a booster dose of 0.5 µg of HBsAg on day 21 ($n = 4$, mean ±s.d).

Stimulation with HBsAg successfully elicited IL-4, IL-6 and IFN-γ secretion. Cytokine production was higher for the nanoparticle formulations when compared with the free antigen and free antigen plus alum or free antigen plus CpG (Figures 4.15 and 4.16).

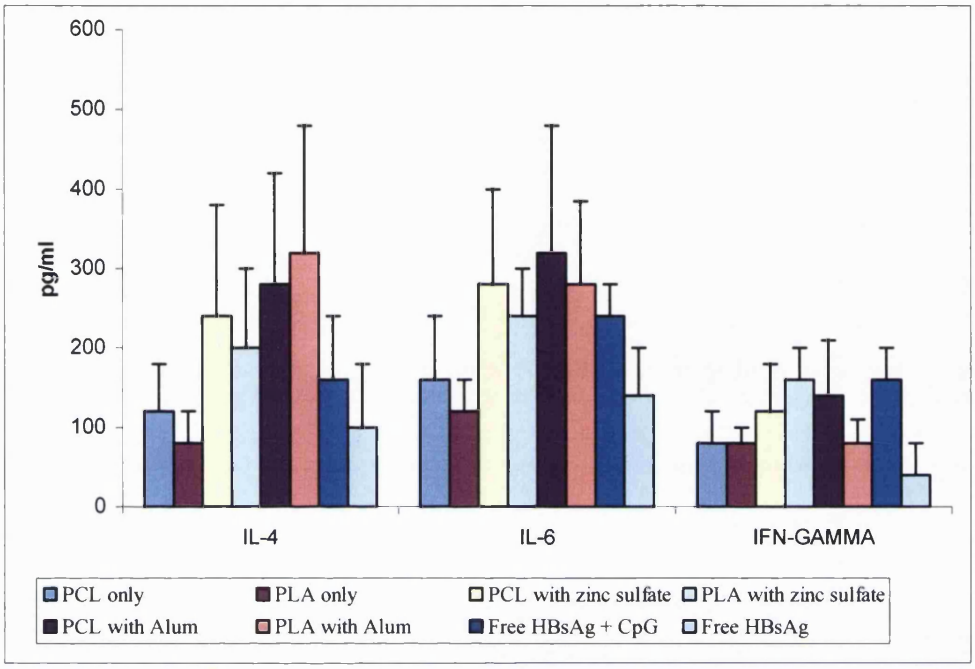

Figure 4.16: Interleukin 4 (IL-4), Interleukin 6 (IL-6) and Interferon gamma (IFN-γ) production by pooled spleens cells after culturing with 2.5 µg/ml concentrations of soluble HBsAg after 90 days. Mice were administered intranasally with PCL or PLA with zinc sulfate or alum nanoparticles or free antigen or free antigen plus CpG. The priming dose was 1 µg HBsAg on day 1 followed by a booster dose of 0.5 µg of HBsAg on day 21 (n = 4, mean ±s.d).

The animals dosed with PCL-zinc sulfate or PLA-zinc sulfate nanoparticles co-encapsulating HBsAg induced higher levels of, INF-gamma and IL-6 compared to naïve mice and those animals dosed with free HBsAg. The production of IFN-gamma may indicate the involvement of the Th1 cascade in the immune response elicited by this group. The same pattern was observed for the animals dosed through the nasal route of administration. For a complete understanding of these analyses the cytokine profiles of each time point may be beneficial. One of the functions of IFN-gamma is the inhibition of viral replication, inducing class switch to the production of IgG2a and enhancing the activity of macrophages. IL-6 is involved in the Th2 cascade and is released from Th2 cells, monocytes and macrophages and promotes the differentiation of proliferating B cells into plasma cells (Goldsby *et al.*, 2002).

4.3.2.2. Immune response following subcutaneous administration of HBsAg loaded PLA microparticles containing alum and chitosan

Microencapsulated antigens are capable of eliciting both humoral and cellular immune responses in naïve animals, probably as a result of targeting the antigen to antigen presenting cells upon release from the microparticles (Johansen *et al.* 2000). Inclusion of additional adjuvants to improve immune responses from polymeric microparticles has been reported previously (O'Hagan *et al.* 1993; Diwan *et al.* 2001; Tobio 2002) and aluminium compounds as adjuvants have been used in combination with liposomes (Alving *et al.* 1993) and biodegradable polymers (Diwan *et al.* 1998). The microencapsulation of alum-adsorbed antigens has also been looked at briefly in order to improve the immunity and stabilise the entrapped antigen (Esparza and Kissel 1992; Johansen *et al.* 2000; Diwan *et al.*, 1998; Johansen *et al.* 2000). In addition, alum enhances the hydrophobicity of polymeric microparticles, thereby enhancing uptake by antigen presenting cells, and improving the magnitude of the immune response (Raghuvanshi *et al*, 2002). The enhanced immune responses might be generated as a result of a synergistic effect due to the presence of alum and the PLA microparticles. In a study conducted by Feng *et al* (2006) it was shown that a single subcutaneous injection of HBsAg-loaded microparticles with a dose of 7.5 μg of HBsAg *per* mouse gave comparable serum antibody titers to those elicited by three injections (2.5 μg of HBsAg *per* dose) of the conventional aluminium adjuvant formulated HBsAg vaccine.

The modification of surface characteristics such as the surface charge and mucoadhesive properties, in the form of chemical modifications of the polymer or in the form of coatings, may profoundly affect the efficiency with which the microparticles target the delivery of bioactive agents to mucosally associated lymphoid tissues and to antigen presenting cells. Many coatings have been used and examples include chemicals, polymers, antibodies, bioadhesives, proteins, peptides, carbohydrates and lectins (Baras *et al.* 1999; Delgado *et al.* 1999; Kaiserlian and Etchart 1999). It is well known that chitosan has mucoadhesive properties and has been used in the past as an adjuvant. Chitosan microparticles have been reported to have immune stimulating activity such as: i) increasing accumulation and activation of macrophages and polymorphonuclear cells, ii) suppressing tumour growth, iii) promoting resistance to infections by microorganisms, iv) inducing cytokines, v) augmenting antibody responses and vi) enhancing delayed type hypersensitivity and cytotoxic T lymphocyte

responses (Ishihara *et al.* 1993; Tokumitsu *et al.* 1999). Jaganathan and Vyas (2006) have tested surface-modified PLGA microparticles using chitosan chloride *in vivo* and found that these positively charged particles produced humoral (both systemic and mucosal) and cellular immune responses.

In this study, high molecular weight of PLA (147 K) was used for the preparation of the microparticles so as to assess the long term immune responses of these formulations in the mice. The degradation time for PLA 147 K is 12 to 16 months, due to its high hydrophobicity and molecular weight. Following subcutaneous delivery of HBsAg encapsulated microparticles (F1, F2 and F3 formulations) and solutions of alum-HBsAg, chitosan-HBsAg and free HBsAg which are equivalent to 1 μg of HBsAg, primary and secondary immune responses were assessed in terms of serum anti-HBsAg IgG levels. Incorporation of chitosan or alum onto/into microparticles enhanced the serum antibody response to the subcutaneously administered HBsAg. HBsAg specific IgG antibody titres obtained were significantly higher ($P < 0.034$) for all formulations except F1 than free HBsAg, on the 15th day after immunisation, following one priming dose (Figure 4.17). The high primary immune responses of the control groups of alum-HBsAg solution and chitosan-HBsAg solution as compared to the microsphere formulations can be attributed to the fact that the microparticles are formulated as controlled release devices, and therefore the release of the antigen is in a gradual and controlled manner and therefore the immune responses too would follow this pattern, whilst in the case of the formulations containing free antigen with alum and chitosan, there would be an enhanced initial response, which may be less likely to be maintained over a longer time scale.

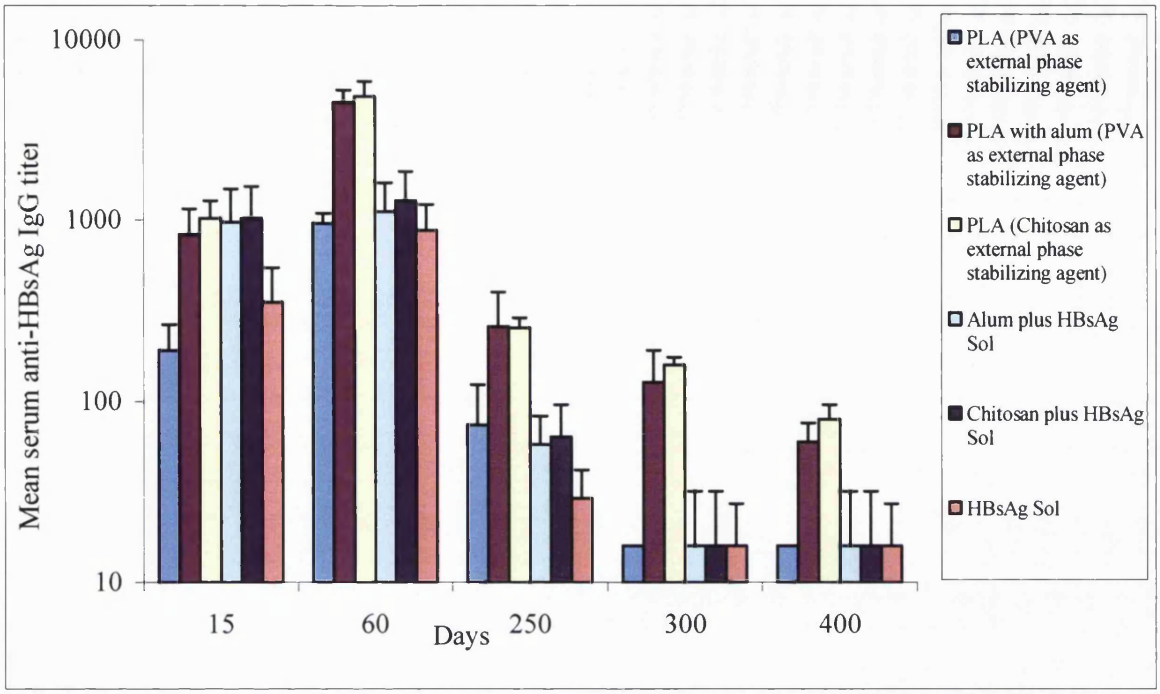

Figure 4.17: Mean serum antibody response to HBsAg loaded PLA nanoparticles. Mice were administered subcutaneously with PLA nanoparticles prepared by prepared by w/o/w solvent evaporation method with or without alum co encapsulation and 2.5% m/v PVA or 0.75% chitosan as external phase stabilizing agent or free antigen plus alum or free antigen plus chitosan or free antigen. The priming dose was 1 μg HBsAg on day 1 followed by a booster dose of 0.5 μg of HBsAg on day 21 (n = 4, mean ±s.d).

After 60 and 250 days, F2 and F3 formulations displayed enhanced immune responses and antibody titres approximately 5-6 times higher than PLA microparticles (F1), alum-HBsAg sol, chitosan-HBsAg sol and free HBsAg sol (Figure 6.3). After 300 days, F2 and F3 formulations gave 8 and 10 times higher antibody titres that other formulations and negative and positive control groups respectively (Figure 4.17). The mode of enhancement of immune response to alum adsorbed HBsAg loaded PLA microparticles or PLA-chitosan microparticles may be due to the macrophage activation properties of chitosan or the combination of the depot nature of alum and activation of immune system by chitosan. Thus, the co-encapsulation of alum in microparticles (F2) or the coating of microparticles with chitosan (F3) led to a significant enhancement in the magnitude of the immune response of microparticles, as compared to PLA-HBsAg microparticles.

For the analysis of HBsAg specific cytokines, splenocytes were stimulated with 5 μg/ml of HBsAg. Stimulation with HBsAg successfully elicited IL-4, IL-6 and IFN-γ secretion. Cytokine production was seen to be dependent on the type of adjuvant which had been incorporated into/onto particles. These cytokines play potentially important

roles in both innate and adaptive immune responses. IL-4 and IL-6 are potentially important cytokines in the Th2 cascade, promoting the proliferation and differentiation of B cells into antibody-producing plasma cells for the production of antibodies. IL-4 promotes IgG_1 and IgE production and decreases IgG_2 and IgG_3 production. IL-6 stimulates immunoglobulin secretion and it can be produced within a few hours of infection by macrophages after ingestion of bacteria (Fraser *et al.* 1998; Singh *et al.* 2006). IFN-γ is secreted by effector Th1 cells after appropriate peptidergic stimuli in conjunction with MHC class II (Mosmann and Coffman 1989) and leads via macrophage activation to extensive inflammatory processes that also enable the killing of intracellular pathogens (Bendelac *et al.* 1997; Singh *et al.* 2006).

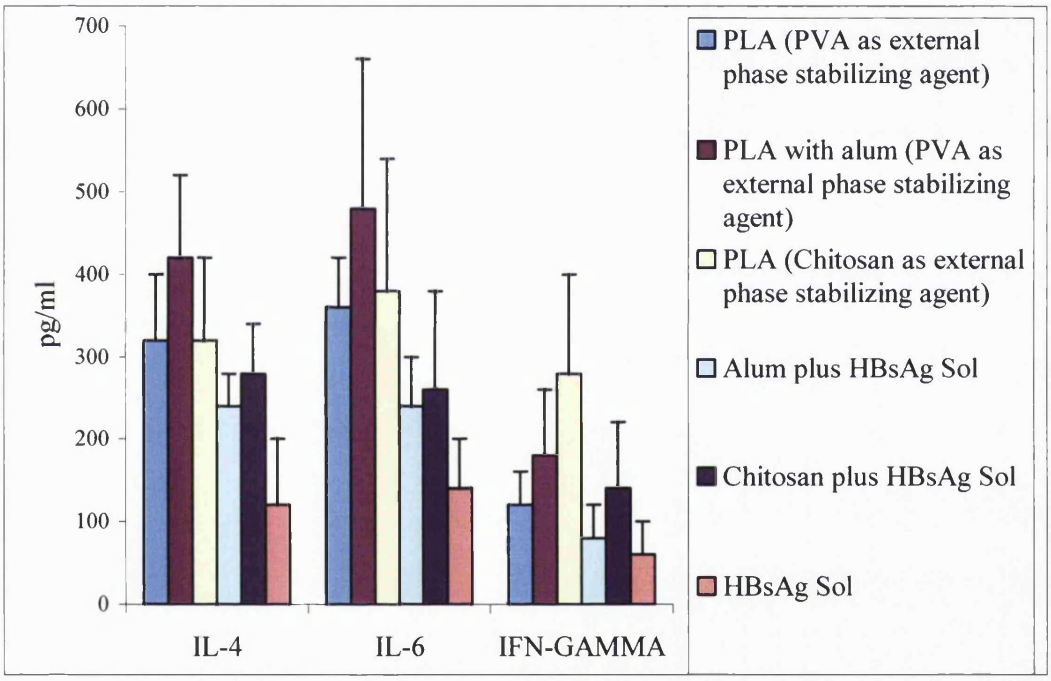

Figure 4.18: Interleukin 4 (IL-4), Interleukin 6 (IL-6) and Interferon gamma (IFN-γ) production by pooled spleens cells after culturing with 5 μg/ml concentrations of soluble HBsAg after 400 days. Mice were administered subcutaneously with PLA nanoparticles prepared by prepared by w/o/w solvent evaporation method with or without alum co encapsulation and 2.5% m/v PVA or 0.75% chitosan as external phase stabilizing agent or free antigen plus alum or free antigen plus chitosan or free antigen. The priming dose was 1 μg HBsAg on day 1 followed by a booster dose of 0.5 μg of HBsAg on day 21 (n = 4, mean ±s.d).

This study demonstrates that IL-4 and IL-6 responses are higher than IFN-γ responses, indicating a Th2 biased response, which can be attributed mainly to the route of administration. Microsphere (F1, F2 and F3) primed spleen cells induced the highest elevated IL-4 and IL-6 productions significantly higher than the solutions of chitosan-HBsAg, alum-HBsAg and free HBsAg primed spleen cells (Figure 4.18). Incorporation

of alum into HBsAg microparticles (F2) has also increased IL-4 and IL-6 production by spleen cells.

The pattern of IFN-γ production by cultured splenocytes was markedly different from those of IL-4 and IL-6, and is shown in Figure 4.18. The use of chitosan either with microparticles or with free antigen has shown a significant increase in the IFN-γ production by spleen cells when compared with the other formulations.

4.4. Conclusions

In this study OVA or HBsAg plus zinc sulfate or zinc oxide or alum co-encapsulated PCL (80 K) or PLA (80 or 147 K) micro/nanoparticles were prepared by the w/o/w double emulsification solvent evaporation method. The effect of co-encapsulation of zinc sulfate or zinc oxide or alum with OVA or HBsAg on the physicochemical characteristics of these particles was investigated. The particles were characterized with respect to size, surface charge and encapsulation efficiency. The antigen loaded particles were tested *in vivo* for their immunogenicity.

The presence of zinc sulfate or zinc oxide or alum increased the particle size of the particles, such that the higher the concentration of these adjuvants, the higher the particle size. The presence of zinc sulfate increased the encapsulation efficiency of OVA or HBsAg in the nanoparticles. This maybe a result of an enhanced stability of the primary emulsion created by the preparation of zinc sulfate in the aqueous phase in the preparation of zinc-PCL or zinc-PLA particles. Studies have shown that the stability of the primary emulsion dictates the final characteristics of the particles produced. The presence of zinc ions in the primary emulsion may reduce the coalescence of smaller emulsion droplets and consequently allow the production of nanoparticles of a reduced size compared with aqueous particles. This may occur by the complexation of zinc with PVA, which would provide greater stearic hindrance when coated around droplets and so enhance stability by reducing coalescence. However, incorporation of zinc oxide decreased the encapsulation efficiency of HBsAg.

For all the formulations, SDS-PAGE confirmed that there was no deterioration of the antigens due to the process parameters and storage. The MTT assay confirmed that the

PCL nanoparticles containing zinc sulfate and alum are not cytotoxic. There was no significant difference in the toxicity of different formulations at any of the concentrations used. The average cell viabilities were between 100 to 120% of control (PEI) at the concentrations studied.

FITC-BSA loaded PCL nanoparticles were prepared using w/o/w double emulsification solvent evaporation method to assess the particle uptake in the macrophage (J774A.1) cells. This *in vitro* experiment showed that zinc sulfate or zinc oxide had no untoward effect on the particle uptake.

In case of the PLA (147 K) microparticles, the inclusion of alum or chitosan significantly enhanced the encapsulation efficiency of microparticles. In realisation with the objectives of the study, *in vivo* investigations were carried out to assess the effect of alum or chitosan inclusion in the microparticles upon immune responses. In analysis of both priming and boosting doses, the highest serum IgG levels are seen in the case of the PLA-HBsAg microparticles, HBsAg-alum-microparticles and HBsAg-chitosan-microsphere formulations when compared with the free antigen, free antigen plus alum and free antigen plus chitosan.

All polymeric nanoparticles induced an HBsAg specific serum IgG antibody response significantly higher than free antigen, both after intramuscular and intranasal administration. The particles containing zinc sulfate gave higher antibody responses when compared with other formulations or free antigen or alum plus free antigen when administered intramuscularly. Intranasally, the particles containing zinc sulfate gave significantly higher antibody responses compared with all the other formulation, free antigen and free antigen plus CpG. The analysis of the cytokine levels showed greater shift towards a Th1 response by the group of animals dosed with the PCL-zinc sulfate or PLA-zinc sulfate particles, although the response was mostly biased towards Th2 type.

Subcutaneous delivery of antigen-loaded microparticles were found to elicit potent primary immune responses in terms of serum IgG, and alum containing HBsAg microparticles displayed higher serum anti-HBsAg IgG titres consistently, demonstrating that admixed alum has a strong impact in enhancing the magnitude of immune responses to PLA microparticles, possibly due to a synergistic immunostimulatory adjuvant effect between the PLA microparticles and alum adjuvant.

However, the surface-modified microparticles with chitosan have also given slightly higher potency and long lasting immune responses when compared with the alum co-encapsulated microparticles. In addition, it was shown that at least to some extent, these different formulations may have the capacity to alter qualitative aspects of the immune response in terms of antigen specific cytokine responses, and hence alter the Th1/Th2 characteristics of the immune response. The mode of enhancement of immune response to alum adsorbed HBsAg loaded PLA microparticles or PLA-chitosan microparticles may be due to the macrophage activation properties of chitosan or the combination of the depot nature of alum and activation of immune system by chitosan.

This study has shown the potential of zinc sulfate as co-adjuvants with PCL or PLA nanoparticles to induce serum IgG antibody responses higher than PCL or PLA only nanoparticles or free antigen or free antigen plus alum/CpG. Therefore, this delivery systems show promise as an alternative strategy to induce robust immune responses.

4.5 Key observations

- Formulations containing zinc sulfate and chitosan were found to be the best among all the tested formulations with respect to improving the adjuvancy of the antigens.

- Co-encapsulation of alum in the polymeric carriers has improved the adjuvancy of the system with sustained release of the antigen.

- The MTT assay confirmed that the particles containing zinc sulfate are not cytotoxic therefore these formulations can be assumed to be safe.

Chapter 5

Bile salts and melittin as adjuvants

5. Co-encapsulation of bile salts and melittin with HBsAg in PLA nanoparticles

5.1. Bile salts and melittin as adjuvants

In the previous chapters (chapter 3 and 4) known (alum) and untried (tocopherol derivatives and zinc) adjuvants were co-encapsulated along with the antigens in the polymeric nano/microparticles. The results have shown that co-encapsulating the adjuvants with the antigen can improve the adjuvancy of these carriers. Penetration enhancers have been used in the past for improving the adjuvancy of the vaccines. Bile salts and melittin (a short peptide with 26 amino acids and a major component of the honey bee venom) are penetration enhancers which can also enhance the adjuvancy of the antigens. Bile salts and melittin has been used in our laboratory as an adjuvant with antigens in the past (Alpar *et al.*, 2001; Bramwell *et al.*, 2003). However, in the past studies bile salts and melittin were only used as solution along with the antigens; therefore it will be interesting to investigate the adjuvant effects of bile salts and melittin if co-encapsulated in the polymeric carriers. We anticipate that the duration and intensity of the adjuvant effects of bile salts and melittin can be improved if they are co-encapsulated in the micro/nanoparticulate systems. The focus of this study is to use melittin, bile salts like cholic acid and lithocholic acid in the polymeric carriers to increase the adjuvanticity of hepatitis B surface antigen (HBsAg). In the previous chapters, high molecular weight polymers of PLA and PCL were used to determine the long term immune responses in the mice. Due to time constraints, it was decided to ascertain the short term (45 days) immunogenicity of the polymeric carriers, therefore low molecular weight PLA (2 K) was used in this study.

5.1.1. Penetration enhancers

Nasal delivery of vaccines is considered to be one of the viable options for the delivery of vaccines. As discussed in chapter 1, the presentation of a suitable antigen with an appropriate adjuvant to the nasal-associated lymphoid tissue (NALT) has the potential to induce humoral and cellular immune responses. The ease of administration and non-invasive nature makes nasal vaccines suitable for mass immunization programmes. The nasal epithelium exhibits relatively high permeability principally because only two cell layers separate the nasal lumen from the dense blood vessel network in the lamina propria. Despite this, the nasal mucosa poses a permeation barrier to high molecular

weight molecules due to the presence of the tight junctions, however, it has been reported that these tight junctions can be reversibly and safely opened (Johnson and Quay, 2005). The mucociliary clearance is probably the most significant physiological factor to adversely effect macromolecule absorption in the nose (Lindhardt *et al.*, 2002). In order to achieve high nasal uptake following intranasal administration, it is desirable to have increased nasal residence of antigens. The molecular weight and polarity of antigens limit their uptake and absorption across mucosal membranes however, if penetration enhancers are used along with the antigens, uptake and absorption can be improved (Alpar *et al.*, 2001). Absorption or penetration or permeation enhancers are widely used in delivery of active compounds to be administered through mucosal sites as they increase the membrane/mucosal permeation/absorption rate of a co-administered drug. Some of the commonly used absorption or permeation or penetration enhancers are listed in the Table 5.1.

Table 5.1: Commonly used and researched mucosal permeation enhancers (Adapted from Natsume *et al.*, 1999; Senel and Hincal, 2001; Davis and Illum, 2003; Remon, 2006)

Type	Examples	Proposed mechanisms of action
Surfactants	sodium lauryl sulphate polysorbate 20 and 80 glyceryl monolaurate saponins (e.g., *Quillaja* saponins)	membrane interaction, extraction of membrane proteins and lipids, solubilisation of peptides
Bile salts	sodium deoxycholate sodium glycocholate sodium fusidate sodium taurodihydrofusidate	denaturation of proteins, decrease of mucus viscosity, decrease of peptidase activity, solubilisation of peptides, formation of reversed micelles
Fatty acids and derivatives	oleic acid caprylic acid lauric acid palmitoylcarnitine	phospholipid acyl-chain disruption
Chelators	sodium EDTA citric acid salicylates	Ca^{2+} complexation (influencing tight junctions)
Inclusion complexes	cyclodextrins and derivatives	increased peptide stability, increased, solubility, enzyme inhibition
Other agents	chitosan	bioadhesive effect (increasing drug retention)

Absorption or Penetration enhancers can influence the absorption in different ways, for example (a) by acting on the mucous layer, i.e. by changing mucus rheology by forming a viscoelastic layer of varying thickness that affecting the absorption; (b) by increasing the fluidity of lipid bilayer membrane, i.e. by disturbing the intracellular lipid packing by interaction with either lipid or protein components; (c) by affecting the components involved in the formation of intercellular tight junctions, some enhancers act on desmosomes, a major component at the tight junctions, thereby increasing absorption;

(d) by overcoming the enzymatic barrier, i.e. inhibiting the various peptidases and proteases, thereby overcoming the enzymatic barrier; (e) by increasing the thermodynamic activity of drugs, some enhancers increase the solubility of a drug thereby altering the partition coefficient (ion-pair formation between the enhancer and the drug). This leads to increased thermodynamic activity which facilitates better absorption (Verhoef *et al.*, 1990; van der Lubben at al., 2001; Sudhakar *et al.*, 2006).

There are two general classes of absorption enhancers: surfactants and calcium chelators. Surfactants like bile acids and salts (derivatives of fatty acids) act by increasing the solubility of hydrophobic macromolecules in the aqueous boundary layer or by increasing the fluidity of the apical (as well as the basolateral) membrane, eventually increasing the transcellular permeability. Calcium chelators reduce the extracellular calcium concentration resulting in the disruption of cell-cell contacts, thereby increasing the paracellular permeability (Artursson *et al.*, 1994; Hochman *et al.*, 1994).

5.1.2. Bile salts

Bile salts are carboxylic acids (C22-C28) with a cyclo-pentenophenanthrene nucleus containing a branched chain of 3-9 carbon atoms ending in a carboxyl group. Their hydrophobic structure is associated with one surface of the steroid nucleus and they form micelles. Primary micelles consist of two to four molecules and secondary micelles are composed of aggregates of the primary micelles (Corrigan and Healy, 2006). The bile salts used in this study were related to cholic acid and lithocholic acid.

Cholic acid ($C_{24}H_{40}O_5$), also known as 3-α,7-α,12-α-Trihydroxy-5-β-cholan-24-oic acid, and lithocholic acid ($C_{24}H_{40}O_3$), also known as 3α-Hydroxy-5β-cholan-24-oic acid, have the molecular weight of 408.57 and 376.57 respectively (Figure 5.1). Studies have shown that cholic acid plays an important role in the absorption of cholesterol and therefore might act as an absorption enhancer (Murphy *et al*, 2005). Bile salts play an important role as physiological surfactants in the intestinal absorption of lipids and lipid soluble vitamins. They have been extensively employed to enhance the absorption of poorly soluble drugs through various epithelia such as nasal, intestinal, rectal, pulmonary, ocular and vaginal (O'Hagan and Illum, 1990).

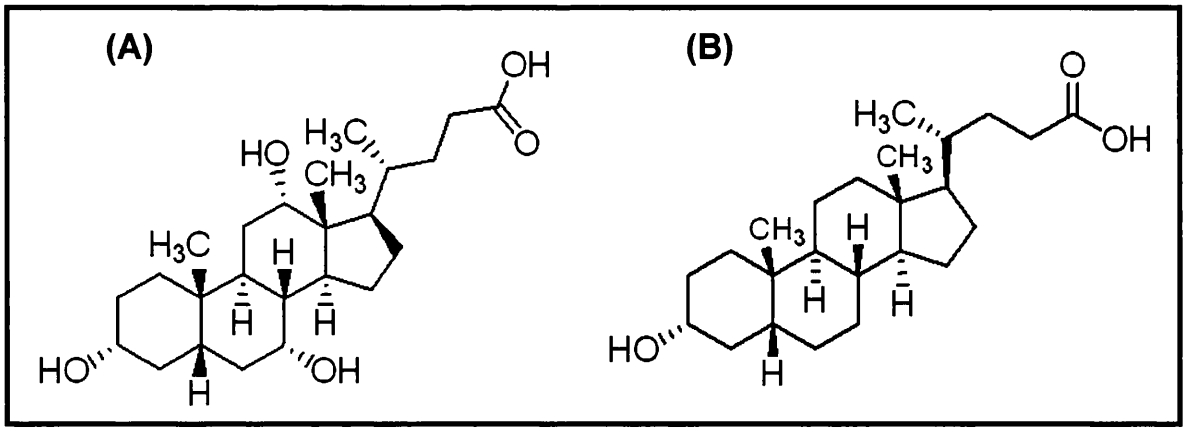

Figure 5.1: Chemical structure of (A) cholic acid; (B) lithocholic acid (from Sigma Aldrich Inc catalogue)

A study by Rafati *et al.* (1997), investigated the use of bile salts as natural emulsifiers to prepare OVA-loaded PLG microparticles by the w/o/w double emulsion technique. It was demonstrated that bile salts can be used as a means of changing the physicochemical characteristics (due to the negative charge of bile salts anions which largely determined the particle surface charge) of antigen-loaded PLG microparticles to improve the adjuvant activity.

5.1.3. Melittin

Melittin is a short peptide with 26 amino acids with amphipatic properties (Figure 5.2). It is a major component of the honey bee venom extracted from *Apis mellifera*. Melittin has no disulfide bridge and the NH_2 terminal part of the molecule is hydrophobic and the C terminal part is hydrophilic and strongly basic. It has been used in the past as an adjuvant in our laboratory wherein it has been shown to increase the immune response of antigens tetanus toxoid (TT) and diphtheria toxoid (DT) solutions when administered intranasally (Bramwell *et al.*, 2003). However, this was used in a solution form rather than co-encapsulating in the nanoparticles. It will be interesting to know if this molecule can further enhance the immune responses when co-encapsulated with the nanoparticles.

Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-
Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-
Lys-Arg-Lys-Arg-Gln-Gln-NH_2

Figure 5.2: Primary amino acid sequence of melittin (from sigma Aldrich Inc catalogue).

5.1.4. Aims and Objectives

The purpose of this study was to investigate the effect of co-encapsulation of the absorption enhancers - cholic acid, lithocholic acid or melittin - along with HBsAg in the low molecular weight PLA (M_W = 2 K) nanoparticles. The low molecular weight polymer has faster degradation time (6-8 weeks) compared to high molecular weight polymers and was therefore used to determine the short term *in vivo* response of these carriers. The formulations were prepared to investigate the following parameters:

- Effect of varying concentrations of cholic acid, lithocholic acid or melittin on the physicochemical properties (size, surface charge, encapsulation efficiency) of HBsAg loaded PLA nanoparticles

- Effect of varying concentrations of cholic acid, lithocholic acid or melittin on the cellular uptake and toxicity of the HBsAg loaded PLA nanoparticles

- The best formulations were chosen for *in vivo* animal studies (in the mouse model) and the antibody responses were measured together with the antigen specific cytokine responses after administering the formulations intranasally.

5.2. Materials and Methods

5.2.1. Materials

Hepatitis B surface antigen (HBsAg)) at a concentration of 1.68 mg/ml was provided as a gift by Shantha Biotechnics, India. Poly (L-lactic acid) (2 K) was obtained from Birmingham polymers Inc, USA. Cholic acid, lithocholic acid, melittin, polyvinyl alcohol (87-89 % hydrolyzed, 13-23 K), L-glutamine and penicillin/streptomycin, QuantiPro® Bicinchoninic acid assay kit was obtained from Sigma Aldrich, UK. RPMI and fetal bovine serum were obtained from Gibco, UK. Horseradish peroxidase conjugated Goat anti-mouse isotype specific immunoglobulin was obtained from Serotec, UK. DuoSet® sandwich ELISA kits were obtained from R&D Systems, UK. Dichloromethane (DCM) and acetic acid were obtained from BDH laboratory suppliers, UK. All other reagents were of analytical grade and were used as received.

5.2.2. Methods

5.2.2.1. Formulation of PLA nanoparticles co-encapsulated with HBsAg and cholic acid, lithocholic acid or melittin

HBsAg loaded PLA nanoparticles were prepared using PVA as a stabilizing agent by w/o/w double emulsification solvent evaporation method as described in chapter 2. The bile salts used are not water soluble and so were dissolved in the organic phase (cholic acid or lithocholic acid were first dissolved in 0.5 ml of acetic acid, and 4.5 ml of DCM was added along with the polymer), whereas, melittin which is water soluble was dissolved in the internal phase stabilizing agent. The theoretical loading of the HBsAg was 1% m/m of the polymer.

The primary emulsion (w/o) was formed by dispersing 10 % m/v PVA along with the protein solution (internal phase) in the organic phase using a high shear homogenizer (Ultra-turrax) at 24,000 rpm for 2 minutes. This w/o emulsion was added drop-wise to 30 ml of 2.5% m/v PVA solution and homogenized for 6 minutes at 10,000 rpm using the Silverson® (Silverson, model L4RT). The formed w/o/w emulsion was left stirring for 4 hours using a magnetic stirrer to evaporate the organic solvent. The emulsion was centrifuged (20,000 rpm, 45 minutes, 4 °C) to obtain a pellet of nanoparticles. The particles were re-suspended in 4 ml water and freeze-dried (Virtis Advantage, England) to obtain a fine powder of nanoparticles. The formulation details are listed in the Table 5.2.

Table 5.2: Formulation details of PLA nanoparticles co-encapsulated with HBsAg and either cholic acid or lithocholic acid or melittin. The external phase stabilizing agent used for all the formulations was 30 ml of 1.25 % m/v PVA

Code	Organic phase (~100mg PLA with DCM)	Amount of melittin in the aqueous internal phase stabilizing agent (1 ml of 10 % m/v PVA with HBsAg)
A 1	-	-
A 2	5 mg cholic acid	-
A 3	10 mg cholic acid	-
A 4	20 mg cholic acid	-
A 5	5 mg lithocholic acid	-
A 6	10 mg lithocholic acid	-
A 7	20 mg lithocholic acid	-
A 8	-	0.1 mg Melittin
A 9	-	0.25 mg Melittin
A 10	-	0.5 mg Melittin

5.2.2.2. Formulation of PLA nanoparticles co-encapsulated with FITC-BSA and either cholic acid or lithocholic acid or melittin

Nanoparticles co-encapsulating FITC-BSA along with either cholic acid or lithocholic acid or melittin were prepared to study the cellular uptake of the particles. The formulation parameters were similar to those described in section (5.2.2.1), except that instead of HBsAg, FITC-BSA was co-encapsulated.

5.2.2.3. Characterization of PLA nanoparticles co-encapsulated with HBsAg and either cholic acid or lithocholic acid or melittin

The nanoparticles were characterized with respect to their size, surface charge and loading as described in 2.2.2.1, 2.2.2.2, 2.2.2.3, 2.2.2.5. The morphology of the nanoparticles was studied using SEM as described in chapter 2.2.2.4. Cellular uptake studies and MTT assay were performed as described in chapter 2. Statistical analysis was preformed using an analysis of variance (ANOVA) general linear model with SPSS software (Version 17, Microsoft) assuming P values of $P \leq 0.05$ as significant.

5.2.2.4. Immunization schedule of PLA nanoparticles co-encapsulated with HBsAg and either cholic acid or lithocholic acid or melittin by nasal route

Six groups (n=4) of female BALB/c mice (25g, 4-6 weeks old) were anaesthetized with an inhaled gaseous mixture of 3% v/v halothane (RMB Animal Health, UK) in oxygen (300 cm^3 min^{-1}) and nitrous oxide (100 cm^3 min^{-1}) for intranasal dosing procedures. The particles were administered intra-nasally to the mice using a micropipette. Each mouse received 50 µl volumes (25 µl *per* nostril), of antigen loaded particle suspension or protein solution alone as described in Table 5.3. Blood samples from the tail vein were taken on days 15, 30, and 45 and the serum specific antibody response was assessed by ELISA as described in chapter 2. The cytokine analysis was carried out as described in chapter 2 for the determination of endogenous cytokines levels, interleukin-2 (IL-4), interleukin-6 (IL-6) and interferon-γ (IFN-γ).

Table 5.3: Immunization schedule of PLA nanoparticles co-encapsulated with HBsAg and either cholic acid or lithocholic acid or melittin by nasal route. The primary dose was on day 0 (each mouse received an equivalent dose of 1 μg of HBsAg). The secondary dose was on day 21 (each mouse received an equivalent dose of 0.5 μg of HBsAg)

Group	Formulation
1	PLA nanoparticles
2	PLA plus cholic acid nanoparticles
3	PLA plus lithocholic acid nanoparticles
4	PLA plus melittin nanoparticles
5	Free HBsAg plus 10 μg Cholera toxin B (CTB)
6	Free HBsAg

5.3. Results and discussion

5.3.1. PLA nanoparticles co-encapsulated with HBsAg and either cholic acid or lithocholic acid or melittin

The PLA nanoparticles were prepared by the w/o/w double emulsion solvent evaporation method and the particles were characterized with respect to the size, surface charge and encapsulation efficiency (Table 5.4).

Table 5.4: Characterisation of size, loading and zeta potential for PLA nanoparticles co-encapsulated with HBsAg and either cholic acid or lithocholic acid or melittin. (n=1, analysis was done 3 times on same formulation, mean ±s.d).

No.	Enhancer in the formulation	Yield % ±s.d	Z average particle size (nm) ±s.d	Poly-dispersity ±s.d	Zeta potential, mV ±s.d	Actual protein content (μg/mg) ±s.d	Encapsulation efficiency % ±s.d
A1	-	63.3 ±1.32	263.3 ±3	0.245 ±0.016	-5.76 ±2.2	5.40 ±0.21	34.2 ±1.3
A2	5 mg cholic acid	67.3 ±2.43	276.6 ±4	0.265 ±0.013	-4.25 ±1.4	5.16 ±0.07	34.7 ±0.5
A3	10 mg cholic acid	64.4±2.45	316.2 ±6	0.245 ±0.021	-6.76 ±1.5	5.65 ±0.20	36.4 ±1.3
A4	20 mg cholic acid	67.8 ±2.45	451.6 ±6	0.345 ±0.023	-7.37 ±2.3	5.71 ±0.10	38.7 ±0.7
A5	5 mg lithocholic acid	63.8 ±1.34	377.7 ±3	0.256 ±0.016	-4.37 ±1.2	5.24 ±0.24	33.4 ±1.5
A6	10 mg lithocholic acid	68.7 ±2.43	439.4 ±4	0.287 ±0.023	-8.26 ±1.7	4.72 ±0.49	32.4 ±3.4
A7	20 mg lithocholic acid	67.8 ±1.43	663.2 ±7	0.378 ±0.034	-7.37 ±2.7	4.94 ±0.34	33.5 ±2.3
A8	0.1 mg melittin	62.3 ±1.32	267.4 ±2	0.278 ±0.023	-6.34 ±1.7	6.00 ±0.26	37.4 ±1.6
A9	0.25 mg melittin	66.5 ±2.34	257.6 ±3	0.226 ±0.012	-7.45 ±1.3	5.79 ±0.29	38.5 ±1.9
A10	0.5mg melittin	67.8 ±3.39	269.2 ±4	0.375 ±0.023	-6.65 ±2.7	5.83 ±0.35	39.5 ±2.4

The encapsulation efficiency of the particles was in the range 34 to 39%. There was no significant difference in the encapsulation efficiency of the formulations irrespective of the bile salts or melittin. The actual antigen content in the particles ranged from 4.72 – 6 μg/mg of the particles. The Z average diameter of the particles with cholic acid was found to be around 276 to 451 nm. The particle size increased with increasing concentration of cholic acid. A similar trend was observed for the particles with lithocholic acid as the particle size ranged from 451 to 663 nm. The bile salts were dissolved in the organic phase during the formulation of these particles therefore increasing the viscosity of the organic phase. Increasing the viscosity of the matrix dispersion tends to increase the final particle size of the particles because higher shear forces are necessary for droplet disruption (Jeyanthi *et al.*, 1997; Viswanathan *et al*, 1999; Yang *et al.*, 2000). This increase in the particle size of the formulations with a higher concentration of bile salts is not unexpected. On the other hand, the particles containing melittin had no significant change in the size (257 to 269 nm) as the melittin was used in the primary aqueous phase rather than in the organic phase.

The zeta potential values of the nanoparticles were low for all the preparations. Surface charge values were between -4.25 and -8.2 mV were measured, particles with bile salts giving the highest values. It has been reported that surface charge plays an important role in the antigen presenting cells (Tabata *et al.*, 1988). Some studies have shown that the surface charge has a great influence for nasal uptake of particles due to electrostatic interaction, with positive formulations such as chitosan binding efficiently to the negatively charged nasal mucosal surface (Illum *et al.*, 1994). However negatively charged PLGA formulations have also been shown to be taken up successfully by mucosal surfaces (Alpar *et al.*, 1994). This indicates that the charge of the particles has only a minor influence on the uptake of the particles following mucosal administration. The morphology of the polymeric nanoparticles was observed by SEM. The SEMs (Figure 5.3) show the formation of spherical nanoparticles; however the increase in the bile salt concentration in the formulations affected the polydispersity of the particles.

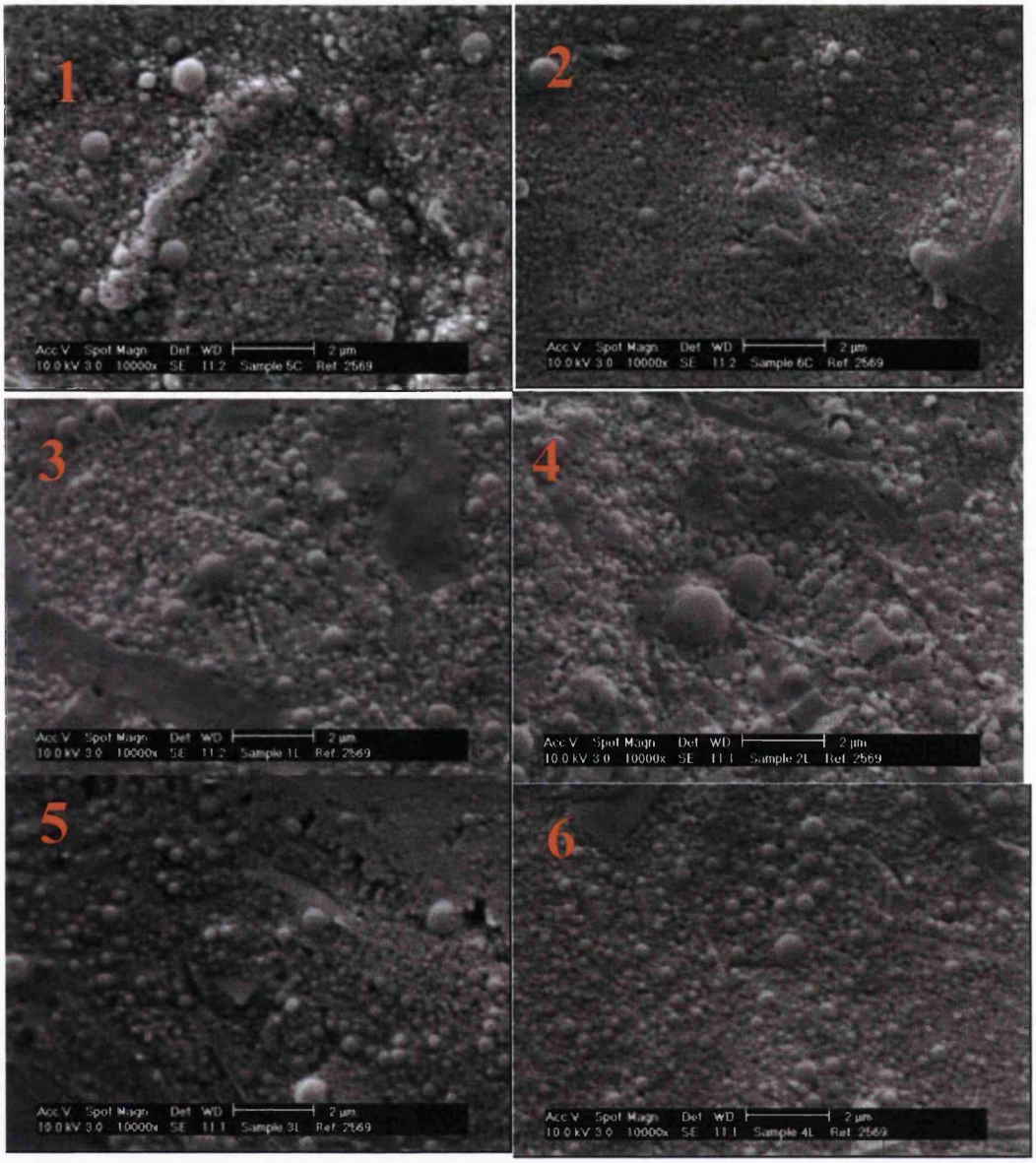

Figure 5.3: SEMs of HBsAg loaded PLA nanoparticles prepared by a w/o/w solvent evaporation method; (1) particles with 5 mg cholic acid; (2) particles with 20 mg cholic acid; (3) particles with 5 mg lithocholic acid; (4) particles with 2 mg lithocholic acid; (5) particles with 0.1 mg melittin; (6) particles with 0.5 mg melittin

5.3.2. MTT assay of the formulations to assess cytotoxicity

Based on the satisfactory encapsulation efficiency it was decided that PLA only nanoparticles, PLA plus 20 % m/m cholic acid nanoparticles, PLA plus 20 % m/m lithocholic acid nanoparticles and PLA plus 0.5 % m/m melittin nanoparticles will be used in the *in vivo* animal studies. As many penetration enhancers have surface active properties, there is a risk that in addition to their desired effects they may also mediate irrevocable damage to membranes or intracellular organelles, following mucosal application. However, for the purposes of nasal vaccination, penetration enhancers would be applied to the nasal membranes very infrequently. Nevertheless, these

formulations have to be tested for their toxicity; therefore MTT assay was carried out for these formulations. The MTT assay is a colorimetric assay system which measures the reduction of a tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into an insoluble formazan product by the mitochondria of viable cells. *In vitro* toxicity of nanoparticles was evaluated by MTT assay in CHO-K1 cells using increasing concentration of nanoparticles (Figure 5.4).
Poly ethyleneimine (PEI) was used as the positive control. PEI is positively charged polyplex which interacts with the blood components activating the complement system and thereby is highly toxic at the cellular levels (Merdan *et al.*, 2002). Due to this known toxic properties of PEI, it was used as the positive control.

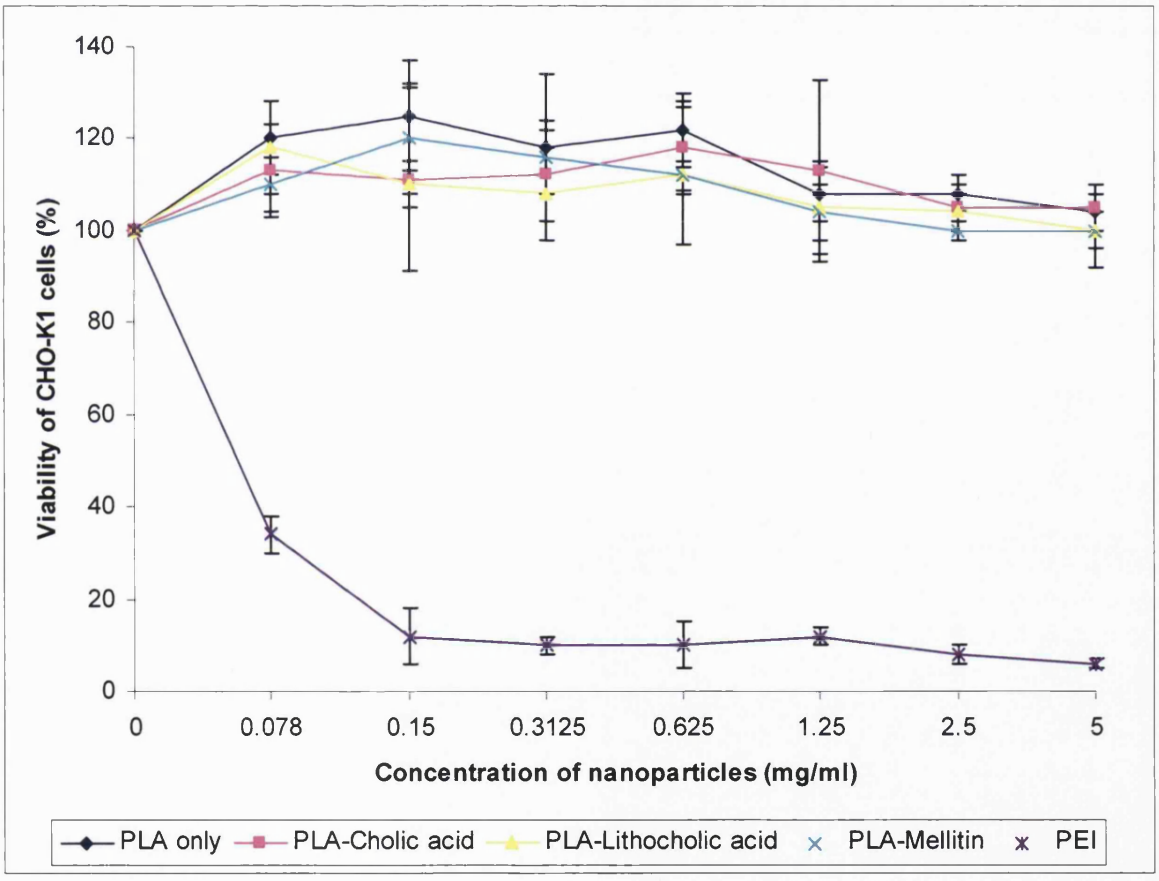

Figure 5.4: Cytotoxicity of PLA nanoparticles co-encapsulating HBsAg and cholic acid or lithocholic acid or melittin analyzed by MTT assay in CHO-K1 cells. PEI was used as a positive control. Cells incubated in cell culture medium were used as a control to assay 100% viability. Viability of cells exposed to the formulations was expressed as percentage of the viability seen in control untreated cells (n=3, mean ±s.d).

The nanoparticle formulations were found to be non-toxic at the majority of concentrations studied. There was no significant difference ($p > 0.05$) in the toxicity of different formulations at any of the concentrations used. There was a slight reduction in

cell viabilities at higher concentrations of 5 mg/ml of the particles. The average cell viabilities were between 90 to 125% of control (PEI) at the concentrations studied.

The concentration of the bile salts and melittin in the formulations is very low i.e. 200 μg/mg of bile salts and 0.5 mg/mg of melittin in the particles. Moreover, as these enhancers were co-encapsulated in the particles, there is a possibility that not all of the bile salts and melittin was encapsulated in the formulation. However, the exact encapsulated quantity of these molecules was not investigated. The MTT assay has shown that the concentrations of these bile salts and melittin used in the formulations are non-toxic. So these formulations can be further evaluated for their adjuvanticity in the animal models.

Some studies have reported severe damage to the mucosa by bile salts. Deoxycholate had the most ciliotoxic effect, whereas taurocholate had the least ciliotoxic effect (Ennis *et al.*, 1990). As most of the penetration enhancers promote drug absorption by perturbating membrane integrity, their major side effects are irritation of mucosal tissue, extent of damage on mucus, cilia and in the mucosal cells and enhancement of the transmucosal passage of noxious materials and bystander antigens (Alpar *et al.*, 2001; Senel and Hincal, 2001). However, the use at sub-toxic levels should preclude this as a limiting factor in the further evaluation of bile salts as mucosal adjuvants. In the case of melittin, when used as a solution, it has been shown to be toxic at 20 μg/ml concentrations but found to be non-toxic at 2 μg/ml concentrations (Bramwell *et al.*, 2003). The concentration used in the formulations is very low and moreover the dilution effect of nasal secretions, as well as short period of time this molecule will be present at the mucosal surface, has to be taken into consideration, therefore this can be safely used for the *in vivo* study.

5.3.3. Studies investigating the interaction/uptake of different nanoparticle preparations by Caco-2 cells

5.3.3.1. Characteristics of nanoparticles employed in the cell uptake studies

PLA nanoparticles co-encapsulated with FITC-BSA and either cholic acid or lithocholic acid or melittin were prepared for cellular uptake studies in the Caco-2 cell lines. The particles were characterized with respect to size and surface charge (Table 5.5).

Table 5.5: Characterization of size and zeta potential for PLA nanoparticles co-encapsulated with FITC-BSA and either cholic acid or lithocholic acid or melittin (n=1, analysis was done 3 times on same formulation, mean ±s.d).

Code	Absorption enhancer in the formulation	Z average particle size (nm) ±s.d	Poly-dispersity ±s.d	Zeta potential, mV ±s.d
B1	-	276.4 ±3	0.232 ±0.013	-6.45 ±2.7
B2	5 mg cholic acid	286.5 ±4	0.245 ±0.034	-8.87 ±1.5
B3	10 mg cholic acid	304.2 ±6	0.283 ±0.032	-7.86 ±1.4
B4	20 mg cholic acid	381.7 ±6	0.245 ±0.024	-8.34 ±2.4
B5	5 mg lithocholic acid	287.4 ±3	0.345 ±0.034	-6.67 ±2.5
B6	10 mg lithocholic acid	375.7 ±4	0.432 ±0.056	-6.78 ±1.6
B7	20 mg lithocholic acid	387.8 ±7	0.243 ±0.056	-5.87 ±2.9
B8	0.1 mg melittin	265.4 ±2	0.258 ±0.076	-7.25 ±2.3
B9	0.25 mg melittin	287.6±3	0.245 ±0.012	-8.56 ±2.4
B10	0.5 mg melittin	283.6 ±4	0.265 ±0.012	-7.98 ±2.3

The Z average diameter of the particles with cholic acid was found to be around 276 to 305 nm. The particle size increased with the increasing concentration of cholic acid. A similar trend was observed for the particles with lithocholic acid as the particle size ranged from 287 to 387 nm. The particles containing melittin were similar in size (265 to 283 nm). The zeta potential of all the formulations was negative with surface charge values between -5 to -8 mV.

5.3.3.2. Cellular uptake of the PLA nanoparticles co-encapsulated with FITC-BSA and either cholic acid or lithocholic acid or melittin

The particle uptake studies were carried out using the FITC-BSA loaded PLA nanoparticles with varying quantity of the cholic acid or lithocholic acid or melittin by Caco-2 cells to assess the binding and uptake of nanoparticles to the intestinal epithelium. Caco-2 cells are an epithelial human colon adenocarcinoma cell line which has been used widely to predict intestinal absorption of polymeric particles. The Caco-2 cell lines are used as an *in vitro* test method for the determination of oral particulate uptake. Therefore use of these intestinal epithelial cells is unlikely to serve as a complete substitute for the more complex nasal epithelium. It is preferable to use the nasal epithelial cell line; however this was used as a model cell line for the present study to give an understanding of the uptake of the particles through the mucosal sites. In a study conducted by Singh *et al* (2006), Caco-2 cells were used to study the uptake of the particles although the formulations were delivered nasally (Figure 5.5).

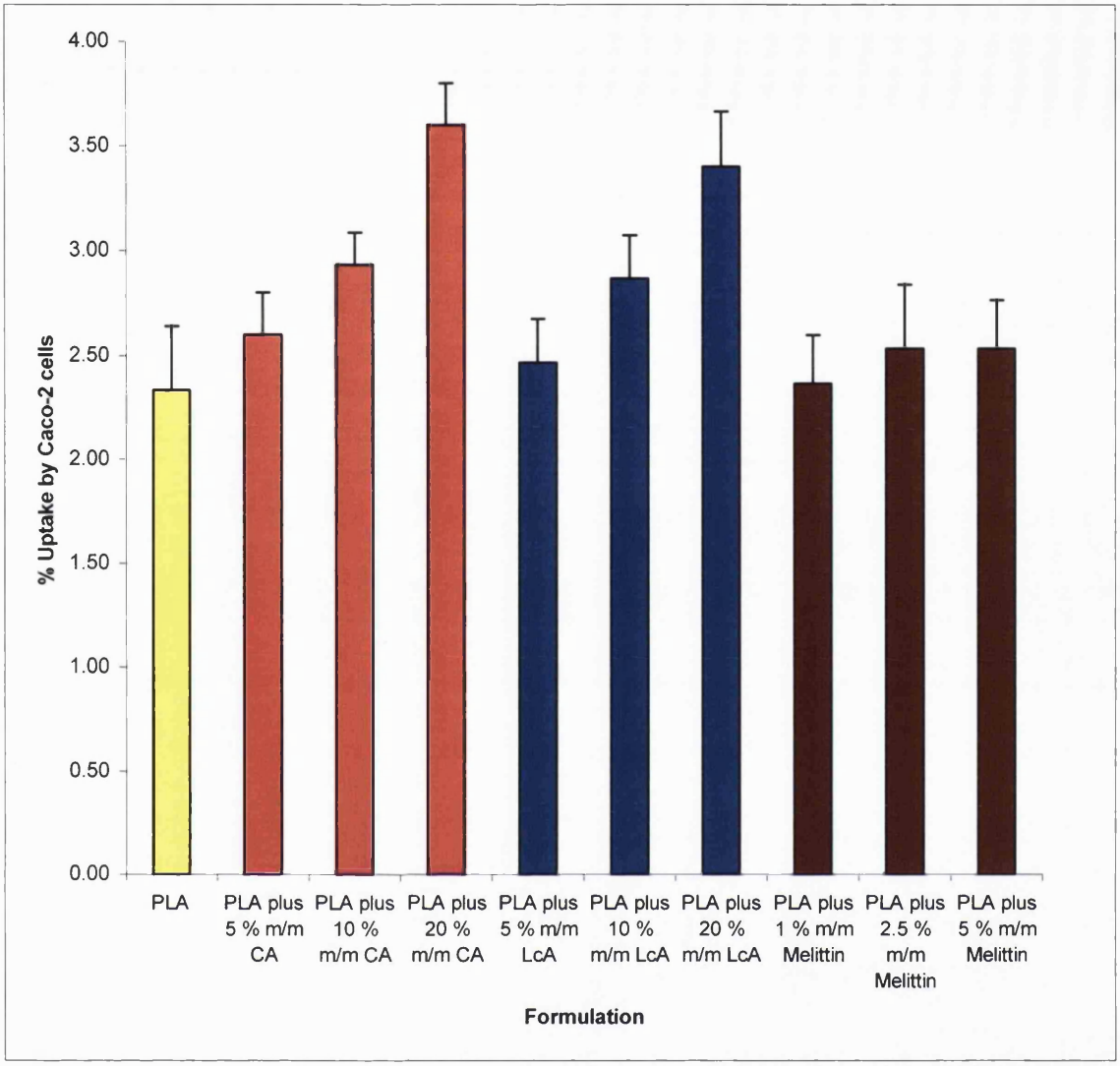

Figure 5.5: The percentage cellular uptake of the loaded PLA nanoparticles co-encapsulated with FITC-BSA and either cholic acid or lithocholic acid or melittin by Caco-2 cells (n=3, mean ±s.d).

It was determined whether the nanoparticles interact with the cells, and whether they were internalized or strongly surface bound. The results are expressed as the percentage of fluorescence associated to the cells and determined using spectrophotometry at 535 nm after subtracting uptake of unbound (free) FITC–BSA. The influence of different release rate of FITC-BSA from the nanoparticles into the media was not deemed to influence the uptake determinations, since all free FITC-BSA and non-adsorbed nanoparticles containing FITC-BSA were removed by washing before uptake determination.

The uptake of the particles is affected by particle size, surface charge, particle hydrophobicity and also by the adjuvants associated onto the particles such as lectin and B-12, lipids (Thomas *et al.*, 1996; Russel-Jones *et al.,* 1999; Mladenovska *et al.*, 2003; Alpar *et al.,* 2005). The particle size and the surface charge are similar for all the

formulations in this study and the only difference being the presence of bile salts or melittin. The uptake of the FITC–BSA loaded nanoparticles by the Caco-2 cells was between about 2.3 and 3.6% of the total fluorescence. The uptake of nanoparticles co-encapsulated with cholic acid and lithocholic acid was higher than for PLA only particles. The presence of melittin did not affect the cellular uptake when compared with PLA only particles.

Several studies have shown the uptake of the particles by Caco-2 cells when using the micro/nanoparticles for the routes other than oral. In the study conducted by Singh *et al* (2006) it was found that hydrophobicity had effected the uptake of the particles with the PCL nanoparticle uptake was significantly higher than for PLGA, PLGA–PCL blend and co-polymer nanoparticles; the more hydrophobic the nanoparticles, the higher the uptake by the Caco-2 cells. Gref *et al* (2003) observed the uptake of PLA and PEG-PLA radio-labelled particles by Caco-2 cells and the uptake were found to be around 0.7 to 1.5%. It was claimed that the possible lower uptake was due to the hydrophilic and neutral PEG coating. The predictions of particle uptake using Caco-2 cells can be over-estimated because Caco-2 cells share features of both absorptive intestinal cells and M cells but do not have the overlying interfering mucous layer, and are exposed to much more particles *per* cm^2 than they would *in vivo* (Brayden *et al.,* 2001).

5.3.4. Immune response following intranasal administration of PLA nanoparticles co-encapsulating HBsAg along with either cholic acid or lithocholic acid or melittin

Cholera toxin (CT) has been used in a number of studies as a mucosal adjuvant; however its toxicity limits its usefulness as an adjuvant. CT consists of two subunits, the monomeric A (CTA) and the pentameric B (CTB) subunit. The CTB subunit is considered to be safer than CT and therefore can be used as an adjuvant. The CTB subunit alters the T cell regulation in mucosal lymphoid follicles thereby acting as an adjuvant. CT is considered to induce a strong Th2 biased response to itself and accompanying antigens (Goto *et al.*, 2000; Lavelle *et al.*, 2004). In the present study, the CTB subunit along with HBsAg was used as a positive control to compare the immune responses obtained for the PLA nanoparticle formulations containing cholic acid, lithocholic acid and melittin after intranasal administration in mice. The polymer used for preparation of the nanoparticles was low molecular weight PLA (2 K) which has a degradation time of 6-8 weeks, therefore this study was conducted for only 45 days.

Figure 5.6 shows serum immune responses to the intranasally administered PLA nanoparticles free HBsAg and free HBsAg plus CTB. The free HBsAg elicited a low HBsAg specific systemic IgG, whereas significantly higher ($P < 0.05$) antibody titers were obtained for all the formulations. On day 15, the PLA only nanoparticles gave comparatively similar responses to free antigen plus CTB; however the formulations containing the bile salts and melittin gave higher antibody titers than free antigen plus CTB.

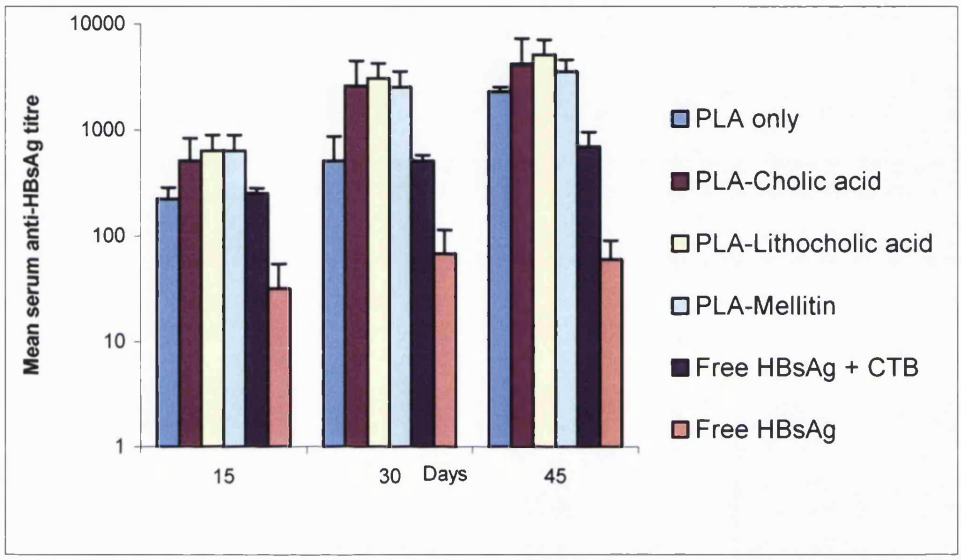

Figure 5.6: Mean serum antibody response to intranasally administered HBsAg loaded PLA nanoparticles with or without cholic acid or lithocholic acid or melittin. Mice were administered intranasally with PLA nanoparticles prepared by w/o/w solvent evaporation method with or without cholic acid or lithocholic acid or melittin or free antigen plus CTB or free antigen. The priming dose was 1 µg HBsAg on day 1 followed by a booster dose of 0.5 µg of HBsAg on day 21 ($n = 4$, mean ±s.d)

This trend was continued for day 30 as well. After 45 days, formulations containing cholic acid and lithocholic acid displayed enhanced immune responses; the antibody titres were ~ 80-85 times than free antigen which was statistically significant ($P < 0.008$). Although the nanoparticles containing the bile salts gave higher antibody responses than PLA only nanoparticles, this was not statistically significant ($P < 0.1$). Formulations with melittin also showed 2-4 fold higher immune responses compared to PLA only nanoparticles which was not significant ($P < 0.08$). CTB plus antigen was used as a positive control in this study and the antibody titers of the formulations containing bile salts and melittin were higher than CTB plus free antigen. This clearly states the importance of these absorption enhancers as an adjuvant. Out of all the formulations, lithocholic acid has shown better immune responses than the others.

Alpar *et al.* (2001) tested the potential utility of deoxycholic acid for nasally applied DT and TT in the BALB/c mouse. Co-administration of the enhancer was found to markedly improve the humoral responses evoking specific serum IgG. The bile salts act by extraction of membrane protein (denaturation) or lipids (phospholipids and cholesterol), solubilisation of peptides, membrane fluidization (i.e. decreasing the mucus viscosity), and formation of reversed micelles, creating aqueous channels. They have also shown the capacity of complexing Ca^{2+} and inhibitory effects on peptidase activity: sodium glycocholate was found to inhibit insulin metabolism in homogenates of rabbit nasal, buccal, rectal and intestinal mucosal membranes. The enhancing effect of bile salts is dependant on its lipophilicity and it has been shown that enhancers having C12 chain lengths have the greatest effect on the nasal route (Remon, 2006).

In a study conducted by Bramwell *et al* (2003) melittin has shown to impart adjuvanticity for diphtheria toxoid. The pseudostratified columnar epithelium of the nasal mucosa is composed of ciliated cells and mucous-secreting goblet cells. Cilia are present at the apical surface and mucous adorns the apices of the flask shaped goblet cells. The epithelium rests on a lamina propria of loose connective tissue, containing mucous glands. Whilst tight junctions seal intercellular pathways, agents which disrupt these may facilitate transport of molecules through to the underlying connective tissue. Melittin is able to affect the paracellular permeability. It leads to an increase in cell debris and destruction of intestinal tight junctions and cell-cell adhesion (Maher and McClean, 2006).

5.3.5. Cytokine responses

The animals were sacrificed after 45 days of immunization and spleens were removed for the determination of endogenous cytokines levels, interleukin-2 (IL-4), interleukin-6 (IL-6) and interferon-γ (IFN-γ). The $CD4^+$ and $CD8^+$ T cells which mediate the cellular immunity play an important role in protection against infectious pathogens. They respond to the antigen processed and presented by antigen presenting cells (APC) by two different ways. The $CD4^+$ and $CD8^+$ T cells recognize the antigen in association with major histocompatability complex (MHC) class II and MHC class I molecules respectively. The $CD4^+$ T cell population is further divided into subpopulations based on their function and the cytokines they secrete. These secreted cytokines play an important role in both innate and adaptive immune responses. For example, IFN-γ is

produced by effector Th_1 cells in conjunction with MHC class II after appropriate stimuli (Mosmann *et al.*, 1989; McNeela and Mills, 2001).

This study shows that the IL-4 and IL-6 responses are higher than IFN-γ responses for the PLA only nanoparticles and free antigen, indicating a Th2 based response (Figure 5.7). IL-6 is an important cytokine in the Th_2 cascade, promoting the proliferation and differentiation of B cells into antibody-producing plasma cells.

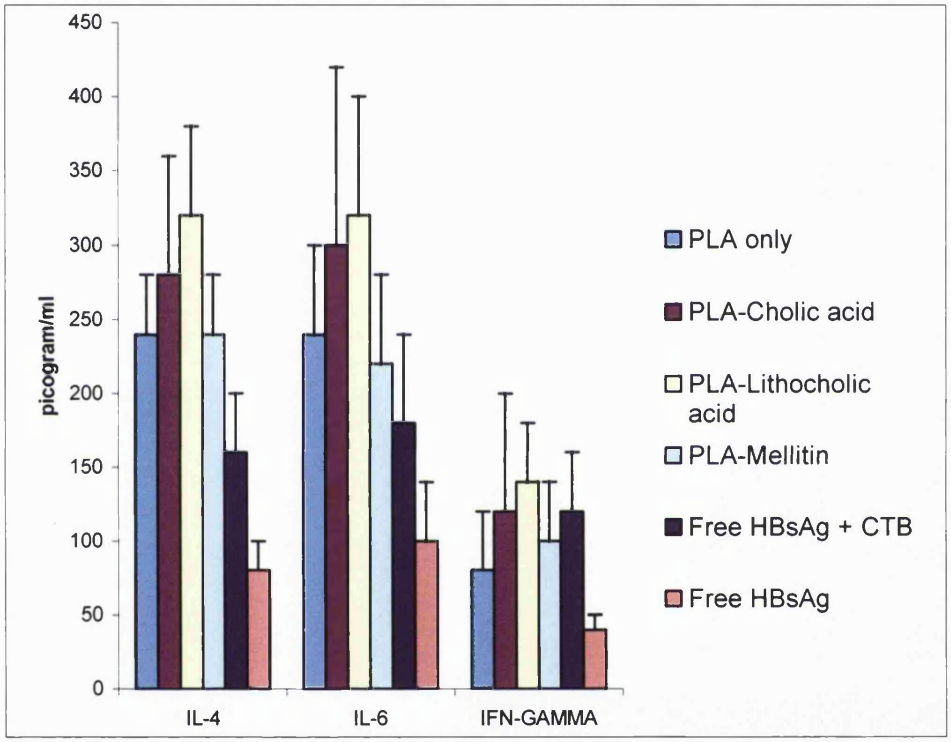

Figure 5. 7: Interleukin 4 (IL-4), Interleukin 6 (IL-6) and Interferon gamma (IFN-γ) production by pooled spleens cells after culturing with 5 μg/ml concentrations of soluble HBsAg after 45 days. Mice were administered intranasally with PLA nanoparticles prepared by w/o/w solvent evaporation method with or without cholic acid or lithocholic acid or melittin or free antigen plus CTB or free antigen. The priming dose was 1 μg HBsAg on day 1 followed by a booster dose of 0.5 μg of HBsAg on day 21 (n = 4, mean ±s.d)

However for the formulations with absorption enhancers like lithocholic acid, cholic acid and melittin the intensity of IL-4 and IL-6 responses were higher than IFN-γ responses. The nanoparticles with lithocholic acid primed spleen cells induced the highest elevated IL-4, IL-6 and IFN-γ productions significantly higher than the free HBsAg and CTB plus free HBsAg primed spleen cells. Incorporation of cholic acid and melittin has also significantly increased IL-4 and IL-6 production by spleen cells. The use of lithocholic acid has shown an increase in the IFN-γ production by spleen cells when compared with the other formulations however, it not statistically significant ($P <$

0.08). IFN-γ responses for the PLA plus lithocholic acid particles were higher than free antigen plus CTB.

5.4. Conclusions

In this study PLA nanoparticles co-encapsulating HBsAg with cholic acid or lithocholic acid or melittin were prepared by the w/o/w double emulsification solvent evaporation method. The concentration of the bile salts increased the particle size of the particles whereas melittin did not have any significant influence on the particle size of the particles. An *in vitro* experiment using Caco-2 cells showed significant higher uptake of PLA nanoparticles containing the bile salts when compared with the PLA only nanoparticles. The highest uptake of the nanoparticles containing lithocholic acid by the Caco-2 cells was mirrored in the *in vivo* studies following nasal administration with the highest serum immune response.

All polymeric nanoparticles irrespective of the presence of the bile salts or melittin induced an HBsAg specific serum IgG antibody response significantly higher than free HBsAg after intranasal administration. PLA nanoparticles containing bile salts and melittin induced HBsAg serum specific IgG antibody responses significantly higher than PLA only particles. The particles containing lithocholic acid induced significantly higher serum responses when compared with the free antigen plus CTB. The cytokine assays indicated that the serum IgG antibody response induced is mainly a $Th1/Th_2$ mixed response with elevated IL-4, IL-6 than IFN-γ responses compared with the free antigen and free antigen plus CTB. The nanoparticles eliciting the highest IgG antibody responses elicited the highest levels of cytokine IL-6 and IFN-γ.

This study has shown the potential of bile salts and melittin as co-adjuvants along with PLA nanoparticles to induce serum IgG antibody responses higher than PLA only nanoparticles and free HBsAg.

5.5 Key observations

- There was no significant difference in the immune responses obtained with PLA only nanoparticles and particles containing bile salts or melittin

- Presence of lithocholic acid remarkably improved the immune responses of the HBsAg loaded nanoparticles

- Melittin and bile salts containing nanoparticles gave better serum antibody responses than free antigen

Chapter 6

Overall Conclusions and Future work

6. Overall conclusions and future work

The overall conclusions are discussed in this chapter. At the end, a discussion of the future work needed is also presented

6.1 Tocopherol derivatives as adjuvants

The aim of this study was to investigate the effect of tocopherol derivatives such as TPGA, TA and TN on the physicochemical characteristics and immunogenicity of antigen loaded (OVA or DT or HBsAg) PCL or PLA micro/nanoparticles. In this study OVA or DT or HBsAg loaded PLA or PCL micro/nanoparticles along with tocopherol derivatives were prepared by the w/o/w double emulsification solvent evaporation or spray drying method. The concentration of TPGS in the organic phase did not affect the particle size and the encapsulation efficiency of OVA loaded PLA nanoparticles. TPGS has shown to improve the encapsulation efficiency of HBsAg in the particles. When DT loaded PCL microparticles were prepared with varying concentration of TPGS in the organic phase by the spray drying method, there was no significant differences between the particle size and encapsulation efficiency. For all the formulations, SDS-PAGE confirmed that there was no deterioration of the antigens due to the process parameters. The MTT assay confirmed that the particles containing TPGS or TA or TN are not cytotoxic.

One of the other aims of this study was to investigate the long term immune response of HBsAg loaded PLA nanoparticles (TPGS in organic or aqueous phase) administered subcutaneously. It was found that TPGS containing formulations elevated HBsAg specific serum IgG antibody titres when compared to the free HBsAg. Moreover, the duration of the response was shown to be maintained for in excess of 400 days. The particles containing TPGS gave better serum specific antibody responses when compared with alum plus free antigen. The immune responses were higher for the particles containing TPGS in the organic phase when compared with particles made using TPGS in the internal phase stabilizing agent. The nanoparticles eliciting the highest IgG antibody responses elicited the highest levels of cytokine IL-6 and IFN-γ.

The potential of TA and TN as adjuvants was investigated by administering HBsAg loaded PLA nanoparticles containing TA and TN in mice intramuscularly and

intranasally. Although the immune response obtained with these molecules was lower when compared with TPGS, nevertheless PLA-TA and PLA-TN have been shown to act as better adjuvants when compared with PLA only nanoparticles and the free antigen.

DT encapsulated PLA microparticles containing TPGS were also investigated for their immunogenicity. Both intramuscular and intranasal administered mice showed significantly higher immune responses for formulations containing TPGS compared to the free antigen. The gut washes of mice dosed with DT loaded PLA microparticles containing TPGS exhibited high IgA and IgG titres compared to lung washes of control mice, where antigen specific IgA responses were very low. The cytokine analysis showed that the IL-6 responses were particularly higher for the PLA-TPGS blend particles when compared with the other formulations and the free antigen.

DT-loaded PCL microparticles prepared by spray drying method with varying concentrations of TPGS were administered intranasally in mice. After 45 days PCL-10% TPGS and PCL-20% microparticles gave a 5 and 13 fold higher serum specific antibody responses compared to PCL only microparticles.

This study has shown the potential of TPGS, TA and TN as co-adjuvants together with PLA or PCL micro/nanoparticles to induce serum IgG antibody responses higher than PLA or PCL only micro/nanoparticles and free antigen. The overall objective of this study was achieved showing that formulations containing TPGS were found to be the best among all the tested formulations with respect to improving the adjuvancy of the antigens. Moreover, the unknown adjuvants, TA or TN were also better than plain micro/nanoparticles as well as free antigen. The tocopherol derivatives also improved the encapsulation efficiency of the micro/nanoparticles.

6.2. Co-encapsulation of mineral compounds

The aim of this study was to investigate the effect of mineral compounds such as alum and zinc sulfate on the physicochemical characteristics and immunogenicity of antigen loaded (OVA or HBsAg) PCL or PLA micro/nanoparticles. It was found that the presence of zinc sulfate increased the encapsulation efficiency of OVA or HBsAg in the PCL (80 K) or PLA (80 K) nanoparticles. This maybe a result of an enhanced stability

of the primary emulsion created by the preparation of zinc sulfate in the aqueous phase in the preparation of zinc-PCL or zinc-PLA particles. For all the formulations, SDS-PAGE confirmed that there was no deterioration of the antigens due to the process parameters and storage. The MTT assay confirmed that the PCL nanoparticles containing zinc sulfate and alum are not cytotoxic. This *in vitro* experiment showed that zinc sulfate or zinc oxide had no untoward effect on the particle uptake.

All polymeric nanoparticles induced an HBsAg specific serum IgG antibody response significantly higher than free antigen, both after intramuscular and intranasal administration. The particles containing zinc sulfate gave higher antibody responses when compared with other formulations or free antigen or alum plus free antigen. The analysis of the cytokine levels showed greater shift towards a Th1 response by the group of animals dosed with the PCL-zinc sulfate or PLA-zinc sulfate particles, although the response was mostly biased towards Th2 type.

In case of the PLA (147 K) microparticles, on subcutaneous delivery, the microparticles were found to elicit potent primary immune responses in terms of serum IgG, and alum containing HBsAg microparticles displayed higher serum anti-HBsAg IgG titres consistently, demonstrating that admixed alum has a strong impact in enhancing the magnitude of immune responses to PLA microspheres, possibly due to a synergistic immunostimulatory adjuvant effect between the PLA microspheres and alum adjuvant. However, the surface-modified microparticles with chitosan have also given slightly higher potency and long lasting immune responses when compared with the alum co-encapsulated microspheres. In addition, it was shown that at least to some extent, these different formulations may have the capacity to alter qualitative aspects of the immune response in terms of antigen specific cytokine responses, and hence alter the Th1/Th2 characteristics of the immune response. The mode of enhancement of immune response to alum adsorbed HBsAg loaded PLA microparticles or PLA-chitosan microparticles may be due to the macrophage activation properties of chitosan or the combination of the depot nature of alum and activation of immune system by chitosan. This study has shown the potential of zinc sulfate as co-adjuvants with PCL or PLA micro/nanoparticles to induce serum IgG antibody responses higher than PCL or PLA only nanoparticles or free antigen or free antigen plus alum/CpG. Therefore, this delivery systems show promise as an alternative strategy to induce robust immune responses.

6.3. Bile salts and melittin as adjuvants

The aim of this study was to investigate the effect of varying concentrations of cholic acid, lithocholic acid or melittin on the physicochemical properties (size, surface charge, encapsulation efficiency) of HBsAg loaded PLA nanoparticles. PLA nanoparticles co-encapsulating HBsAg with cholic acid or lithocholic acid or melittin were prepared by the w/o/w double emulsification solvent evaporation method. The concentration of the bile salts increased the particle size of the particles whereas melittin did not have any influence on the particle size of the particles. An *in vitro* experiment using Caco-2 cells showed significant higher uptake of PLA nanoparticles containing the bile salts when compared with the PLA only nanoparticles. The highest uptake of the nanoparticles containing lithocholic acid by the Caco-2 cells was mirrored in the *in vivo* studies following nasal administration with the highest serum immune response.

All polymeric nanoparticles irrespective of the presence of the bile salts or melittin induced an HBsAg specific serum IgG antibody response significantly higher than free HBsAg after intranasal administration. PLA nanoparticles containing bile salts and melittin induced HBsAg serum specific IgG antibody responses significantly higher than PLA only particles. The particles containing lithocholic acid induced significantly higher serum responses when compared with the free antigen plus CTB. The cytokine assays indicated that the serum IgG antibody response induced is mainly a $Th1/Th_2$ mixed response with elevated IL-4, IL-6 than IFN-γ responses compared with the free antigen and free antigen plus CTB. The nanoparticles eliciting the highest IgG antibody responses elicited the highest levels of cytokine IL-6 and IFN-γ. This study has shown the potential of bile salts and melittin as co-adjuvants along with PLA nanoparticles to induce serum IgG antibody responses higher than PLA only nanoparticles and free HBsAg.

6.4. Future work

There is a lot of scope for future work in this project. The most important is the use of appropriate factorial design to minimise the optimization studies and using different techniques like spray drying and super critical fluid technology for preparation of these

formulations. These techniques have a scope for large scale manufacturing with asceptic conditions.

The SDS-PAGE showed that there was no deterioration of the antigen; however this is not sufficient with respect to the biological activity. It would be relevant to run western blotting on the antigens extracted from the polymeric particles which would confirm that the immunogenicity of the antigens following exposure to solvents during the formulation process. *In vitro* release of these micro/nanoparticles can also be studied; however the *in vitro in vivo* correlation is still a debatable aspect as the biological microenvironment can be critical, making the correlation difficult.

It would be relevant to investigate the uptake of these particles by macrophages and dendritic cells, which could be confirmed *in vitro* by fluorescence and confocal microscopy, which would help in understanding the biodistribution of the particles after administration via different routes.

During this work the IgG sub classes were not evaluated. The extent of subclass response will give a better idea about the Th1 and Th2 orientation of these carrier systems. Moreover, the mucosal washes will further give an idea about the extent of this carrier system's mucosal adjuvancy.

In a recent study, co-polymer of PLA and TPGS was prepared (Lee *et al.*, 2007). This co-polymer can be further evaluated with the encapsulation of the antigens as the degradation characteristics will be altered and which it turn affect the adjuvancy of these carriers. This might also influence the manner in which antigen is presented to APC. The effects of such physicochemical characteristics can be evaluated *in vivo* and a better particulate delivery system can be formulated.

Publications

Publications

Papers

Effect of vitamin E TPGS on immune response to nasally delivered diphtheria toxoid loaded polycaprolactone microparticles
S.Somavarapu, **S.Pandit**, G.Gradassi, M.Bandera, E.Ravichandran, O.H.Alpar (2005), International Journal of Pharmaceutics, 298 (2), 344-347

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Abstracts

Microencapsulation of diphtheria toxoid in PCL and vitamin E TPGS blends using spray drying
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32nd Annual Meeting and Exposition of the Controlled Release Society (2005), Miami Beach, USA

Adjuvant effect of vitamin E TPGS on HBsAg loaded PLA microspheres
S.Pandit, G.Gradassi, S.Somavarapu, O.H.Alpar
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Immune response induced by S.equi enzymatic extract adsorbed to surface modified polycaprolactone microspheres
H.F.Florindo, **S.Pandit**, S.Somavarapu, A.J.Almeida, O.H.Alpar
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HBsAg Loaded PLA Nanoparticles: Adjuvancy of Tocopherol Acetate and Tocopherol Nicotinate in Mice
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In vitro characterization and immune response of nanoencapsulated Streptococcus equi antigens
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Encapsulation of Streptococcus equi antigens in PLA nanoparticles: Production and characterization
H.F.Florindo, **S.Pandit**, L.M.D.Gonçalves, O.H.Alpar, A.J.Almeida
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