NOVEL FORMULATIONS FOR ANTIGEN DELIVERY USING BIODEGRADABLE POLYMERS: NEW APPROACHES FOR THE USE OF NEW AND ESTABLISHED ADJUVANTS

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THESIS SUMMARY

The use of immunological adjuvants has been established since 1924 and ever since many candidates have been extensively researched in vaccine development. The controlled release of vaccine is another area of biotechnology research, which is advancing rapidly with great potential and success. Encapsulation of peptide and protein drugs within biodegradable microspheres has been amongst the most successful of approaches within the past decade. The present studies have focused on combining the advantages of microsphere delivery systems composed of biodegradable polylactide (PLLA) and polylactide-co-glycolide (PLGA) polymers with that of safe and effective adjuvants. The research efforts were directed to the development of single-dose delivery vehicles which, can be manufactured easily, safely, under mild and favourable conditions to the encapsulated antigens. In pursuing this objective non ionic block copolymers (NIBCs) (Pluronics® L101 and L121) were incorporated within poly-dl-lactide (PDLA) micorospheres prepared with emulsification-diffusion method. L101 and L121 served both as adjuvants and stabilising agents within these vaccine delivery vehicles. These formulations encapsulating the model antigens lysozyme, ovalbumin (OVA) and diphtheria toxoid (DT) resulted in high entrapment efficiency (99%), yield (96.7%) and elicited high and sustained immune response (IgG titres up to 9427) after one single administration over nine months. The structural integrity of the antigens was preserved within these formulations. In evaluating new approaches for the use of well-established adjuvants such as alum, these particles were incorporated within PLLA and PLGA microspheres at much lesser quantities (5-10 times lower) than those contained within conventional alum-adsorbed vaccines. These studies focused on the incorporation of the clinically relevant tetanus toxoid (TT) antigen within biodegradable microspheres. The encapsulation of both alum particles and TT antigen within these micropheres resulted in preparations with high encapsulation efficiency (95%) and yield (91.2%). The immune response to these particles was also investigated to evaluate the secretion of serum IgG, IgG1, IgG2a and IgG2b after a single administration of these vaccines. The Splenic cells proliferation was also investigated as an indication for the induction of cell mediated immunity. These particles resulted in high and sustained immune response over a period of 14 months. The stability of TT within particles was also investigated under dry storage over a period of several months. NIBC microspheres were also investigated as potential DNA vaccine delivery systems using hepatitis B plasmid. These particles resulted in microspheres of 3-5 μm diameter and were shown to preserve the integrity of the encapsulated (27.7% entrapment efficiency) hepatitis B plasmid.

Keywords: Adjuvants, Microspheres, Non ionic block copolymers, Alum, Tetanus toxoid,

To My Dear Father

In the loving and everlasting memory of my mother

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ABBREVIATIONS

ABTS	(2,2' azino-bis) 3-ethylbenzthia-zoline-6-sulphnic acid
Ag	antigen
AMPS	ammonium persulphate
AF	antigen formulation
APCs	antigen presenting cells
BALT	bronchial-associated lymphoid tissue
BCA	bicinchoninic acid
BSA	bovine serum albumin
CMIS	common mucosal immune system
CPM	counts per minute
CRT	cathod ray tube
CTL	cytotoxic T lymphocytes
DC	denditic cells
DCM	dichloromethane
DT	diphtheria toxoid
DTH	delayed-type hypersensitivity
DTP	diphtheria, tetanus and pertussis
EDTA	ethylenediamine-tetraacetic acid
EFC	eosinophil chemotactic factor
EIVA	equine infectious anaemia
ELISA	enzyme-linked immunosorbent assay
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
FDA	Food and Drug Administration
GALT	gut associated lymphoid tissue
γ-HPCD	γ-hydroxypropylcyclodextrin
GI	gastrointestinal
HBV	hepatitis B virus
Hib	haemophilus influenza type b
HLB	hydrophile-lipophile balance
HPLC	high performance liquid chromatography
HSA	human serum albumin
Ig	immunoglobulin
i.m.	intramuscular
i.n.	intranasal
IL	interleukin
IFN	interferon
ISCOMs	immune stimulating complexes
MCA	Medicines control agency
M cells	microfold cells
MHC	major histocompatability complex
MPS	mononuclear phagocyte system
MS	microspheres
Mw	molecular weight
NALT	nasal associated lymphoid tissue
NIBC	nonionic block copolymer
OD	optical density

OVA	ovalbumin
o/w	oil-in-water
PBS	phosphate-buffered saline
PBST	0.05% v/v Tween 20 in PBS
pI	isoelectric point
PCLN	posterior cervical lymph nodes
PDLA	poly(dl-lactide)
PLA	polylactide
PLGA	polylactide-co-glycolide
POE	poly oxyethylene
POP	poly oxypropylene
PP's	Peyer's patches
pU	partially unfolded
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
RES	reticuloendothelial system
RCTB	recombinant cholera toxin B
rpm	revolutions *per* minute
RT	room temperature
SAF	Syntex antigen Formulation
S-IgA	secretory IgA
s.c.	subcutaneous
SCLN	superficial cervical lymph nodes
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S/e	single emulsion
SEM	scanning electron microscopy
SIV	simian immunodeficiency virus
T_C	cytotoxic T cells
TEMED	N,N,N,N'-tetramethylene diamine
TGF-β	transforming growth factor beta
Th	T helper cells
TNF-β	tumor necrosis factor beta
Tris	tris(hydroxymethyl)-aminomethane
TT	tetanus toxoid
WHO	world health organisation
w/o	water-in-oil
w/o/w	water-in-oil-in-water
v/v	volume *per* volume
w/v	weight *per* volume
w/w	weight *per* weight

1.0. Introduction

1.1.Vaccine formulations

Vaccination is the most cost effective way of disease prevention and has had a large impact on the protection and also reduction of mortality rate from infectious diseases in both humans and domestic animals. Most current vaccines are highly effective, producing long-term protection against infectious diseases (Clemens *et al.*, 1996). These vaccines are predominantly attenuated live, replicating organisms which although protect the host from the infective pathogens, suffer from many disadvantages. These are mainly their reduced safety due to reversion to virulence, their limited shelf life and the requirement for refrigeration during storage and transport. For example immunisation for smallpox and measles has failed in some cases due to inadequate refrigeration before use (Kissel and Koneberg, 1995). All live and attenuated agents are particularly thermo-sensitive. These limitations have focused an intense interest on the investigation into identification and isolation of more effective and safer vaccine formulations. The development of inactivated, and subunit vaccines has focused on the production of formulations which meet all the necessary requirements of the ideal vaccines, such as, efficacy, safety, convenient application and cost.

1.2. Subunit vaccines

Subunit vaccines, which contain fragments of pathogen components are much safer and are more stable at ambient temperatures. Their safety is due to the lack of the infectious agents in their formulations. The safety issue has been further addressed with development of a new generation of recombinant subunit and peptide vaccines (Powel and Newman; 1995).

1.2.1. Characteristic advantages and disadvantages of subunit vaccines

As stated in the above section, the subunit vaccines are much safer than vaccines containing live, attenuated organisms. The new generations of recombinant subunit vaccines which mimic small regions of the microbial proteins, are defined at the molecular level, eliciting specific immune responses which contribute to their greater safety. However, the measures taken to improve the safety of subunit vaccines by purification steps in their preparation have led to their poor immunogenicty. Various immunological adjuvants have been incorporated in the design of subunit vaccines, rendering them stronger and more efficient (Zhao and Leong, 1996). Aluminium hydroxide and aluminium phosphate and recently liposomes (Gregoriadis, 1998, 1990) and an emulsion formulation (MF59) are the only approved adjuvants for human use. Immunostimulatory compounds (ISCOMs) and muramyl dipeptide (MDP) are amongst the new generation of more effective adjuvants which may be used as substitutes for alum compounds. A large number of genetically engineered cytokines have been identified more recently. These have been known to manipulate the host immune system in producing antigen-specific type, which can be used to optimise vaccination efficacy (Schopf *et al.*, 1999). The exact mechanism by which the adjuvants exert their effects is not yet clearly understood. Formulations containing the antigen in a controlled release delivery system have shown that the antigen can be directed efficiently to antigen-presenting cells (APCs) to produce both cellular and humoral immnue responses (Mestecky *et al.*, 1991, Eldrige *et al.*, 1989). The following section introduces the nature and the requirements of immunological responses to subunit vaccines.

1.2.2. Immunological response to subunit vaccines

To produce an effective immunogenic reaction, the activation of both T cells and B cells is important. This is necessary since they interact with one another in a complex way either

directly or through cytokines. There are three types of effector T cells: inflammatory $CD4^+$ T cells activating macrophages, helper $CD4^+$ T cells, activating antibody-producing B lymphocytes and cytotoxic $CD8^+$ T cells which kill their target cells. The ''principal orchestrators'' of the immune response are the T-helper (T_H) cells (Stite *et al.*, 1991). They are the necessary requirements for the activation of the major effector cells in the immune response [i.e., cytotoxic (T_C) cells and antibody–producing B cells]. Antigen presentation is mediated by specialized APC (macrophages, dendritic cells and B cells)(Yong *et al.*, 1997).

1.2.3. Antigen presentation and humoral immunity

Following antigen uptake, immuno-relevant epitopes are presented on the surface of the APC, in combination with a major histocompatibility complex (MHC) molecule. Figure 1.1. represents a schematic diagram of the initiation of immune response to the antigen following its internalisation. The combination of APC and MHC attracts the T_H cells and activates them in two ways: 1) binding the T-cell antigen receptor to the MHC complex and 2) production of interleukin-1 by APC. The activation of T_H cells triggers a complex cascade, which is initiated by the release of lymphokines. The lymphokines stimulate B lymphocytes, to proliferate, differentiate and produce specific antibodies. The differentiated B lymphocytes also produce memory cells. The released lymphokines activate granulocytes, macrophages, and natural killer cells. T_H cells activate the cytotoxic T cells directly. Some long-lived T_H cells seem to provide a memory function in the T-cell compartment of the immune system (Stites *et al.*, 1991).

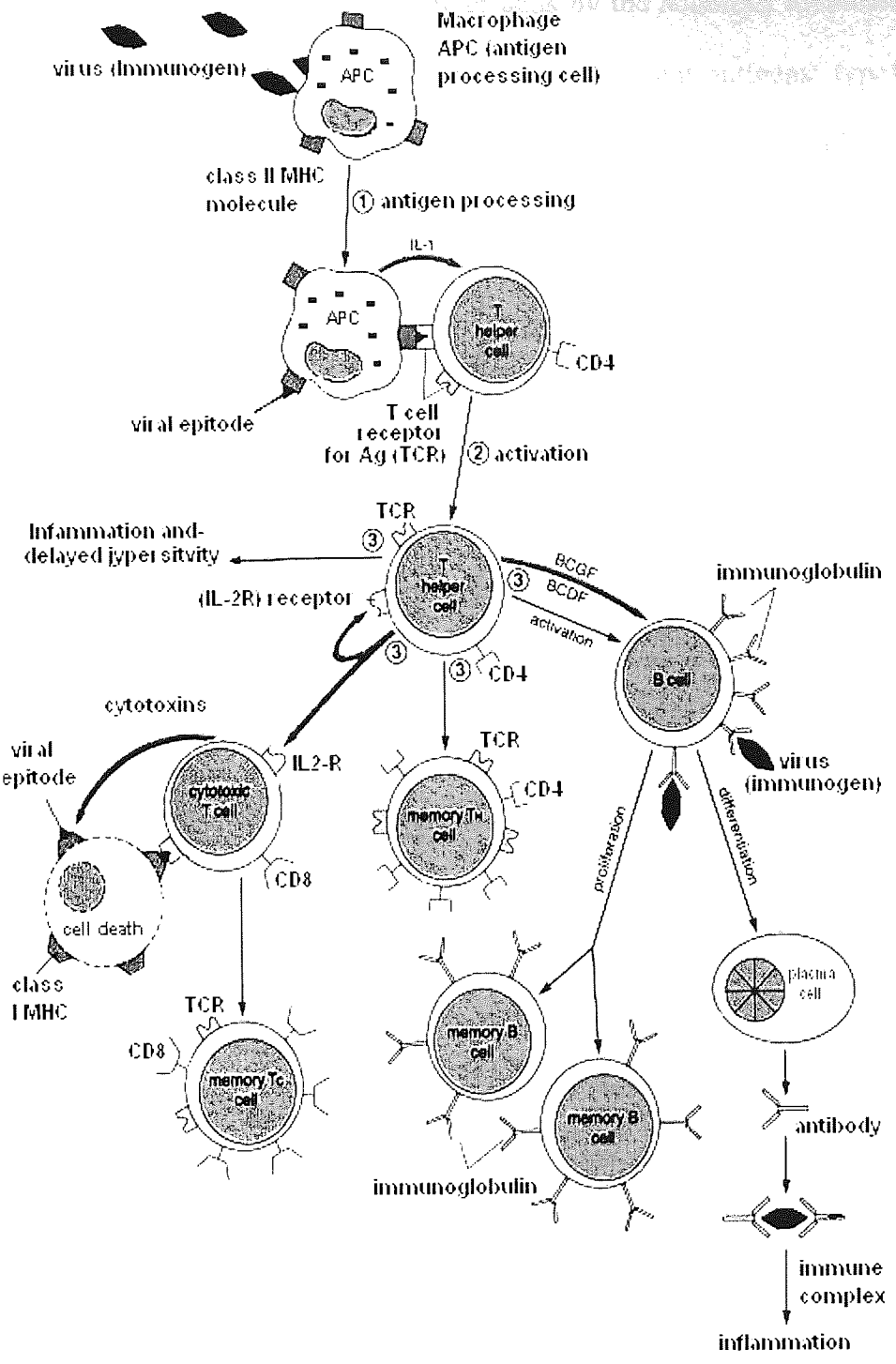


Figure 1.1. Schematic diagram representing the immune response to the internalization of antigen. (adapted from Stites and Terr, 1997)

B lymphocytes seem to be activated directly by certain molecules (thymus-independent antigens) or through the cooperation of T helper cells by the action of thymus-dependent antigens. There are two different types of thymus-independent antigens; type1 thymus-independent antigens, which are polyclonal activators, focused onto the specific B-cells by surface immunoglobulin (sIg) receptors and type2 thymus-independent antigens. The latter are polymeric molecules capable of cross-linking many sIg receptors and through their long half-lives, provide a persistent signal to the B-cells (Roitt and Delves, 2001). The cross-linking of surface Ig receptors (e.g. by type 2 thymus-independent antigens) activate B-cells. Thymus-dependent antigens require the involvement of T helper cells to stimulate antibody production by B-cells. Antigen captured by specific surface Ig receptors is taken into the B-cells, processed and expressed on the surface as a peptide in association with MHC II. This complex is recognised by the T helper cells which activate the resting B-cells (Roitt and Delves, 2001).

1.2.4. T- helper cells and cytokines

Evidence of the existence of two T_H cell subsets, was first presented by Mosmann and co-workers in 1986. These researchers reported that, cloned murine helper T lypmphocytes could be divided into two functional subsets on the basis of the immunoregulatory cytokines they produced (Mosmann *et al.*, 1986). T helper cells type1 (Th1) were found to produce gamma-interferon (IFN-γ), whereas T helper cells type2 (Th2), produced interleukin 4 (IL-4) (Mosmann *et al.*, 1986 and 1987). Since then, numerous studies have confirmed the above authors' original findings. Th0 and Thp clones were also identified. The former could produce both types of Th1 and Th2 cytokines and the latter has been shown to serve as precursor to the other Th clones (Paul *et al.*, 1994). Type 1 and 2 cytokines have subsequently been shown to demonstrate cross-regulatory properties, such

that they exert a positive as well as a negative feed back on one another (Mosmann *et al*, 1989a and 1989b, Seder *et al.*, 1992). For example, IL-4 inhibits the production of IFN-γ by Th1 clones whereas IFN-γ inhibits the production of IL-4 by the Th2 clones. These cross-regulatory properties were later linked to a number of observations made in as early as the 1960's and were best summarised by Parish in 1972. The author described an inverse relationship between cell-mediated immunity (CMI) and humoral immunity in response to antigenic stimuli (Parish, 1972). Th1 clones were recognized to produce better helper activity for CMI whereas Th2 clones have been shown to be more important for B-cell development and antibody production. One of the exceptions to the above observation is the enhancement of the immunoglobulin G2a (Ig G2a) antibody production by the Th1 clones. The interactions between different cytokines produced by Th1 and Th2 subsets, is represented in figure 1.2. Although cloned T cells provide important insight into the regulation of immune system, they however fall short of representing the *in vivo* mechanisms that regulate the immune system. Thus the Th1 cytokines now include IFN-γ, IL-2, and tumor necrosis factor beta (TNF-β), whereas Th2 cytokines include IL-4, IL-5, IL-6, IL-10, IL-13 and possibly IL-9. Other cytokines such as IL-12 have now been shown to be important contributors to the regulation of the immune system (Trinchieri, 1993, 1994). In addition to the helper T cells, it has now been recognised that other immunological sources produce several of the Th1 and Th2 cytokines. Bloom and co-workers therefore introduced the Type1 and Type 2 nomenclature in 1992 to include The $CD8^+$ as well as the $CD4^+$ T cells as sources of Th1 and Th2 immunoregulotry cytokines (Bloom *et al.*, 1992).

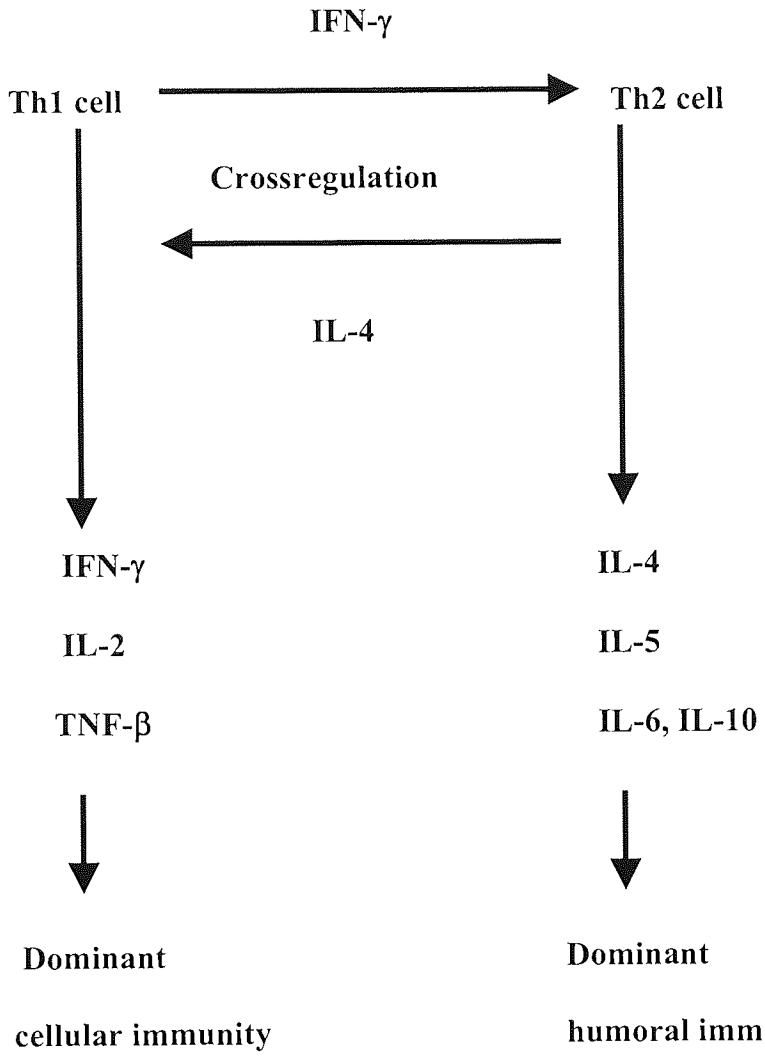


Figure 1.2. The interactions between Th1 and Th2 subset cytokines (adapted from *Lucey et al.*, 1996).

Other authors have chosen to define the Type 1 and Type 2 cytokines to include cytokines produced by non-T-cell leukocytes (Lucey *et al.*, 1996)(Table1.1.).

Table 1.1. Leukocyte sources of various cytokines

Cell Source	Cytokines	
	Type1	Type2
$CD4^+$ T cell	IL-2, IFN-γ, IL-12, TNF-β	IL-4, IL-5, IL-6, IL-13, IL-10
$CD8^+$ Tcell	IL-2, IFN-γ,	IL-4, IL-5, IL-10
NK cell	IFN-γ, TNF-β	
Monocyte/Macrophage	IL-12	IL-6, IL-10
B cell	IL-12, TNF-β	IL-6, IL-10
Dendritic cell	IL-12	
Neutrophil	IL-12	
Mast cell		IL-4, IL-5, IL-6,
Eosinophil		IL-4, IL-5, IL-6,

In this definition, ''Type 1 response is a strong cellular immune response with normal or increased levels of IL-2, IFN-γ, and TNF-β, and or IL-12 while a Type 2 is, an impaired cellular response with an increase in one or more B-cell activities (e.g., hypergammaglobulinemia, autoantibody production or hyper–IgE) and, an increase in the level of IL-4, IL-5, IL-6, IL-10 and/or IL-13''. In the process of antigen presentation by professional APCs, the combination of the APCs' preference for a certain T-helper cell subclass with the direct induction of cytokines by APCs, can determine the nature of the immune response. For example dendritic cells take up the antigens as they infiltrate the injection site. These cells will express and present peptides to $CD8^+$ cells following transport to the lymph nodes or spleen. On the other hand APCs may activate CD4+ cells, through MHC class II presentation of the antigen. Such events would lead to both cellular and humoral immunity (Manickan *et al.*, 1997). Both T helper cell types can drive autoimmune diseases in which a dysregulation of immunity to self has occurred. However, many autoimmune diseases, including multiple sclerosis (MS), are mediated by Th1 cells, and in several animal models of Th1-mediated autoimunity it is possible to block the disease by inducing a Th2 response to the autoantigen (Rook *et al.*, 2000).

Morokata and co-workers have shown that the antigen dose regulates Th1 and Th2 responses in the lungs of C57BL/6 and BALB/c mice independent of the splenic responses (Morokata *et al.*, 2000). Low-dose sensitisation (less than 8 μg of OVA) elicited Th1-type immunoglobulins (Igs) secretion, including low levels of IgE, IgG1 and high levels of IgG2a. The pattern of immune response was opposite to that observed in C57BL/6 mice, where high levels of IgE, IgG1 and low levels of IgG2a were secreted. In contrast, high-dose sensitisation (more than 50 μg) reversed the pattern of immune response in the two strains of mice. In the design of the subunit vaccines the targeting of the relevant immune responses is essential in ensuring the induction of the protective immunity. This however is not an easy task and various studies have shown that the subunit vaccines could stimulate irrelevant or even harmful immune responses (Graham *et al.*, 1993).

1.2.5. Antigen presentation and cytotoxic T lymphocytes

In addition to the T helper cells, the immune system controls diseases by the induction of cytotoxic T lymphocyte (CTLs). Cytotoxic T cells kill the infected cells and are therefore targeted in designing tumour immunotherapy and vaccines for viral infections. Most conventional vaccines (including subunit vaccines) fail to elicit CTL responses. T lymphocytes use an antigen receptor to monitor the surface of the APCs for the presence of foreign peptides. The antigen receptor on the T_H cells recognise the peptides bound to the MHC class II molecules whereas CTLs' receptors recognise the antigens displayed on MHC class I molecules (Germain *et al.*, 1996). To become fully activated the T cells require additional stimulation, which takes place through the interaction of other receptors (e.g. CD28 and CD40L) on the T cells and certain ligands (e.g. B7 and CD40) present on the surface of the APCs (Chambers *et al.*, 1997). Amongst the APCs, dendritic cells are believed to be the most potent stimulators of the immune responses (Banchereau *et al.*,

1998). Dendritic cells were first visualised as the Langerhans cells (LCs) in the skin in 1868 but their characterisation took place only 25 years ago. They are efficient stimulators of B and T lymphocytes (Steinman, 1998). Located in most tissues, dendritic cells capture and process antigens, and display large amounts of MHC-peptide complexes at their surface. While regulating their co-stimulatory molecules, they migrate to lymphoid organs, the spleen and the lymph node. There they liase with and activate antigen-specific T cells (Banchereau *et al.*, 1998, 2000; Bell *et al.*, 1999). *In vivo* or *in vitro*, only few of these cells are required to induce a strong T-cell response. Dendritic cells stimulate the outgrowth and activation of a variety of T cells depending on different conditions. These cells can induce CTLs (which express the accessory molecule CD8 and hence interact with MHC class I bearing cells) to proliferate (Bender *et al.*, 1995). In the presence of mature dendritic cells and due to their IL-12 production, CD4-expressing T-helper cells turn into interferon-γ-producing Th1 cells (Cella *et al.*, 1996). The effect of IL-12 and interferon-γ is to promote the differentiation of T cells into killer cells. Dendritic cells in the presence of IL-4 however induce T cells to differentiate into Th2 cells, which secrete IL-5 and IL-4. These cells activate eosinophils and help B cells to make the appropriate antibodies, respectively. Dendritic cells are composed of three distinct subsets, which can induce different types of immune responses. At least three distinct pathways of DC development exists in mice: myeloid DC, lymphoid DC and Langerhans DC (Pulendran *et al.*, 2000; Banchereau *et al.*, 1998, 2000). In mice, the lymphoid DC make a higher levels of IL-12 and induce naïve T cells to produce IFN-γ thus promoting type1 responses (Pulendran *et al.*, 1999; Ohteki *et al.*, 1999). Myeloid DC induce T cells to produce IL-4 and favour type2 responses (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999). While human DC can also polarise naïve T cells, the type of responses seem opposite, when myeloid DC have been shown to secrete large amounts of IL-12, and favour a type1 immune response

(Rissoan *et al.*, 1999; Cella *et al.*, 1999). The extent of T cell polarisation may be related to DC differentiation and maturation stages (Cella *et al.*, 1999). Dendritic cells have therefore been targeted for potentiating immune responses by immunization approaches which uses these cells once exposed to the antigens *ex vivo* or by directly targeting antigens into them *in vivo*. In contrast to MHC class II, the MHC class I, molecules display peptides from genes that are expressed inside the cell, which enables the immune system to monitor for the presence of foreign antigens such as those from viruses or tumour cells (York *et al.*, 1996). Most of these antigens are produced in the cytoplasm by the action of a proteolytic particle known as proteasome (York *et al.*, 1996). They are then transferred to the endoplasmic reticulum where, they are bound by the newly synthesised MHC class I molecules and transported to the cells. The conventional vaccines fail to stimulate a CTL response since they are injected into the extracellular fluids and thus are presented on MHC class II molecules, which stimulate the T_H cell responses. Since the antigens are contained within membrane-bound vesicles, their entry to the cytoplasmic compartment is inhibited, thus preventing their accessibility to MHC class I molecules (Watts, 1997). Several approaches have been used to stimulate the CTL responses with vaccine antigens. One approach is to introduce antigen genes into the host so that the APC will synthesise the antigen and therefore present its peptides to MHC class I molecules. Viral or bacterial vectors have been used to introduce antigens into the APC. The use of plasmids and RNA as vaccine delivery systems have been described which would allow repeated immunisations to be effective and seem likely to be safer than viral vectors. Other approaches include the use of adjuvants such as ISCOMs, emulsions with monophosphoryl lipid A, an oil-in-water emulsion composed of pluronic, squlane and Tween to elicit CTL responses. These approaches have been developed empirically.

1.3.Enhancement of antigen immunogenicity

Adjuvants were originally described by Ramon in 1924 as "substances used in combination with a specific antigen that produced more immunity than the antigen alone", and this definition remains valid to date (Ramon, 1924). The use of adjuvants in the modern subunit vaccines is necessary as described before due to their weak immunogeneity (section.1.2.1). Although alum has an excellent safety record, it fails to induce an appreciably high immune response both through antibody production and cell-mediated immunity (Gupta *et al.*, 1995). This necessitates the need for development of new generation of adjuvants which in addition to their safety records, would lead to induction of high levels of immune response. The balance between the safety and the potency of the adjuvants is the most important factor in their development. For their use in standard prophylactic immunisation procedures in healthy individuals, they should only induce a minimum number of side-effects if any (O'Hagan, 1997). However, for more chronic viral, bacterial, neoplastic or autoimmune diseases more pronounced side effects might be acceptable. The characteristics of the "ideal" adjuvant comprise a long list (Table 1.2).

Table 1.2. Some characteristics of the "ideal" adjuvant (adapted from O'Hagan, 1997).

Safety
Biodegradability and biocompatibility
Efficacy
Single dose administration
Induction of both humoral and systemic immunity
Orally administered
Stability
Ease of production
Cost-effective
Long shelf life

1.3.1. Immunostimulating compounds

1.3.1.1. Bacterial adjuvants

Lipopolysaccharides of gram negative bacteria are another second class of immunostimulatory compounds, which have been shown to possess adjuvant activities. The most extensively evaluated member of this group is monophosphoryl lipid A (MPL), which is obtained from *Salmonella minnesota*. This compound has been defined as penta- and hexaacyl derivatives of diglucosamine monophosphate, and in its clinical use with a variety of antigens has produced adjuvant activity with tolerable side effects (Ulrich *et al.*, 1995). Bacillus firmus, a non-pathogenic gram-positive bacterium of the external environment, has also been investigated for immunomodulatory properties. This has been shown to stimulate an increase in anti-ovalbumin IgG in sera, bronchoalveolar lavages and intestinal washings after both intranasal and intratracheal immunisation (Milckova *et al.*, 2001). The bacterium resulted in an enhanced anti-ovalbumin IgA in intestinal secretions and bronchoalveolar lavage fluid after intranasal and intratrachael immunisation, respectively. It has been found that the immunomodulatory effect of B.Firmus on antibody formation was antigen specific (Milckova *et al.*, 2001).

1.3.1.2. Particulate antigen delivery systems

The development of particulate delivery systems which, enhance the adjuvanticity of the associated antigens, stems from the fact that the infectious agents (parasites, bacteria and viruses) which are particulate in nature are recognised and efficiently taken up by professional APCs in the natural course of an infection. Different delivery systems (e.g. ISCOMs, liposomes, microparticles and emulsions) can be manufactured to mimic certain characteristics of pathogens necessary for the induction of immune responses such as size, surface hydrophobicity, etc.

1.3.1.3.1. ISCOMs™

Saponins are a heterogeneous group of glycosides, which occur widely though, not exclusively in plants (Price *et al.*, 1987). They share a common structural basis, which comprises either a steroidal or triterpenoid aglycone to which one or more sugar chains are attached. Many saponins are surfactants forming stable foams when shaken in water. Many form complexes with cholesterol both free and membrane-bound. The adjuvant properties of saponins were first recognised in the 1920s (Ramon, 1926). ISCOMs™ are complexes of saponins, cholesterol, lipid and immunogen, which can aggregate into the typical 40 nm cage-like structure. They can be formed using mixtures of Quillaia saponins (Ronnberg *et al.*, 1995), defined saponin fractions or purified components (Sjolander *et al.*, 1998). Numerous studies have reported the ability of ISCOMs to potentiate antibody- and cell-mediated immunity to incorporated antigens after parenteral administration (Barr *et al.*, 1998, Kensil, 1996). Mowat and co-workers presented evidence that oral immunisation of ovalbumin (OVA) in ISCOMs produced strong antibody and cellular responses. The pattern of cytokine production was similar to that observed after parenteral immunisation procedures of these compounds. OVA in ISCOMs, given by oral route activates, both Type 1 and Type 2 cytokine responses (Mowat *et al.*, 1993). However the requirement for repeated immunisations and large amounts of antigen which induce only an immune response of very short duration could limit their practical use in routine immunisation procedures (Mowat *et al.*, 1993). Although the results with OVA as the model antigen have been very successful, ISCOMs have not been able to produce positive results with some antigens (Sjolander *et al.*, 1998).

1.3.1.3.2. Non ionic block copolymers

Oxide types of block copolymers have since the introduction in the early 1950s been used intensively in industrial applications of aqueous systems (Mortensen, 2001). Most studies and applications concern triblock copolymers based on poly(ethylene oxide), POE, as the water-soluble block and with poly(propylene oxide). POP, as the hydrophobic block. These copolymers are known commercially as Pluronics® or superionics or through the generic name as polaxomers. The potential of Pluronics® to generate a systemic and local immune response has been evaluated in a recent study using Pluronic F127® resulting in enhanced immune response to intranasally administered tetanus toxoid (Westerink *et al.*, 2002). These Pluronics®copolymers were the subject of part of the studies in this thesis and will be discussed further in chapters three and four of this manuscript.

1.3.1.3.3. Aluminium compounds

Aluminium compounds are the only adjuvants used for human vaccines and thus become the benchmark or reference preparations for evaluating new adjuvant formulations for human vaccines (Gupta and Siber, 1995). Aluminium compounds used as vaccine adjuvants include aluminium phosphate, aluminium hydroxide and alum-precipitated vaccines. The detailed discussion of these adjuvants will be presented in chapters five and six of this manuscript.

1.3.1.3.4. Mineral Adjuvants

The first evidence of the use of oil emulsions as adjuvants in vaccine formulations dates back to 1916, when Le Moiginic and Pinoy immunised mice with heat-inactivated *Salmonella typhimurium* in an emulsion of water and vaselin oil, using lanolin as an emulsifier (Le Moignic and Pinoy, 1916). The oil emulsions however, were not fully

appreciated until Jules Freund and co-workers decades later combined a paraffin (mineral) oil emulsion and heat-killed mycobacteria to produce an extremely potent adjuvant, Freund's complete adjuvant (FCA) (Freund *et al.*, 1937, Freund, 1956). Until then, adjuvants had been held by the general view to be able to raise high antitoxin titres. In contrast, FCA proved to be a highly efficient stimulator of cell-mediated immunity in addition to its ability to augment the humoral immune response. FCA, however, had a profile of adverse side effects (table 1), severe enough to restrict its use to experimental immunology in laboratory animals. A modified version of FCA is known as Freund's incomplete adjuvant (FIA). In this formulation the antigen is administered in a similar water-in-oil (W/O) emulsion, but without mycobacterial components. This formulation induces a potent humoral immune response, but it is not efficient enough in stimulating delayed-type reactions (Raffel, 1948). FIA has been included in veterinary, as well as human vaccines. The veterinary vaccines included such vaccines against foot-and-mouth disease (McKercher *et al.*, 1977), equine influenza virus (Street, 1967) and rabies (Freund *et al.*, 1948). In humans, FIA was used for a period of about two decades, particularly with vaccines against influenza virus (Salk *et al.*, 1952), tetanus toxoid (Pittman, 1967), and killed polio-myelitis virus (Salk *et al.*, 1953), whereas it failed to increase vaccine efficacy when used with adenovirus (Miller *et al.*, 1965) and trachoma (Woolridge *et al.*, 1967). In Britain alone, approximately 900,000 doses of a mineral oil-adjuvanted influenza vaccine were administered to humans in the early 1960s (Lindblad, 1997). The use of FIA adjuvanted vaccines was brought to a halt in mid-1960s, because of the concerns about the safety of the adjuvant (table 1). The mechanism of action of the mineral oil emulsions is thought to be three fold: a) the establishment of a repository antigen-containing locus at the site of injection allowing a gradual and continuos release of antigen (Herbert, 1967), b) provision of a vehicle capable of transporting emulsified antigen through the lymphatic

system to distant sites (e.g., draining lymph nodes and the spleen), creating additional foci of antibody formation and c) interaction with mononuclear cells, such as phagocytic cells and antigen presenting cells (Lindblad, 1997). Freund's adjuvants are used in priming immunisations. FCA with its mycobacterial components is able to skew a humoral immune response toward Th1 profile with pronounced IgG2a stimulation with high titres in mice.

Table 1.3. Side effects in laboratory animals historically attributed to the use of Freund's adjuvants

Freuend's Complete Adjuvant	Sterile abscesses Granulomas Muscle indurations Plasma cell neoplasia in BALB/c mice Ascites in BALB/c mice Amyloidosis Adjuvant arthirits in Lewis rats Experimental allergic encephalomyelitis in guinea pigs
Freuend's Complete Adjuvant	Sterile abscesses Granulomas Muscle indurations Plasma cell neoplasia in BALB/c mice Ascites in BALB/c mice

The antibody profile, however, is not entirely restricted to IgG2a; other subclasses are seen as well. The ability to stimulate Th1 immunity is further sustained by a number of studies where FCA has been tested alone or in comparison with aluminium hydroxide, which is well documented as a Th2 adjuvant (Grun and Maurer, 1989; Lindblad *et al.*, 1997).

1.3.1.3.5. Microspheres

The term 'microsphere' is generally implied when describing spherical particles within the size range of 50 nm-2 mm (Arshady, 1993). The term nanosphere is used to indicate smaller sizes than 1 μm. Polymeric microspheres used for biomedical applications are manufactured from a wide range of natural and synthetic sources, such as polysaccharides

(Arshady, 1991) and proteins (Arshady, 1990). It is a well-established fact that the association of antigens with polymeric microparticles leads to the potentiation of immune response (O'Hagan, 1997). The adjuvant effect of microparticles appears to be largely a consequence of their uptake into dendritic cells, macrophages and local lymph nodes following intramuscular injection (O'Hagan *et al.*, 2001). Microparticles facilitate the entry of the encapsulated antigens to the lymph nodes, provide their high local concentration over an extended time-period and also promote their interactions with antigen presenting cells. PLGA microparticles have been shown to be effective inducers of CTL responses in rodents against entrapped antigens (Nixon *et al.*, 1996; Maloy *et al.*, 1994). One of the main factors influencing the above adjuvanticity is the particle size. Smaller particles (< 10 μm) have been shown to be significantly more immunogenic than larger particles (> 10 μm)(Eldrige *et al.*, 1991). Microspheres have been demonstrated to exert an adjuvant effect for cell-mediated immunity after both systemic and mucosal administration (Maloy *et al.*, 1994) (Moore *et al.*, 1995). Recent studies have shown that microparticles could induce immune responses over a time period of one year after one single-dose administration (Cleland *et al.*, 1994) (O'Hagan *et al.*, 1995). Singh and co-workers have recently demonstrated that a single immunization in rats of tetanus toxoid entrapped in microparticles produced immune responses which were comparable to that after three doses of tetanus toxoid adsorbed on alum (Singh *et al.*, 1997).

1.4. Polymeric microspheres for delivery of vaccine antigens

1.4.1. The lactide/glycolide polymers

Over the past decade many controlled release systems for drug delivery have been extensively explored (Langer, 1990). Encapsulation of peptide and protein drugs in biodegradable microspheres have been amongst the most successful of these approaches

investigated in the literature (Maulding, 1987, Johnson *et al.*, 1996). L-lactic and its copolymer with D-lactic acid and glycolic acid (PLGA) have been widely utilised in development of biodegradable microspheres (Cleland, 1998; Jung *et al.*, 2002; Johansen *et al.*, 2001). Their extensive use in microsphere manufacturing is due to their excellent biocompatibility (Yamaguchi *et al.*, 1993), biodegradability and variable mechanical processability (Park, 1994). The release rate of proteins from these polymers can vary between a few days to well over a year. Protein release could take place in three ways: (1) diffusion from or through the system; (2) a chemical or enzymatic reaction leading to the degradation of the polymers and (3) solvent activation through osmosis or swelling of the polymers (Langer, 1990). The addition of glycolide to lactide accelerates the degradation rate of the polymer. PLLA and PLGA polymers are easily synthesised and the monomers and polymers are commercially available. These polymers are stable if kept dry and below 40 °C, which is important for achieving a prolonged shelf life in microsphere preparation (Aguado and Lambert. 1992). The biodegradation, biocompatibility and the mechanisms of protein release of the above polymers will be dealt with in the following sections.

1.4.1.1. Biodegradation and biocompatibility of lactide/glycolide polymers

The generally held view on the degradation of aliphatic polyester microspheres is that of hydrolytic degradation (Okada, 1995, Li *et al.*, 1995). Although the possibility of enzyme catalysed degradation has been considered in several studies, the results have not been as convincing (Schakenraad *et al.*, 1990). Vert and co-workers have studied the size dependence of the hydrolytic degradation of poly lactic/glycolic acid polymer-based delivery systems. They demonstrated that in large particles (> 300 microns), the heterogeneous hydrolytic degradation is characterised by a higher rate of degradation in the

core of the system compared with the surface (Vert *et al.*, 1994, Grizzi *et al.*, 1995). In contrast, smaller particles undergo a homogeneous degradation with the rate of the degradation being equivalent both for the core and surface of the system (Spenlehauer *et al.*, 1989). Many factors contribute to the hydrolytic degradation of biodegradable polymers which include, chemical composition, additives (acidic, basic, monomers, solvents, drugs), porosity, molecular weight and site of implantation (Anderson and Shiva, 1997). Altering the composition by increasing the glycolide mole ratio in a ploy(DL-lactide-co-glycolide) copolymer system leads to an increase in its rate of degradation (Tice *et al.*, 1992). The basic and acidic nature of the incorporated agents in the polymeric microspheres could lead to their decreased or increased degradation. Maulding and co-workers have found that basic compounds can catalyse ester linkage scission and thus accelerate polymer degradation. On the other hand the incorporation of appropriate amount of basic compounds can neutralise the carboxyl end-groups and thus decrease the rate of degradation (Li *et al.*, 1995). Porosity of the microspheres plays an important role in accelerating the rate of degradation, especially when the dimensions of the pores are such that would allow cellular migration into the pores. This influence is demonstrated in the studies carried out by Isobe and co-workers. They found that 75:25 poly(DL-lactide-co-glycoside) porous microspheres underwent degradation within three weeks following implantation, whereas non-porous microspheres of 74:26 poly(DL-lactide-co-glycolide) were expected to degrade over a time period of 20 weeks (Isobe *et al.*, 1996, Tice *et al.*, 1992). The molecular weight and the molecular weight distribution of the polymer may play a role in the degradation of the microspheres in the way that a large molecular weight distribution might facilitate the autocatalytic degradation of the polymer chains. A large molecular weight distribution would indicate relatively large numbers of carboxylic end groups, thus leading to accelerated rate of degradation. In comparison, a narrow molecular

weight distribution would lead to a slower rate of degradation due to the lower number of carboxylic acid end groups available for autocatalysis (Anderson and Shive, 1997). In a recent study Tracy and co-workers investigated the factors affecting the degradation rate of PLGA microspheres *in vivo* and *in vitro*. Their results indicate that degradation is faster in vivo than in vitro in a HEPES buffer at physiological pH and temperature (Tracy *et al.*, 1999). The authors found that amongst all the polymer chemistry variables tested, the PLGA end group had the greatest effect on degradation with uncapped (free carboxyl end group) PLGA degrading faster than capped (lauryl ester is the standard end group but methyl ester can be used to lower the inherent viscosity). They also showed that there was a trend towards faster degradation with lower molecular weight polymer microspheres. The addition of sparingly soluble salts such as zinc carbonate was shown to impair degradation *in vivo* and *in vitro* of capped PLGA only (Tracy *et al.*, 1999). The authors indicate that the entrapped protein (in this case, recombinant human growth hormone) had no significant effect on rate of degradation, whereas Giunchedi and co-workers found that diazepam-loaded PLGA microspheres had a significantly higher degradation rate than the corresponding empty microspheres *in vitro* (Giunchedi *et al.*, 1998). In their investigations of the *in vitro* degradation of polyester microspheres, Giunchedi and co-workers have found that preparation methods play an important role in determining their degradation behaviour. Their results show that spray-dried empty microparticles degrade faster than microspheres prepared by solvent evaporation (Giunchedi *et al.*, 1998). In evaluating the biocompatibility of the polymeric microspheres, it is important to have an understanding of the inflammatory and healing responses of these materials. Those responses are initiated by injury which in the case of the microspheres is their injection to the body. The extent of the pathophysiologic responses by the host are thought to be tissue-dependent, organ-dependent and species-dependent (Anderson, 1994). The sequence of events following the

injection of microspheres is listed in Table 1.4. Factors that might effect the intensity and time duration of the inflammatory and wound healing responses include the size, shape, and chemical and physical properties of the biomaterial and the physical dimensions and properties of the material, prosthesis, or delivery device.

Table 1.4. Sequence of events following microsphere implantation (The tissue response continuum) (adapted from Anderson and Shive, 1997).

Injury- injection, implantation
Acute inflammation- polymorphonuclear leukocytes
Chronic inflammation- monocytes and lymphocytes
Granulation tissue- Fibroblasts and New Blood Capillaries
Foreign body reaction- macrophages and FBGCs at the material-tissue interface
Fibrosis- fibrous capsule

Injection of the microspheres results in the implantation of the microspheres which have a large surface area to volume ratio into a given tissue volume. Thus, the implant (volume of microspheres) is comparable to implant sites seen with open structured foams, porous biomaterials and knitted or woven fabrics (Anderson, 1994). The sequence of events which follows implantation is generally considered as the tissue response continuum and is called so, because each individual event leads into the next event.

1.4.2. Immune response to polymeric microspheres

The fate of microspheres following injection has been discussed briefly in earlier sections (section 1.2.4.). This section places emphasis on the role of macrophages and the fate of microspheres following their encounter with those cells. The most important cell in the mononuclear phagocyte system (MPS) is the macrophage, which plays an important role in the induction of immune responses following the entry of foreign antigens to the body. Macrophages are a diverse group of cells, which operate at many different levels in response to foreign antigenic material. Their immunoregulatory functions include the

development of host resistance to parasitic attacks, tumours and, a wide variety of micro-organisms (Carr, 1973). Macrophages are all derived from bone marrow precursors. They first circulate as monocytes and following physiological or inflammatory conditions, they enter tissues where they mature into macrophages (Carr, 1973). The inflammatory giant cells are also derived from macrophages following appropriate environmental stimuli in areas of chronic inflammation. During the development of host immune response to an inflammation, the macrophages respond to mediators released by other leukocytes and they release mediators (IL-1, IL-6, IL-8, IL-12 and TNF-α) themselves, which have primary or secondary effects on the development of host responses (Janeway *et al.*, 1999). Biomedical polymers fall under two categories: one is the bioinert polymer, which should not be recognised as foreign material by the host immune system. The second category includes the bioactive polymers, which should induce an immune response (Tabata *et al.*, 1990). Most types of microspheres are cleared rapidly from body fluids by cells of the mononuclear phagocyte system (MPS). In site specific drug delivery where the localisation of microspheres in certain sites is required their rapid clearance by the MPS cells must be prevented.

1.4.2.1. Phagocytosis of polymer microspheres *in vitro*

There are three subsequent steps in the process of phagocytosis. These are the attachment, ingestion and digestion of the particles. The attachment of the particle is a necessary requirement for its internalisation by the phagocyte. The mechanism of recognition of the foreign antigens by the cells is not known yet but the presence of specific surface receptors has been suggested. Following attachment, a phagosome is formed so that the particles are internalised and transported to the inside of the cell membrane (Simon *et al.*, 1973). The ingestion step is characterised by the engulfing plasma membrane, which surrounds the particle during the entry to the macrophages. During this process the amount of fluid

entering the cells is minimised due to the close contact between the plasma membrane and the surface of the particle (Silverston *et al.*, 1977). The exact mechanism of the ingestion is unclear as yet, but the action of the cytoskeleton system is strongly associated with the ingestion phase (Stossel *et al.*, 1982). The cytoplasm beneath the area of the particle associated-plasma is enriched with a network of actin microfilaments. Small particles are swept by the active ruffling of the cell membrane into vacuoles, which then pass deep into the cell. In the case of larger particles, a few macrophages adhere together and become very close to the particle with the subsequent engulfing of the particle. After ingestion of the vesicle, which surrounds the particle, the phagosome fuses with one or more lysosomes to form a secondary lysosome or phagolysosome. The hydrolytic enzymes, contained in the lysosme are then discharged into the vacuoles to degrade the contents. These enzymes include proteases, nucleases, glucosidases, lipases, phospholipases, phosphatases and sulfatases. All of these are acid hydrolases with optimal activity at pH 5. The environment of the phagosome becomes acidic rapidly leading to the degradation of and dissociation of ligand-receptor complexes and retrieval of receptors and membrane constituents by recycling (Mellman *et al.*, 1984). Studies with incubation of macrophages with PLA and PLGA microspheres *in vitro* have shown that the microspheres are well phagocytosed within 6 hours. Observing the degradation of the PLGA microspheres by scanning electron microscopy, shows that, after the contour of the microspheres decreases gradually after the 6 hour incubation and that at day 7 no microsphere could be seen inside the cells. In contrast, the PLLA microspheres could still be observed after 7 days inside the cells. The release of substances from microspheres was observed when a fluorescent dye had been incorporated inside the microspheres. The PLGA microspheres had a clear contour and their incorporated dye was seen to occupy the space inside the cell (except for the nucleus) after 4 hours of incubation. With increasing incubation time, the dye diffused out

into all of the internal space of the cell, resulting in the staining of the entirety of the cell (Tabata *et al.*, 1990). These results indicate that microspheres phagocytosed by macrophages degrade gradually leading to the slow release of their incorporated substances in the cells.

1.4.2.2. Factors regulating microsphere phagocytosis

The factors regulating the uptake of the particles by macrophages include, size, surface charge, surface hydrophobicity and the adsorption/encapsulation of proteins and other additives. Studies carried out by Kawaguchi and co-workers, have demonstrated that particles with a diameter between 0.4 and 1.0 μm were the most readily ingested by leukocytes, when the volume of particles added was fixed (Kawaguchi *et al.*, 1986). Robert and co-workers had found that particles with a diameter ranging between 0.3 to 2.6 μm had the largest uptake by the leukocytes (Robert *et al.*, 1963). Macrophages seem to have the ability of recognising the size of the particles attached to them by mechanisms, which are as yet unclear. Although the size is an important factor, the surface property of the particles seems to be the dominating factor in their uptake by macrophages. Zeta potential of the particles has a direct and pronounced effect on their phagocytosis. As the absolute value of zeta potential increases both for negatively and positively charged surfaces, the phagocytosis of the particles is increased. The lowest phagocytosis is observed for particles with surface zeta potential of zero (Tabata and Ikada, 1990). The dependence of the phagocytosis on the zeta potential of the charged microspheres seem to be related to the negative charge of the macrophage membrane and the presence of the divalent cations such as Ca^{2+} and Mg^{2+} in the culture medium (Tabata and Ikada, 1990). Numerous studies have demonstrated the direct effect of physicochemical properties of particle surface such as hydrophobicity on their phagocytosis (Goodwin *et al*, 1974) (Van

Oss, 1978) (Kozel *et al.*, 1980). Generally, the higher the surface hydrophobicity of the particles, the higher uptake by macrophages (Van Oss *et al.*, 1972). In addition there seems to be an optimal surface hydophobicity for phagocytosis which does not depend on the bulk properties of the microspheres such as their degradability, but by their surface properties. The presence of proteins and other additives associated with the particles affects macrophage phagocytosis. Immunological proteins such as immunoglobulin G (IgG and its four subclasses; IgG1, IgG2, IgG3 and IgG4) and the third component of complements (C3b) enhance phagocytosis of the particles. This process is known as opsonisation. Complement was discovered many years ago as a heat-labile component of normal plasma that augments the opsonisation of bacteria by antibodies and allows some antibodies to kill bacteria. Proteins such as methylated albumin (Gambrill *et al.*, 1973), fibronectin (Mclain *et al.*, 1976) fibrinogen or fibrin (Van Oss, 1975) and macrophage migration inhibition factor (Dy *et al.*, 1974) possess opsonizing potential. Tabata and co-workers carried out studies on the effect of various proteins grafted on the surface of cellulose microspheres on their phagocytosis. They found that cellulose microspheres grafted with BSA underwent the least phagocytosis. The addition of other proteins such as gelatin and IgG significantly increased the phagocytosis of cellulose microspheres (Tabata *et al.*, 1990). They also found that lowering the hydrophobicity of the microsphere surface by pre-coating with non-proteinaceous macromolecules such as polyvinyl alcohol (PVA), dextran and polyvinlypyrolidone (PVP) led to a reduction in macrophage phagocytosis.

1.4.2.3. Phagocytosis of microspheres *in vivo*

The size of the microspheres plays a very important role in their distribution and fate after intravenous injection, regardless of the nature of the matrix. Microspheres with a diameter larger than 7 μm are mainly cleared by simple entrapment or filtration in the capillary bed

of the lung (Poste *et al.*, 1983). Smaller microspheres with a diameter between 0.1 to 7 μm will normally pass through the lung and accumulate in the liver and the spleen. There appears to be a clear relationship between particle size and uptake in the spleen; the greater the size, the more efficient the uptake process (Moghimi *et al.*, 1991). Moghimi and co-workers have shown that 250 nm particles coated with poloxamine 407 are taken up at a significantly higher rate than 60 nm and 150 nm particles (Moghimi *et al.*, 1991). Some histological studies have demonstrated that microspheres of 3 to 5 μm were found consistently in the vascular channels, Kupffer cells, and the sinusoids of the liver and spleen. The same studies show a predominant distribution of microspheres of 12 μm in the capillaries of the alveolar walls and, occasionally free in the alveolar lumina in the lung (Schroeder *et al.*, 1978; Hoshioka., 1981). Microspheres smaller than 100 nm could leave the systemic circulation through fenestrations in the cells lining the blood vessels. These fenestrations vary in size depending on the capillary beds. The capillary endothelium of pancreas, intestines, and kidney has fenestrations of 50-60 nm while that of liver, spleen, and bone marrow has about 100 nm. It has been thought that under normal circumstances, particles being recognised as foreign will be removed largely by the macrophages resident in the liver. The spleen uptake is thought to be low since only a small fraction of the blood (7%) circulates through the spleen (Davis *et al.*, 1993). The physicochemical properties of the microspheres govern their uptake by macrophages residing at liver, spleen, and bone marrow. These physicochemical properties include surface charge and surface hydrophobicity (Illum *et al.*, 1982) similar to that observed *in vitro* experiments. Microspheres with more hydrophobic surfaces are cleared more rapidly by macrophages compared with those with less hydrophobic surfaces (Van Oss, 1975). Polystyrene microspheres, which possess hydrophobic surfaces, are normally taken up by MPS cells of the liver and spleen after i.v. injection (Kanke *et al.*, 1983). The rapid and rather efficient

clearance of microspheres is a result of two interrelated processes. The first is the coating of particles by opsonising factors such as IgG and complements, and the second is the adhesion of the particles to the surface of macrophages and their subsequent engulfment. Altering the surface hydrophobicity could also affect the specific uptake of particles by different organs. Wilkins and co-workers found that the positively charged gelatin–coated polystyrene microspheres accumulated initially in the liver and then in the spleen, whereas negatively charged microspheres of the same sized were observed in the liver and spleen. Coating of polystyrene microspheres with non-ionic surfactant Poloxamer 338 and 188 has been shown to result in a significant increase in the uptake of the number of microspheres reaching the lung and a corresponding reduction in their number reaching the liver (Illum and Davis, 1983, 1984).

1.4.2.4. Antigen release profiles from biodegradable polymeric microspheres

Sah and co-workers have carried out extensive studies on the release behaviour of bovine serum albumin from biodegradable microcapsule formulations (Sah *et al.,* 1994). They found that polymer composition and the ratio of polymer to protein were important factors influencing the release of the protein. Increased amount of the polymer in dense, less porous polymeric microspheres lead to formulations, which inhibit the burst effect. In contrast, an initial burst effect was related to the porosity of the microspheres. Studies with ovalbumin as model protein demonstrated that in two polymers with different degrading properties, the immune response was polymer dependent (O'Hagan *et al.*, 1994). Both polymers showed increased serum IgG and salivary IgA antibody responses compared to immune responses induced by soluble ovalbumin. The polymer with a higher degradation rate, induced higher levels of salivary IgA antibodies, whereas the more slowly degrading polymer induced higher levels of serum IgG antibodies. The release

mechanism of encapsulated antigens within PLA/PLGA microspheres is a combination of surface and bulk erosion of the polymer, diffusion of the drug through the polymeric matrix and release through the pores (Aguado and Lambert, 1992). The approximate time for biodegradation of D,L and DL isomers of the PLA/PLGA polymers is summarised in table 1.5. The biodegradation times and therefore the release of the encapsulated antigens may vary depending on the implant surface area, porosity, and molecular weight (Lewis, 1990). The degradation rates for PLGA polymers have been shown to be influenced by their crystallinity and hydrophobicity (factors that affect the polymer chain packing) (Gombotz and Pettit, 1995).

Table 1.5. Approximate time for the biodegradation of Lactide/Glycolide polymers (adapted from Lewis, 1990).

Polymer	**Approximate time for biodegradation (months)**
Poly (L-lactide)	**18-24**
Poly (DL-lactide)	**12-16**
Poly (glycolide)	**2-4**
50:50 (DL-lactide-co-glycolide)	**2**
85:15 (DL-lactide-co-glycolide)	**5**

The PLGA degradation (catalysed by hydrolysis) and thus drug release kinetics are affected adversely by a crystalline or hydrophobic polymer composition. There are four specific factors that influence the copolymer crystallinity and hydrophilicity which are: (i) the ratio of lactide to glycolide monomer in the copolymer, (ii) the steroregularity of the monomer units in the polymer, (iii) randomness of lactide and glycolide repeat units in the copolymer backbone which decreases the ability of chains to crystallise (Giliding *et al.*, 1981), and (iv) low molecular weight (Gombotz and Pettit, 1995). Copolymers with approximately 20-70% glycolide content are amorphous where as copolymers, which are

rich either in lactide, or glycolide are crystalline (Giliding *et al.*, 1981). The stereochemistry of the monomers, affects the polymer chain packing which causes the D,L-poly(lactic acid) to exist as a more amorphous solid than L-poly(lactic acid) (Migliaresi *et al.*, 1994). Low molecular weight polymers tend to be more hydrophilic and degrade faster than high molecular weight polymers (Chawla *et al.*, 1985). Surface hydrophobicity of the polymer has been shown to influence the induced immune response (Alpar *et al.*, 1994). It can therefore be concluded that various factors such as the above-mentioned influence the release of proteins from polymeric microspheres and a knowledge of this, could influence the design of formulations for specific requirements. Table 1.6. illustrates the list of some of the factors influencing protein release kinetics.

Table. 1.6. Various factors influencing protein release kinetics (adapted from Crotts and Park 1998)

Polymer molecular weight
Polymer composition (hydrophobicity/hydrophilicity, amorphous/crystalline)
Uncapped or capped terminal end groups
Residual amount of metallic catalyst
Protein molecular weight
Isoelectric point of the protein
Amino acid composition
Size and distribution of microspheres
Surface and internal morphology of microspheres
Protein loading amount within microspheres
Residual moisture content in microspheres
Addition of excipients
Buffer capacity of incubation medium
Volume of buffer solution for the incubation of microspheres
Amount of microspheres in the incubation medium
Method of sampling for released protein

1.4.3. Microsphere technology and formulation of PLLA/PLGA microspheres

1.4.3.1. Emulsification and microsphere preparation

Double emulsions have always been considered to have a high potential for sustained and prolonged release of active materials, mainly in the field of water-soluble drug delivery. Double emulsions have been used to prepare formulations delivering a variety of drugs (Cole and Whateley, 1997; Ghosh *et al.*, 1997; Safwat *et al.*, 1994; Khopade *et al.,* 1996). Drugs can be encapsulated within polymers using the of w/o/w double emulsion solvent evaporation method or w/o/o solvent extraction (coacervation). Polylactides are insoluble in water and must be dissolved in an organic solvent such as ethyl acetate or methylene chloride (oil phase). The antigen is then added to this either in a solid form or an aqueous solution to form a fine suspension or emulsion. This emulsion is then used to form the microspheres (Cleland, 1995). The traditional process of producing PLA/PLGA microspheres involves the process of solvent evaporation in order to remove the organic solvent, which is required to solubilise the polymer. The solvent used is typically methylene chloride or ethyl acetate. In a typical solvent evaporation process, microspheres are formed by first dissolving the polymer in the organic solvent. This is then mixed with a liquid or solid form of the protein to produce an emulsion (liquid protein) or suspension (solid protein). The mixture is then added to a solution of an emulsifying agent such as polyvinyl alcohol in water, which results in the formation of polymeric microspheres. The nascent microspheres are then added to excess of water to facilitate the removal of the organic solvent (Cleland, 1998). The final microspheres are then ultracentrifuged and washed to remove surface adsorbed particles. The final stage of the preparation is the drying of the microspheres which could be done either, by lyophilization or air-drying. Figure1.3 is a diagrammatic representation of the w/o/w solvent evaporation method. For coacervation, the first antigen/oil emulsion is transferred to a solvent in which the polymer

has a low or negligible solubility, non-solvent (e.g., silicone oil). Mixing the non-solvent with the first emulsion results in the production of microspheres, forming a second emulsion (solid or water in –oil-in-oil). Another non-solvent such as heptane is then added to the final emulsion to extract the organic solvent from the first oil phase. The excess solvents in the supernatant are then removed and the final microspheres are dried.

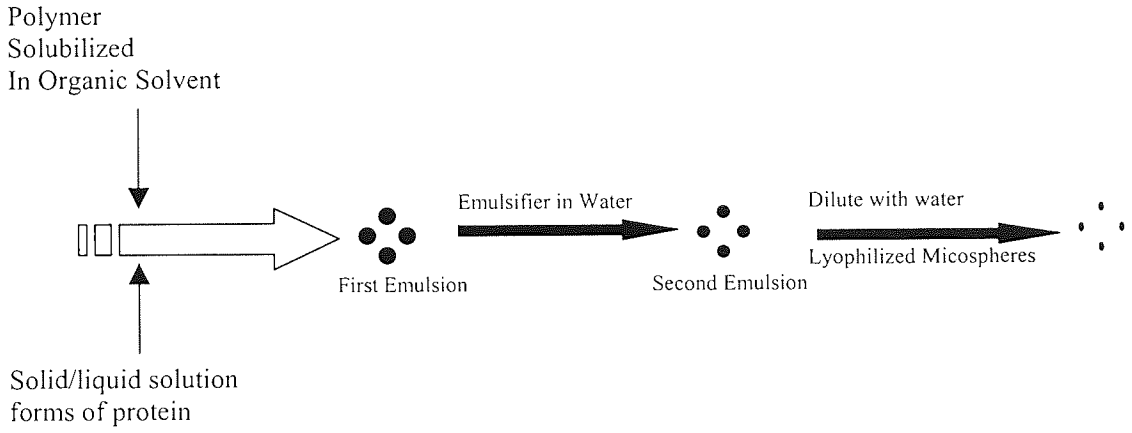


Figure 1.3. Solvent evaporation method for the production of polymeric microspheres adapted from Cleland, 1998).

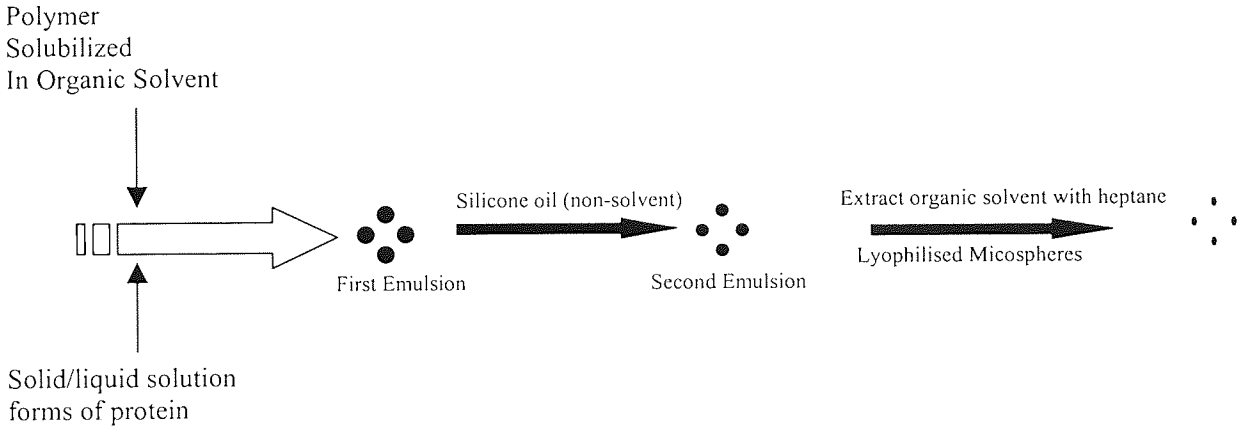


Figure 1.4. Coacervation method for the production of polymeric microspheres (adapted from Cleland, 1998).

Compared with solvent evaporation, the coacervation-phase separation method (Fig 1.4.) is probably as versatile in terms of the range of drugs and polymers which can be used. However, there are more stages of manufacturing processes involved. Coacervation technique has the advantage of allowing the entrapment of liquids such as oils, since the polymer is deposited as a coat around the material to be encapsulated. A feature which is lacking in the manufacture of microspheres using the solvent evaporation method. The structure of microspheres formed using solvent evaporation method is essentially drug dispersed through a matrix as a solid or a molecular dispersion, whereas those produced by coacervation are essentially capsular in structure (Watts *et al.*, 1990). Therefore, in coacervation-phase separation, coating integrity is critical for preserving the microsphere properties.

1.4.3.1.1. Double emulsion solvent evaporation method (w/o.w)

The double emulsion solvent evaporation method also known as in-water drying method, is one of the most useful methods for the entrapment of water-soluble compounds (Couvreur *et al.*, 1997; Leo *et al.*, 1998; Schugens *et al.*, 1994). In microspheres prepared using single o/w emulsion the water-soluble molecules are not entrapped efficiently due to their rapid dissolution in the aqueous continuous phase. The double emulsion solvent evaporation method, overcomes this problem. The w/o/w double emulsion is generally used for the entrapment of proteins and peptides. The introduction of these particles to the internal phase results in the formation of microcapsules which have increased encapsulation efficiency compared with those particles produced by the single emulsion solvent evaporation method (Garti *et al.*, 1998). The particular location of the proteins within the two emulsions leads to its stabilising effect on the double emulsion and the loading. Liquid-liquid emulsification is a critical step in the double emulsion (w/o/w or o/w/o) entrapment of particles. Many factors influence the characteristics (size,

entrapment efficiency, release kinetics) of the formed particles using the double emulsion method. Recent studies have shown the dependence of the particle size on homogenisation intensity and duration. It has been found that an increase in the intensity and/or duration of homogenisation process leads to a decrease in the size of microspheres (Zambaux *et al.*, 1998, Maa *et al.*, 1997, Nihat *et al.*, 1995). The other factors that influence the particle size are as follows: viscosity, total volume size and volume ratio of the continuous phase to the dispersed phase, and the rotor design of the homogeniser. The entrapment efficiency of the microspheres formed by w/o/w emulsion system is also affected by various factors. Jeffery and co-workers studied the effect of varying several parameters on the entrapment efficiency of a model protein OVA in a double emulsion solvent-evaporation technique. They found that an increase in the volume of the external aqueous, an increase in the volume and viscosity of the internal aqueous phase led to an increase in the entrapment efficiency of OVA (Jeffery *et al.,* 1993). The stability of the double emulsion system is also affected by several parameters. Recent studies have shown that the pH of the inner emulsion plays a role in its stability and also the entrapment efficiency (Leo *et al.*, 1998). The surfactant used in the emulsion plays an important role in its stability (Nianxi *et al.*, 1992). The authors found that ester compounds such as Span 80 and triethanolamine oleateon (FM) are less successful in stabilising the emulsion compared to compounds such as E644 (polyamine). The concentration of the internal phase is also an important factor in the stability of the double emulsion systems. Nianxi and co-workers have found that an increase in the concentration of the internal phase decreases the stability of the emulsion system. The addition of electrolytes has been shown to improve the stability of the w/o/w emulsions by counter-balancing the pressure differences between the inner water droplets and also by playing a critical role in the balancing osmotic pressure effects and between the two water phases (Rosano *et al.*, 1998). Although the double emulsion method is a

very useful method in encapsulation of water-soluble proteins, it suffers from many drawbacks in relation to protein stability. Since proteins are frequently exposed to cavitation, heat, organic solvent, or shear during the microencapsulation process, their integrity and functional activity may be compromised (Sah, 1999). The first emulsification step to make the w/o emulsion is considered to cause significant protein aggregation and denaturation (Morlock *et al.*, 1997; Cleland *et al.*, 1996). In order to maintain the stability of encapsulated antigens and promote their release in an intact form, initial studies of antigen stability may be performed. These initial studies should include screening of stabilisers to prevent denaturation during encapsulation and incubation at physiological pH, ionic strength and temperature over the desired release time (Hanes *et al.*, 1997). Cleland and co-workers have devised a rapid method for screening stable formulation in encapsulating two model proteins. These studies involved the formulation of proteins in different buffers and excipients, followed by emulsification of protein solution in an organic solvent. After this step, the protein was extracted from the organic solvent by addition of excess buffer. The extracted protein was characterised by analytical methods that shed light onto the physical and chemical state of the protein. The findings of these studies and other similar investigations have shown that excipients such as mannitol, trehalose, gelatin and human serum albumin can stabilise proteins during their emulsification in organic solvents (Cleland and Jones, 1996; Chang and Gupta, 1996). It is thought that mannitol and trehalose, exert their stabilising effects through their ability to cause preferential hydration of the protein (Arakawa *et al.*, 1993), resulting in a reduced exposure of the proteins to the water-organic solvent interface. Although the general approach of using known stabilising excipients might apply for protein microencapsulation, the suitability of each must be determined for individual antigens in different formulations. It is also important to have an understanding of the stability aspects

of proteins *in vivo* after the administration of formulations. Porous PLGA microspheres typically become fully hydrated within a short period of time (minutes to hours), when placed in aqueous environment such as an intramuscular or subcutaneous injection site (Shah *et al.*, 1992). Therefore, the antigen encapsulated within the above polymers is exposed to an aqueous environment for extended periods of time prior to its release. In assessing the stability of the incubated antigen, it is essential that all conditions are simulated to mimic the physiological environment, including physiological ionic strength, pH and temperature (serum proteases or other macromolecules which can not readily diffuse through the PLGA matrix need not be included). The above conditions may exert detrimental effects on the proteins by subjecting them to chemical reactions and other processes which may lead to protein aggregation (Cleland and Langer, 1994). The most common reactions, which may cause protein degradation, include oxidation, deamidation and aggregation (Arakawa *et al.*, 1993). These reactions are highly antigen-dependent and may affect the immunogenicity of a particular antigen. Many studies have investigated the effects of stabilising agents or the protection of proteins within biodegradable polymers (Audran *et al.,* 1998; Schwendeman *et al.*, 1998; Chang and Gupta, 1996). Cleland and co-workers investigated the stability of recombinant human growth hormone encapsulated within PLGA microspheres and compared its degradation in the microspheres to its degradation in aqueous solution (Clealand *et al.*, 1997). In this study both the physical and chemical degradation of the protein was evaluated. The authors found that the PLGA did not influence the rates of protein degradation and that the pH within the PLGA microspheres was not significantly different from the bulk solution when the release buffer was replenished daily. Under physiological conditions, the microspheres will be exposed to a large excess of buffer ions and salts as well as a continuous flow of fluid both in subcutaneous and muscular tissues. Therefore, ensuring the stability of the antigenic

proteins under these conditions will mean its release in its native form *in vivo* at the desired time.

1.5. Routes of delivery of polymeric microspheres

1.5.1. Parenteral administration

Most vaccine developments have focused on the parenteral route of administration. Although effective in the induction of the systemic immunity there are several disadvantages associated with this delivery route. The systemic immunity may not always provide an optimal protection against several types of infectious diseases. This is particularly the case, for the mucosally contracted diseases such as cholera, influenza and probably AIDS (Mestcky *et al.*, 1994). In contrast to the mucosal immunity, systemic immunity seems to suffer from age-associated dysfunction, which limits its protection for the elderly (O'Hagan, 1998). Also the mucosal immunity is developed earlier than systemic immunity which makes it more attractive to target in vaccination of the very young. The parenteral administration is also associated with the pain and discomfort for the patients and carries the risk of infection due to the use of inadequately sterilised needles. The need for the presence of trained personnel makes this route of delivery expensive. All the above disadvantages associated with parenteral administration of vaccines, makes the induction of mucosal immunity through other routes of delivery such as oral or nasal administration of vaccines extremely attractive.

1.5.2. Mucosal delivery and mucosal immunity

The mucosal surface area of an adult individual is 400 m^2, which is two hundred times larger than the area of the skin (Brandtzaeg *et al.*, 1997). Mucosal surfaces are the most frequent portals of entry of infectious agents, allergens and carcinogens (McGee *et al.*,

1990). The main humoral mediators operating on the mucosal surfaces are secretory IgA (SIgA) and secretory IgM (SIgM) antibodies (Mestecky *et al.*, 1987). These antibodies are regulated and transported by mechanisms, which are quite distinct from those operating within the systemic immunity. The main sources of precursor cells, which are committed to the synthesis of IgA are organised lymphoepithelial structures found along the gastrointestinal and respiratory tracts. These are gut- and bronchus-associated –lymphoid tissues (GALT and BALT), which function as sources of antigen-sensitised and IgA-committed plasma cell precursors, populating the remote mucosal tissues and glands and constitute what is known as the common mucosal immunity (CMI). There is currently a great interest in designing vaccine delivery systems, which would target this common mucosal immune system for oral, intranasal, intra-vaginal, intra-tracheal and also rectal delivery of vaccines (O'Hagan, 1997).

1.5.2.1. Gut-associated lymphoid tissue

The gut-associated lymphoid tissue is a major component of the common mucosal immunity. Specialised lymphoreticular tissues in gastrointestinal tract possess the capacity to take up environmental antigens (Rudzik *et al.*, 1975). Oral administration of antigens stimulates T helper cells and IgA precursor B cells in the inductive regions of GALT (particularly in Peyer's patches). This leads to the dissemination of B and T helper cells to mucosal effector sites such as the lamina propia of the gastrointestinal tract to secretory glands, which leads to the induction of antigen specific secretory IgA antibody responses.

1.5.2.2. Nasal-associated lymphoid tissue

The nasal administration of drugs takes advantage of the higher permeability of the nasal mucosa compared with other mucosal surfaces (Watanabe *et al.*, 1980; Illum *et al.*, 1988).

Its leaky epithelium allows the restricted passive permeation of high molecular weight molecules through intercellular pathways. The advantages which are associated with this surface as a delivery route for drugs are, the large surface area, the accessibility and easy administration that increases patient compliance (Almeida and Alpar, 1996). The nasal mucosa is highly vascularised and the venous flow that drains it, escapes the portal system allowing the blood to reach various tissues and organs before the liver, thus preventing a first-pass metabolism (Wuthrich and Buri, 1989). Nasal administration of drugs causes an immediate onset of pharmacological action, which makes it an ideal route of delivery for products that undergo degradation in the gastrointestinal tract (Duchene and Ponchel, 1993). The equivalent of nasal-associated lymphoid tissues (NALT) in humans is known as Waldeyer's ring. These consist of the adenoid or nasopharangeal tonsils, the bilateral pharyngeal lymphoid bands, the bilateral tubal and palatine tonsils and the bilateral lingual tonsils (Kuper *et al.*, 1992). The NALT are covered by an M-cell lymphoepithelium. The stimulation of NALT is initiated by the uptake of antigens. The particulate antigens are taken up mainly by the M cells of the lymphoid tissues, whereas, soluble antigens are mainly absorbed at the nasal epithelium (Kuper *et al.*, 1992). Particulate antigens are processed at the NALT and preferentially are drained to the posterior cervical lymph nodes (PCLN). Soluble antigens are contacted by the APCs associated with the nasal epithelium and are carried out to the superficial cervical lymph nodes (SCLN), which in turn drain to PCLN. Soluble antigens may induce a systemic immunity or a status of specific tolerance at the SCLN. Since they are also drained to the PCLN, which are involved in the enhancement of a secretory immune response, they could also induce secretory immunity in this way. The balance between the activation of the posterior or superficial cervical lymph nodes determines the nature of the immune response following nasal stimulation (Hameleers *et al.*, 1991). The nasal delivery route has been used in recent years to

immunise animal models (Gallinchan *et al.*, 1993, Roberts *et al.*, 1993). Nasal delivery route has been sought as a potential route of immunisation with microspheres. In the recent years a number of studies have demonstrated the benefits of this route for protection against some infections (Alpar *et al.*, 2001; Klinguer *et al.*, 2001; McNeela, *et al.*, 2001; Eyles *et al.*, 1998; Yen *et al*, 1996).

1.5.2.3. Oral delivery routes of vaccines

Oral delivery is the easiest and the most convenient way for delivery of drugs, especially in repeated or routine administration (Florence *et al.*, 1993). The potential advantages of this route are limited due to the degradation of orally administered drug by digestive enzymes in the gastrointestinal tract (GI). Microspheres, which encapsulate the desired antigen, may prove to be a protecting measure against the destructive effects of the digestive enzymes. Two important factors influence the successful delivery of particles through oral route: the first is the nature of the delivery vehicle, which should prove resistant to degradation in the GI tract, protecting the encapsulated drug (Chen and Langer, 1998). The second factor is the effective absorption rate of the encapsulated drugs in the GI tract. The absorption of the particles takes place predominantly at the intestinal lymphatic tissues (i.e. Peyer's patches) as evidenced by the majority of the available literature (Hillery *et al.*, 1994, LeFevre *et al.*, 1978, 1989). Particle uptake stimulates the T helper cells and IgA precursor B cells leading to the dissemination of B and T helper cells to mucosal effector sites such as the lamina propria of the GI tract and to secretory glands. This results in antigen specific secretory IgA antibody responses (Mestecky, 1987, Philips-Quagliata *et al.*, 1988). The epithelial cell layer overlying the Peyer's patches contains specialised M cells, which transport antigens from the surface luminal membrane to the pocket region of the Peyer's patches by transcytosis, apparently with little degradation or chemical

alteration (Sneller and Strober, 1986). The particle absorption mechanism and rate in the GI-tract has been shown to be size-dependent. Small (submicron) colloidal particles are thought to be absorbed and transported via the intercellular pathway through the endocytes, while larger particles (several microns) are absorbed exclusively by M cells of the Peyer's patches (Chen and Langer, 1998). Studies investigating the effect of particle size on the uptake, have shown that 5.7 μm particles were taken up into the Peyer's patches, whereas 15.8 μm were not (Lefevre *et al.*, 1980). Eldrige *et al.*, 1990, have demonstrated that the upper limit for particle uptake into the Peyer's patches was about 10 μm. In a recent study Desai *et al*, 1996, showed the existence of a size exclusion phenomenon in the GI tract absorption of particles, by demonstrating a significantly higher uptake (10-250 fold higher) for 100 nm particles than the larger particles. Eldrige *et al.*, 1990, have also shown that the nature and surface characteristics of the particles affect their uptake. They demonstrated that hydrophobic particles were taken up to a greater extent than hydrophilic particles. Other factors that affect particle uptake include, particle dose (Ebel, 1990), the administration vehicle (Le Ray *et al.*, 1994), the volume and tonicity of the administration vehicle (Eyles *et al,* 1995), age (La Fevre *et al.*, 1989), the fed-state of the animal (Ebel, 1990), and use of targeting agents on particles (Pappo *et al.*, 1991, Porta *et al.*, 1992). The oral delivery of drugs entrapped in PLA/PLGA microspheres has been shown to induce immune responses (Eldrige *et al.*, 1990, O'Hagan *et al.*, 1993, 1994). O'Hagan and co-workers,1993 have shown that oral immunisation in mice with OVA (1mg) encapsulated in PLGA microspheres induced potent serum and salivary antibody responses. The same group also showed that lower doses of OVA (100 μg) were effective in the induction of potent immune responses (O'Hagan *et al.*, 1997). The immunogenicity of PLGA microspheres entrapping OVA has been shown to depend both on the total number of particles *per* dose and the dose of antigen administered (Uchida *et al.*, 1994). It has been

demonstrated that the immunogenicity of PLGA particles with entrapped OVA also depend on the size of the particles with size 4 μm particles being optimal in the induction of serum immune responses, while 7 μm particles being optimal for mucosal IgA responses (Tabata *et al.,* 1996). PLGA microparticles have also been used for the oral delivery of DNA in mice, resulting in the induction of serum and mucosal immunity (Jones *et al.*, 1997). Recent studies have shown that protective immunity may be achieved following oral delivery of certain antigens (Jones *et al.*, 1997, Kende *et al.*, 1995).

1.6. Aims and Objectives

The objectives of this project have been to focus on the main aspects of novel vaccine formulations, namely the development of novel vaccine formulations using easy, mild manufacturing conditions, single-dose delivery of antigens, mucosal administration, stability of the entrapped antigen, improvement on the current application of established adjuvants, and new delivery vehicles for DNA vaccines. These objectives are addressed in chapters 3 to 8.

1. Microencapsulation of model protein antigens in polylactic acid and non ionic block copolymer microspheres. The objective of this study was the assessment of non ionic block copolymers for the development of novel biodegradable antigen delivery systems based on PLA polymers. These preparations were designed to use mild manufacturing conditions, which would be both easy to set up and also ensure reproducibility (chapter 3).
2. Immunisation with non ionic block copolymers/ poly (DL-lactide) microspheres encapsulating model antigens. The objective of this chapter was to assess the potential of the above particles for a single-dose delivery of clinically-relevant antigens such as

DT. This work also examined the mucosal delivery of the vaccine formulations and evaluated the effects of combination dosing with saponins in order to modulate the immune response into induction of a more pronounced cell-mediated immunity (chapter 4).

3. Encapsulation of alum-associated antigens within PLA and PLGA microspheres. The aim of this study was to develop solid powder formulations incorporating alum particles in order to apply them as controlled release delivery vehicles, increase their shelf life and also to facilitate their use at room temperature (chapter 5).
4. Immunisation studies with alum-loaded microspheres. The objective of this chapter was to evaluate the immune response to encapsulated alum particles in association with TT. The study followed the immune response to the above particles over a long period of time after one single administration. The effect of microencapsulation within biodegradable polymeric microspheres was also investigated for cell mediated immunity (chapter 6).
5. Storage and stability of alum-loaded PLA/PLGA microspheres. This study evaluated the stability aspects of TT antigen encapsulated in different biodegradable polymers in association with alum particles. The stability studies were carried out both in terms of the immune response and also antigen structural integrity after a long period of dry storage of the particles. In this chapter the co-encapsulation of protein stabilisers was also investigated (chapter 7).
6. Microspheres for the delivery of Hepatitis B plasmid DNA. This study examined the suitability of optimised NIBC/PDLA microspheres as delivery vehicles for model plasmid DNA. The main focus of this chapter was the maintenance of the structural stability of the plasmid DNA (chapter 8).

2.0. Materials and Methods

2.1. Freeze-drying of microspheres

2.1.1 Background

Lyophilisation (freeze-drying) is the most common process for making solid protein pharmaceuticals (Cleland *et al.*, 1993; Fox, 1995). The freeze-drying process consists of two major steps: freezing of a protein solution and drying of the frozen solid under vacuum. The drying step is further divided into two phases: primary and secondary drying. The primary drying removes the frozen water and the secondary drying process removes the non-frozen 'bound water' (Arakawa *et al.*, 1993). The amount of non-frozen water for globular proteins is about 0.3-0.35 g g^{-1} protein (Rupley and Careri, 1991). In understanding and subsequent design of a robust lyophilisation cycle, several critical temperatures such as glass transition temperature (T'_g), collapse temperature (T_{col}), crystallisation temperature (T_{cry}), eutectic temperature (T_{eut}) and devitrification temperature (T_{dev}) should be understood and determined. During cooling of a protein solution, ice formation concentrates all solutes. Solute concentration eventually changes the solution from a viscous liquid to a brittle glass, which contains about 20-50% water (Pikal, 1990). The glass transition temperature (T'_g), refers to this reversible transition for the maximally freeze-concentrated solution. The collapse temperature (T_{col}), is the temperature at which the interstitial water in the frozen matrix becomes significantly mobile (Jennings, 1999). This temperature is closely related to the (T'_g), and had been considered to be equivalent to T_g (true glass transition of a pure polymer) of an amorphous system or to the eutectic melting temperature of a crystalline system (Slade *et al.*, 1989; Pikal, 1990). When the temperature of an aqueous protein formulation drops below 0°C, water usually crystallises out first, and then the crystalline component (usually having the least solubility in the formulation), may crystallise out. This temperature is termed crystallisation temperature.

Cooling the aqueous protein formulation beyond the crystallisation temperature leads to the crystallisation of the least soluble component causing its crystallisation with water at the same time as a mixture. This temperature is termed eutectic crystallisation/ melting temperature. Devitrification is a process by which a metastable glass forms a stable crystalline phase on heating above T'_g (Slade *et al.*, 1989; Chang and Randall, 1992). Further increase above T'_g may lead to an exothermic event, corresponding to the recrystallisation of a component such as mannitol (Meredith *et al.*, 1996). This temperature is termed devitrification temperature. The freezing step is thought to be as important as the drying step due to its potential effect on proteins during lyophilisation (Willemer, 1992). Cooling rate (v) is a critical parameter during this step and can be defined as:

$$v = \frac{\delta T\,(r,\, t)}{\delta t}$$

where $T\,(r,\, t)$ is the temperature field which is a function of both time, t, and location, r, and therefore, the rate varies temporally and spatially (Hartman *et al.*, 1991). In general, a faster freezing rate generates small ice crystals (Eckhardt *et al.*, 1991), whereas a slower cooling rate results in the formation of larger ice crystals. The size of crystals determines the pore size to be created during subsequent drying. Larger ice crystals generate large pores, resulting in the rapid water sublimation during primary drying (Willemer, 1992), but the secondary drying may slow down due to smaller surface areas, limiting water desorption during secondary drying (Bindschaedler, 1999). A moderate rate of supercooling (10-15 °C) has been recommended to ensure a balance (Pikal, 1990). As mentioned earlier there are two stages in the drying process. When the shelf temperature is fixed, the drying / sublimation rate, v of a frozen solid can be expressed as:

$v = A_p (P_p - P_o) / R_p$

Where, A_p = cross sectional area of a product
P_p = product vapour pressure at sublimation front
P_o = partial vapour pressure in a product vial
R_p = resistance of a dried product layer to vapour flow

R_p may be different under different freezing conditions. Only through adjustment of P_o the rate of drying can be changed in a given cycle, since all the other parameters are usually fixed at a freezing rate for a particular protein formulation. The driving force for water sublimation during lyophilisation is the temperature difference between the product and the condenser. The commonly used condenser temperature is – 60 °C, allowing a minimum of 20 °C lower than the product temperature during primary drying (Franks, 1990). To achieve a high drying rate, product temperature is set as high as possible. The shelf temperature controls the product temperature and as such, effective heat transfer between shelf and product is essential, which is affected by the degree of vacuum in the drying chamber. A moderate increase in chamber pressure often increases the drying rate due to more effective heat transfer, leading to higher product temperature (Bindschaedler, 1999). It has been suggested that a chamber pressure at one-fourth to one-half of the saturated vapour pressure over ice usually lead to high sublimation rate (Bindschaedler, 1999). To have an efficient drying step and to reduce probability of product collapse, a formulation should be designed such that its T'_g is as high as possible. To ensure a high T'_g during secondary drying, the primary drying cycle should be completely finished so that only a minimum amount of bound water is left in the formulation. The end of primary drying process is when all the frozen water is removed and the rate of water sublimation is significantly reduced.

2.1.2. Procedure

Aliquots (5ml) of diluted aqueous suspensions of washed microspheres were dispensed into glass drying tubes and frozen at –70 °C for 60 minutes. After this pre-freezing, the microsphere samples were placed on the cooled (-40 °C) shelf of the freeze-dryer (VIRTIS ADVANTAGE, Biopharma process system, Winchester, UK). The samples were allowed to equilibrate for a while before setting the shelf temperature at –20 °C. The cycle was continued for 48 hours at a chamber pressure of ~0.2 Torr. Dried microspheres encapsulating subunit and model antigens were stored desiccated.

2.2. Particle size analysis by laser diffraction

2.2.1. Background

Laser diffraction has become the predominant technique for controlling particle size within the pharmaceutical industry. Laser diffraction measures particle volume, which is generally the most appropriate variable for the pharmaceutical industry. The laser diffraction method, more correctly termed Low Angle Laser Light Scattering (LALLS) relies on the fact that diffraction angle is inversely proportional to particle size. Instrumentation consists of a laser source of coherent intense light (helium-neon gas laser with wavelength of 633 nm in Mastersizer E), a suitable detector such as photosensitive silicon, and a means of passing the sample through the laser beam. The particles pass through an expanded and collimated laser beam in front of a lens in whose focal plane is positioned a photosensitive detector, consisting of a series of concentric annular rings. The unscattered portion of the laser beam is brought to focus in the detector plane and is usually allowed to pass through an aperture at the centre of the detector and effectively out of the system. However, the annular rings centred around the axis of the incident laser beam record the intensity of the light scattered over a range of angles and the distribution

of scattered intensity is analysed by a computer size distribution (Rawle, 1995). The software uses Mie theory to deduce the volume of the particle from the light scattering observed. Mie theory predicts all paths of light around and through the particle and copes with partially absorbing particles including those which are totally opaque or clear. Therefore, it may be applied over the entire range of particle types and sizes to predict scattering at any angle from forward diffraction to back scatter. Many thousands of particles may be illuminated at any one time and the diffraction pattern will change in time as the number distribution of particles in each size class varies with particles entering and leaving the beam. This temporal variation is usually integrated to give an average for a truly representative sample of the particles present. The amount of light scattered by a particle is proportional to the sixth power of its diameter. Thus in the 0.1 to 1.0 μm range, the ratio of scattered intensities is in the order of 10^6 (Malvern Masrersizer E, Handbook Malvern Instruments, Malvern, UK). The LALLS technique generates a volume distribution for the analysed light energy data. This volume distribution can be converted to another length or number, but such a derivation involves assumption about the form of the particle. The data may be heavily skewed by a small number of large particles or aggregates.

2.2.2. Procedure

Microspheres were analysed using the MasterSizer/E™ (Malvern, Worcs., UK). Freeze-dried samples were suspended in distilled water and made into a slurry. The suspension was then ultrasonicated for 30-60 seconds. Particle sizes were determined using the multimodal and monomodal analysis (suitable for spherical particles) of the MasterSizer/E™ fitted with a 45 mm angle lens and a flow cell and analysed by the 2OFD presentation. Results were expressed as volume and number mean (±SD). Before each

presentation. Results were expressed as volume and number mean (±SD). Before each size measurement the instrument was checked using polystyrene standards dispersed in aqueous solution.

2.3. Scanning electron microscopy

2.3.1. Background

The history of electron microscopy stretches back about fifty years. A major driving force for the development of different types of electron microscopy has been the limitations encountered when using light microscopy. Using electrons with wavelengths much less than 0.1 nm (1 Å) would provide better resolution than light microscopy (limited by visible wavelengths to about half a micrometer). In addition the depth of field and depth of focus of electron microscopes has proved to be much better than those of a light microscope at the same magnification. Scanning electron microscopes (SEMs) are mainly used to study the surface morphology of materials. In SEM, a fine beam of electrons is scanned across the surface of the specimen in synchronism with the spot of the display cathode ray tube (CRT). A detector monitors the intensity of a chosen secondary signal from the specimen and the brightness of the CRT spot is controlled by an amplified version of the detected signal. If for any reason, the intensity of the emitted secondary signal changes across the specimen then contrast will be seen in the image on the CRT. SEM produces images that are readily interpreted because they contain light and shade in much the same way as everyday images, which are familiar to our eyes. However, there are differences between the way in which the human eye collects an image and the way an image is formed in SEM microscopy. One difference is that there is no 'ray path' between the object and the image and this, accounts for the importance of electronics in manipulating and displaying the image before it is displayed on a cathode ray tube. A second significant difference is that

simultaneously (the image points are received in parallel) while an SEM image is collected point by point, that is in a 'serial' fashion. The major components of the SEM which require careful alignment and need to be set up properly are the electron gun (a filament is heated to white heat and gives off many thermoionic electrons), the condenser lens (es), which form the illumination system and generate the fine beam, the scan coils, and the detector. All SEMs also contain small apertures, which limit the angular spread of the electron beam (Goodhew and Humphreys, 1988).

2.3.2. Procedure

Scanning electron microscopy was used to analyse the size and morphology of microspheres. A thin layer of microspheres was mounted onto aluminium stubs using carbon discs. The surface was then coated with 20nm-thick gold film for 90 seconds under an argon atmosphere in a sputter coater (Emscope SC500). Microspheres were examined using a scanning electron microscope (Cambridge Instruments Stereoscan 90B, 25 kV).

2.4. The standard bicinchionic acid protein assay (BCA)

2.4.1. Background

The bicinchionic acid (BCA) assay benefits from both sensitivity and reproducibility and is suitable for working with small sample volumes (Smith *et al.*, 1985). The assay is based on the Lowry protein assay (Lowry *et al.*, 1951). In this assay the reaction of protein with an alkaline copper II (Cu^{2+}) reagent (Reagent B) produces copper I (Cu^{1+}). This copper I ion then reacts with two molecules of reagent A containing 4,4-dicarboxy-2,2-biquinolone (bicinchoninic acid) to form a copper/peptide chelate. The product of the reaction is water-soluble with an intense purple colour, which can be quantified by spectrophotometric measurement at a λ_{max} of 562 nm (non-overlapping with the polymer spectrum). This

assay is subject to less interference than the Lowry assay and is also more sensitive (concentrations of ~ 10 μg ml^{-1} of protein/peptide). Sample volumes can be reduced to 20 μl by performing the assay in a microtitre plate (flat bottomed 96-well microtitre plates, Fisher, Leics, UK).

2.4.2. Procedure

The protein loading of microparticles were determined in triplicate by their digestion (10-20μg) in sodium hydroxide (1M) until the medium lost all turbidity, neutralisation of the solution to pH 7 with hydrochloric acid (10M), then submitting samples and standards (treated in the same manner) to BCA analysis. This included the mixing of one volume of sample or standard (20μl) with ten volumes of (200μl) of freshly-prepared protein assay reagent. The plates were then incubated at 60 °C for colour development, and were then allowed to cool to room temperature prior to spectrophotometry readings. The absorbance of the contents of each well was determined at 562 nm using a microtitre plate reader (Anthos reader 2001, Anthos Labtech Instruments, Austria). A minimum of four samples of each test solution was used. Reagent solution A (Pierce, Rockford, Illinois, USA) and cupric sulphate 4% w/v reagent B were mixed together in the ratio of fifty volumes of solution A and one volume of solution B (typically 20 ml + 0.4 ml respectively). Aliquots (200μl) of the above mixture were added to each well of a 96-well microtitre plate containing 20μl of either standards or unknown concentrations of proteins. It was thus ensured that both the standards and test samples were treated under similar conditions. Following the absorbance reading, a standard graph of absorbance versus protein concentration was constructed for the range of protein standard solutions used, in order to determine the concentration of the test samples by the way of extrapolation from the graph.

Figure 2.1. represents the linear relationship between absorbance and protein concentration for a range of standard protein solution (5-200 μg ml^{-1}).

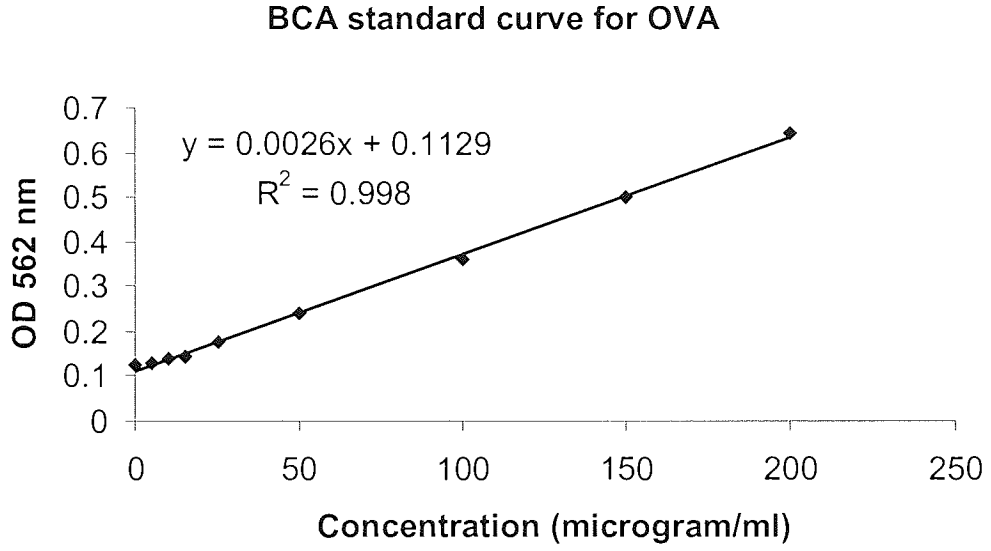


Figure 2.1. Typical calibration curve for protein solutions analysed by BCA assay, n=3.

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

2.5.1. Background

Any charged ion or group migrates when placed in an electric field. Since proteins carry a net charge at any pH other than their isoelectric point, the application of an electric field to a protein mixture in solution results in different proteins migrating at different rates towards one of the electrodes. A gel can be considered as a porous medium in which the pore size is similar to the size of the protein molecules so that a molecular sieving effect occurs and the separation is dependent on both charge density and size. In gradient

polyacrylamide gels the concentration of polyacrylamide increases (and hence pore size decreases) as the protein migrates further from the origin. This results in separation of a wide range of molecular masses (e.g. 14 - 300 kDa). Most proteins bind sodium dodecylsulphate (SDS) in a constant weight ratio (1.4 g of SDS per gram of polypeptide). If excess SDS is added to the protein sample the intrinsic charges of the polypeptide become insignificant and SDS - polypeptide complexes migrate in the polyacrylamide gels strictly according to polypeptide size. In addition to SDS, denaturing buffer systems contain a thiol reagent (to cleave disulphide bonds) and urea (to disrupt hydrogen bonds) with the net result that proteins dissociate into their individual polypeptide units. Proteins can be visualised directly on the polacrylamide gel by staining with Coomassie Blue or silver staining. Alternatively they can be transferred by electroblotting to a support matrix such as a nitrocellulose membrane. The mechanism of protein binding is not fully understood, but it is certainly non-covalent and probably involves hydrophobic interactions.

2.5.2. Procedure

The protein separation was carried out by gel electrophoresis with the Mini Protean system (Bio-Rad laboratories Ltd., Herts, UK). A discontinuous buffer system was used in order to obtain greater sample resolution. This involved a non-restrictive large-pore gel called a stacking gel, which is layered on top of a separating (running gel). The running and stacking gels were prepared as described (table 2.1.). Polymerisation was initiated by ammonium persulphate as the initiator peroxide and the quaternary amine, N,N,N,`N`-tetramethylene diamine (TEMED) as the catalyst under aerobic conditions (by addition of electrode buffer to the exposed upper surface of the gel).

Gel components	Running gel Acrylamide concentration (%gel) 10% Volume (ml)	15% volume (ml)	stacking gel (5%) Mold volume 5 ml
dd water	4.0	2.3	3.4
30% acrylamide	3.3	5.0	0.83
1.0 M Tris (pH 6.8)	-	-	0.63
1.5 M Tris (pH 8.8)	2.5	2.5	-
10% SDS	0.1	0.1	0.05
10% AMPS	0.1	0.1	0.05
TEMED	0.004	0.004	0.005

Table 2.1. Reagents for preparation of Tris-glycine SDS-PAGE gels (modified from Harlow & Lane, 1988).

The separating gel was cast between the glass plates (to ~ 2.5 cm from the top) which were separated by 0.75 mm or 1.5 mm plastic spacers and allow to set under anaerobic conditions for up to 30 minutes. The electrophoresis buffer was removed from the top of the set gel and the stacking gel was cast in order to concentrate the proteins. Denatured samples were prepared by mixing an equal volume of sample buffer (table 2.2.), heated at 100 °C on a solid heating block up to 90 seconds. Samples or molecular weight markers (table 2.3) were then loaded (up to 20 µl per well). The tank electrophoresis buffer (x5) was prepared as described in table 2.4.

Buffer constituents	**Volume**
500mM Tris.HCl pH 6.8	5.0 ml
10% SDS	4.0 ml
Glycerol	5.0 ml
Mercaptoethanol	0.5 ml
Bromophenol blue	2.0 mg
dd water	10 ml

Table 2.2. Reagents for preparation of sample buffer

A current of 60 mA was used for two 1.5 mm gels and maintained until the dye front reached the bottom of the gels.

Buffer constituents	**Quantity**
Tris.Base	15.1 g
10% SDS	50.0 ml
Glycine	94.0 g
dd water	950.0 ml

Table 2.3. Reagents for preparation of electrophoresis buffer

The gels were stained in 0.1% Coomassie brilliant blue in 50% methanol/ 10% acetic acid for up to 2 hours. The destaining solution consisted of 10% acetic acid/ 40% methanol. Following their destaining the gels were scanned using a UV transilluminator (Model White TRW 20 transilluminator model GDS 7500 Darkroom. UVP®, Upland, CA, USA).

Protein marker	**Molecular weight kDa**
Phosphorylase B	97.4
Bovine serum albumin	66.2
Ovalbumin	45.0
Carbonic Anhydrase	31.0
Trypsin inhibitor	21.5
Lysozyme	14.4

Table 2.4. Pre-stained molecular weight markers for SDS-PAGE

All chemicals were electrophoresis grade. AMPS solution (10% w/v) was used fresh and not stored.

2.6. Surface charge determination using zeta potential analysis

2.6.1. Background

There is a net electrical charge at the surface of a particle in liquid medium. There are many origins of this surface charge depending on the surface of the particle and its surrounding medium. These include the ionisation of groups (acidic or basic) on the particle surface or the adsorption of specific ions from the medium. The net surface charge on the particle surface affects the distribution of ions in the surrounding region, with an increased concentration of counter ions close to the surface. Thus an electrical double layer exists round each particle. There are two regions (inner and outer) in the liquid layer surrounding the particle. The inner region (Stern layer) contains a strongly bound concentration of ions where as the outer (diffuse) region is where ions are less firmly associated. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. During the movement of particles (e.g. due to gravity), the ions move with it, as opposed to those ions outside this boundary which remain dissociated from the particle. The potential at this boundary (surface of hydrodynamic shear) is the zeta potential. The exact location of the above boundary is an unknown feature of the electrical double layer and in reality is a region of rapidly changing viscosity (Shaw, 1991). The magnitude of the zeta potential gives an indication of the potential stability of the particles within a given medium. Large positive or negative surface charges result in the repulsion of particles thus minimising aggregation. However, particles with low zeta potential values, tend to come together and flocculate. The general dividing line between stable and unstable suspensions is generally taken to be either + 30 or – 30 mV. Values

more positive than +30 or more negative than –30 indicate the stability of the suspensions. The most important factor affecting zeta potential is pH. Zeta potential values, which are quoted without an indication of the medium pH are virtually meaningless. The zeta potential of particles van be determined by measuring the particle mobility in an applied electric field. The velocity of the article is dependent on the strength of electric field or voltage gradient, the dielectric constant of the medium, the viscosity of the medium and the zeta potential. The velocity of a particle in a unit electric field is referred to as its electrophoretic mobility. Zeta potential is related to the electrophoretic mobility by the Henry equation:

$$U_E = \frac{2\varepsilon z \mathcal{F}(Ka)}{3\eta}$$

where:

z	=	zeta potential (mV)
U_E	=	Electrophoretic mobility
ε	=	dielectric constant
η	=	viscosity
$\mathcal{F}(Ka)$	=	Henry's function

The units of K, termed the Debye length, are reciprocal length and K^{-1} is often taken as a measure of the ''thickness'' of the electrical double layer. The parameter a refers to the radius of the particle and therefore Ka measures the ratio of the particle radius to electrical double layer thickness. Electrophoretic determinations of zeta potential are most commonly made in aqueous media and moderate electrolyte concentration. $\mathcal{F}(Ka)$ in this case is referred to as Smoluchowski approximation. Therefore calculation of zeta potential from the mobility is straightforward for systems that fit the Smoluchowski model, i.e.

particles larger than about 0.2 microns dispersed in electrolytes containing more than 10^{-3} molar salt. For small particles in low dielectric constant media $\mathcal{F}$ (Ka) becomes 1.0 and allows an equally simple calculation. This is referred to as the Huckel approximation. For the present study the zeta potential determinations were carried out using a Malvern Zetamaster (Malvern Instruments, UK). A laser Doppler anemometry instrument such as the Malvern Zetamaster consists of two coherent laser beams derived from the output of a low power laser. These beams intersect within the sample cell, forming a beam crossover pattern of interference fringes. Particles moving across the fringes in response to the applied electric field scatter light with an intensity, which fluctuates at a frequency, related to their velocity. The frequency of the scattered laser light differs from the frequency of the initial laser beam. This shift is caused by the Doppler effect and is a function of the particle velocity. The signal from individual photons of scattered light are detected by a photomultiplier and analysed by a digital correlator to give a frequency spectrum from which the particle mobility and the zeta potential are calculated.

2.6.2. Procedure

Zeta potential of our microsphere preparations were evaluated in potassium chloride (1 x 10^{-3} M) buffer, which had been filtered through a 0.22 μm filter before use. This buffer is a weak electrolyte and provides a suitable environment for the measurement of zeta potential. Standards (Malvern zeta standards AZ 55, Malvern Instruments, Malvern, UK) were used prior to each measurement in order to monitor the operational efficiency of the instrument. The defined zeta potential of this standard was –55 mV at 25 °C, and the acceptable deviation from this value was ±5. Microsphere samples were diluted in potassium chloride (1mM) solution prior to analysis and the resulting zeta potential data

subjected to interpretation by use of the system software (Malvern Zetamaster software version 2.1).

2.7. Enzyme-linked immunosorbent assay (ELISA)

2.7.1. Background

The ELISA technique makes use of the formation of a specific antibody-antigen complex to detect the levels of the antigen under investigation (Engvall and Perlmann, 1971, 1972). For the antigen to be amenable to the ELISA method, it must be capable of evoking an immune response to provide the specific antibodies required in the ELISA protocol. The antibodies used in an ELISA protocol must be labelled in someway to allow the detection of the antigen. Also the antigen must be immobilised somehow either by sticking it directly to the tissue culture plates or if the antigen is a cell surface component then the cells must be immobilised. The ELISA method uses immobilised antigen incubated with an antigen-directed antibody. This is followed by washing to remove unbound antibody and then another incubation with an anti-immunoglobulin antibody conjugated with an enzyme. These two first incubations can be reduced to just one by using a labelled form of the antibody against the immobilised antigen. Finally, incubation with a substrate of the antibody-conjugated enzyme will result in a measurable reaction usually a change in optical density. This reaction can be quantified by setting up a standard curve using known concentrations of the antigen.

2.7.2.Procedure

Blood samples were taken from all mice on days 7, 14, 35, 56 and 105 and serum antibody responses were measured by ELISA. Polystyrene ELISA plates (sterile 96 well plates, Dynatech, Sussex, UK) were coated overnight with the required antigen (OVA, 0.4 mg/ml,

or DT 11.2 µl/ml, 50 Lf units, or TT 3.2µl/ml, 10Lf)(Lf = limes flocculating value and is equal to the amount of toxoid which when mixed with one International unit of anti-toxin produces an optimally flocculating mixture) and kept at 4 °C. Plates were washed three times with PBS-Tween 20 (0.05% v/v), using a multilabel plate reader VICTOR^2 (Wallac instruments, UK). Wells were blocked with 100 µl of BSA (4%w/v) solution at 37 °C for one hour. At the end of this time, plates were washed three times as before. Appropriate serial dilutions of test sera (10µl) were diluted in PBS in microtitre plates (96 well plates, Fisons, Leics, UK) and 60 µl transferred to the corresponding wells of the coated ELISA plates. The plates were incubated for one hour at 37 °C and then washed three times as before. To each well 60 µl of either anti-mouse polyvalent immunoglobulin peroxidase conjugate (Sigma LTD, Poole, UK)(diluted 1:1200 in PBS), or anti-mouse IgG2a (diluted 1:2000 in PBS), IgG2b (diluted 1:2000 in PBS) or IgG1 (diluted 1:1200 in PBS) (Serotec Ltd, Oxford, UK) were added. The plates were then allowed to incubate at 37 °C for one hour. The plates were washed three times as before with PBS-Tween 20 (0.05% v/v). ABTS substrate solution 60 µl (67 ml citrate buffer + 4 ABTS [2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] tablets + 7 µl H_2O_2 solution (30%) was added to wells and plates were incubated at room temperature until sufficient colour (green) had developed. The plates were then read spectrophotometrically at λ_{405nm} using ELISA plate reader (Wallac Instruments, UK). The end-point titre was taken to be the mean absorbance (OD_{405nm}) of negative control sera incubated under the same conditions.

2.8. Splenic cell proliferation assay

Immunised mice were humanely culled and their spleens removed and placed into ice-cold sterile PBS. Individual (not pooled) spleens (crude suspension of spleen cells) in 10ml working media (RPMI 1640, Life technologies), supplemented (to a resultant concentration

of 10% v/v) with foetal bovine serum (Life technologies), 20 mM L-glutamine (Sigma chemicals, Poole, UK), 10^5 U of penicilin litre^{-1}, and 100 mg of streptomycin litre^{-1} (Sigma chemicals, Poole, UK), was prepared by gently grinding the spleeen on a fine wire screen. After allowing the cell suspension to settle for approximately 5 minutes, the liquid was transferred to sterile 20 ml volume 'Falcon' tubes, being careful not to disturb the cellular debris at the bottom. The cell suspension was spun at 1200 rpm for 10 minutes. After centrifugation the supernatant was removed, the cell pellet re-suspended in 10 ml fresh working media and the centrifugation procedure was repeated. Following this final centrifugation the cell pellet was re-suspended in 5 ml fresh working media, and the cell concentration assessed using a haemocytometer and the trypan blue (Sigma chemicals, Poole, UK) exclusion test. The single cell suspensions were used to assess cytokine production and antigen specific proliferative responses.

2.8.1. Evaluation of antigen specific proliferative responses

The proliferation of cells is a complex, multistep process that is regulated by a vast array of extracellular and intracellular signals. Most assays that measure cell proliferation involve measurement of the rate of DNA replication in cells. The conventional method of measuring DNA synthesis involves the incorporation of tritiated thymidine into cellular DNA. Using sterile 96 well tissue culture plates (Fisher, Leics, UK), 100 µl volumes of viable splenic cells (1 x 10^7 cells ml^{-1}) were seeded onto 100 µl volumes of sterile serially diluted antigen (DT or TT in working media) to give a resultant concentration range of 0 to 1 Lf. As a positive control, cells were co-cultured with concanavalin A (Sigma chemicals, Poole, UK), at a concentration of 1 µg ml^{-1}. Covered plates were incubated in a humid (5% CO_2) environment at 37 °C for 72 hours. After this period of incubation, 0.5 µCi of [^{3}H]thymidine (Amersham, UK) in 40 µl volumes of sterile working media was added to

each well, and the incubation continued for a further 24 hours. The well contents were harvested onto glass filter mats (CAMCO, Cambs. UK) using a cell harvester (Titertek). The discs representing each well were punched from the filter mats into 5 ml volumes of scintillation fluid (Optiphase Hisafe, Fisher, Leics. UK) to measure the incorporation of [^{3}H]thymidine into the cultured cells using standard counting procedure. Results were expressed as both counts per minute and stimulation indices (mean activity of cells from immunised mice divided by the mean activity of cells from naïve animals).

2.8.2. Measurement of cytokine production *in vitro*

Interleukin-4 is a cytokine produced primarily by activated T lymphocytes, mast cells and basophils. It's an important regulator of isotype switching, including IgE production in B lymphocytes. It's an important modulator of the differentiation of precursor T helper cells to the Th2 subset that mediates humoral immunity and modulates antibody production. Inteferon gamma is also produced primarily by T lymphocytes and natural killer cells. It induces the production of cytokines and has been shown to augment IL-12 induced Th1 development (Wenner *et al.*, 1996). Cytokine levels (IL-4 and IFN-γ) in culture supernatants were determined after 24 hours incubation of original splenocyte cell cultures. Supernatants from triplicate cultures were pooled and stored at –70 °C until performing the assay. IL-4 and IFN-γ levels in the culture supernatants were quantified using Quantikine capture ELISA's (R&D Systems Europe ltd, Oxfordshire, UK) according to the manufacturers instructions. This assay employs the quantitative sandwich enzyme immunoassay technique. Monoclonal antibody specific for moue IFN-γ and IL-4 have been pre-coated on a microplate. Standards, controls and samples were pipetted into the wells and any mouse IFN-γ or IL-4 present in the samples were bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal

antibody specific for mouse specific IFN-γ or IL-4 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the Stop Solution was added. The intensity of the colour measured is in proportion to the amount of mouse IFN-γ or IL-4 bound in the initial step. The sample values were then read off the standard curve.

2.9. Size exclusion HPLC method

The TT in each microsphere preparation (~10mg) was extracted using 0.3 ml dichloromethane and (3 x 0.3ml and x 0.4 ml) PBS / 5% SDS. The freshly extracted aqueous solutions were then immediately used for HPLC analysis. The stability of TT toxoid antigen was evaluated using HPLC. A protein sample (100μl) was injected into the TosoHaas TSK Gel G3000 SW column (7.5 mm i.d. x 60 cm long) column, eluted by a 50mM phosphate buffer (pH 7.0) at a rate of 0.8 ml/ min and was detected at 280 nm. The changes in the profile were analysed qualitatively. The chromatograms were analysed for the structural integrity of antigenic proteins.

2.10. *In vitro* release of antigens

The release of antigens from microparticles was determined by incubation of particles (~5-10 mg) in 1mL of PBS containing 0.01% w/v sodium azide and 5mM SDS at 37°C. Microspheres were suspended in phosphate buffered saline (pH 7.5) in eppendorf tubes and shaken (~120 cycles min) in a thermostatted water bath at 37 °C. At increasing time points, aliquot samples were (200 μl) removed and centrifuged at (13,000 rpm, 10 min). This was replaced by fresh buffer (200 μl) in the original samples. Protein concentrations were determined by a BCA assay (section 2.4.2). Release studies were carried out in

triplicates samples for each time point. The results are expressed as percent protein released against time point and are the mean of three individual samples for each time point, (± SD). Volume dilutions were corrected for throughout the experiment.

2.11. Statistical analysis

The results were expressed as mean ± SD for all experimental data analysis. Statistical analysis was made using Excel 97 for windows. Student's t-test was performed to assess statistical significance. Results were considered statistically significant if $p < 0.05$.

3.0. Microencapsulation of model protein antigens in polylactic acid (PLA) and non ionic block copolymer (NIBC) microspheres

3.1. Introduction

3.1.1. Molecular structures and physical properties of NIBCs

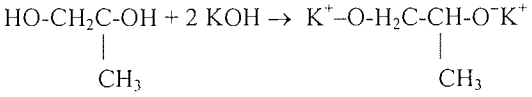
Nonionic block copolymers are a family of compounds consisting of propylene oxide associated with ethylene oxide, which were developed for use as surfactants (Schmolka, 1966, Lunsted and Schmolka, 1976). The most widely studied block copolymers are the Pluronics®. These copolymers usually consist of a hydrophobic poly(oxypropylene) (POP) core associated with hydrophilic poly(oxythylene) (POE) end blocks. The sizes and relative positions of the hydrophobic POP and the hydrophilic POE blocks can be altered during synthesis. In general Pluronics® are more soluble in cold water than in hot water, due to hydrate formation. The initial studies investigating the stabilising effects of these molecules were carried out using low molecular weight copolymers. These were used in oil-based emulsions where the copolymer molecules align at the interface. The surfactant properties of these compounds contribute to the stabilisation of the emulsion (Hunter *et al.*, 1981, 1986). The copolymer-stabilised emulsions have also been shown to offer the additional advantage of intrinsic adjuvanticity to certain model proteins (Hunter *et al.*, 1981).

3.1.1.1. Synthesis of copolymers

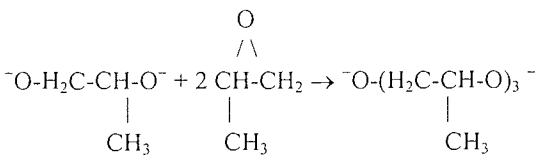
Alkylene oxide polymers are composed of oxirane monomers. These are three member heterocyclic ring compounds containing two carbon atoms and one oxygen atom. These compounds which are also known as 1,2 epoxides (more commonly known as alkylene oxides) are generally synthesised by the oxidation of alkenes and substituted alkenes. Ethylene oxide and propylene oxide are common examples of the above molecules and are used mostly for the production of polymers and associated reaction intermediates (Newman *et al.*, 1998). Block copolymers are generally synthesised through ring opening

addition polymerisation using two monomers, propylene oxide and ethylene oxide, added in predetermined sequences and amounts (Lundsted and Schmolka, 1976). The polymerisation involves the sequential addition of the monomers to active hydrogen atoms containing initiators in the presence of a suitable catalyst. The most commonly used initiator is propylene glycol and the base-catalysed (e.g. potassium hydroxide) reactions are the most widely accepted methods due to their simplicity and economic feasibility (Kastens, 1963). The chemical reactions for the base-catalysed polymerisation of propylene oxide and ethylene oxide are summarised below. The initiator is propylene glycol:

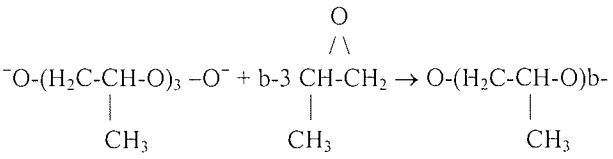
1. Reaction of propylene glycol with potassium hydroxide to form the salt.



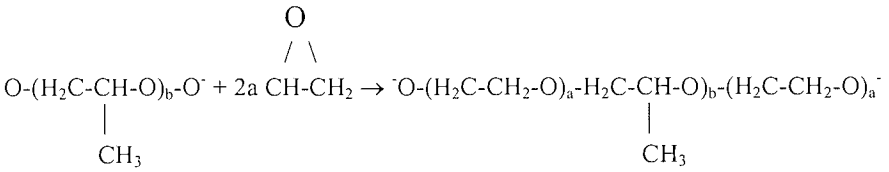
2. Initiator salt opening the 1,2-epoxide (e.g.propylene oxide) and is added to it.



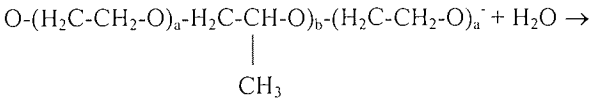
3. The resulting anion reacts with more epoxide molecules in step wise ring opening, and an addition reaction forms the hydrophobe of the desired molecular weight.

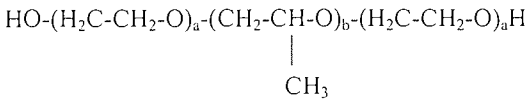


4. The hydrophobe is a "living polymer''chain being reactive at both ends. Thus it reacts with ethylene oxide forming the hydrophilic blocks on both ends of the chain.



5. The final stage involves the hydrolysation of the block copolymer anionic ends with water resulting in the regeneration of the catalyst. The regenerated catalyst can be removed by filtering the product after heating with magnesium silicate and silicone dioxide.





The copolymers were manufactured commercially by Wyandotte Chemicals Corporation and BASF Performance Chemicals (Parsippany, NJ) (Fig.3.1).

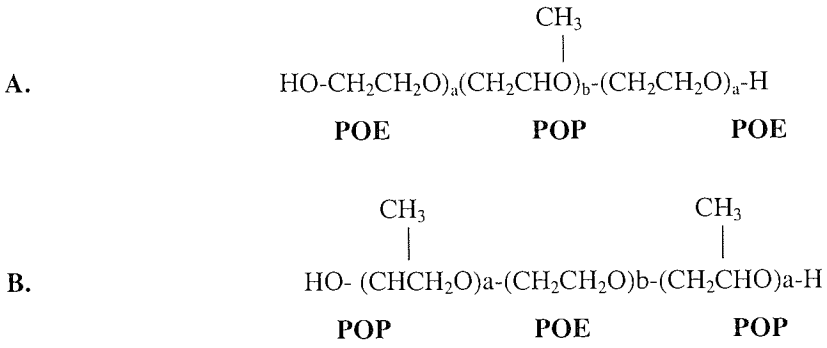


Figure 3.1. Structures of nonionic copolymers developed by BASF Performance Chemicals, A=Pluronic®, B= Reverse Pluronic®.

Pluronic® copolymers are classified according to a descriptive system developed by BASF. This system which is called the Pluronic® grid, is formed by plotting the molecular weight of the POP component against the amount of the POE, which is expressed as a percentage. This percentage is based on the MW of the intact POE-POP-POE product. According to the above grid the first two numerical digits are series numbers, related to the approximate MW of the POP component and the last digit refers to the POE block. The prefix letter L (liquid), P (paste), or F (flake) denotes the physical state of the pure copolymer (Fig 3.2.).

% poly(oxyethylene) in total product →

MW ↓	Pluronic 1st digits ↓	10	20	30	40	50	60	70	80
4000	12	L121	L122	L123				F127	
3625	11								
3250	10	L101		P103	P104	P105			F108
2750	9		L92		L94				F98
2250	8	L81			L84				
2050	7		L72						
1750	6	L61	L62	L63	L64	P65			F68
1450	5								F58
1200	4		L42	L43	L44				F48
950	3	L31				L35			P38

Figure 3.2. The Pluronic® Nomencluture grid developed by BASF (adapted from Newman *et al.*, 1998).

3.1.2. The immunological properties of the non ionic block copolymers

Non ionic block copolymers have been shown to be involved in the induction of both specific (Kast *et al*, 1993) (Li *et al.*, 1995) (Roussel *et al.*, 1995) and non-specific (Howerton *et al.*, 1990) immune responses in laboratory animals. The non-specific immune modulation of these copolymers was evident in the findings of the Howerton and co-workers. They found that the (i.p.) administration of the Pluronic® copolymer L81 (MW, approximately 3kDa) to mice in an oil-based emulsion resulted in the expression of higher levels of Class II major histocompatibility (MHC) antigens by the peritoneal macrophages. Up-regulation in the expression of the Class II MHC is an indication of the macrophage activation (Howerton *et al.*, 1990). The macrophage activation was further investigated by priming the isolated macrophages from treated mice for the production of

superoxide anions and tumour killing capabilities. Their findings suggested that the macrophage functions were enhanced by the Pluronic® treatment. Moghimi and co-workers provided further evidence on the involvement of Pluronic® in macrophage activation (Moghimi *et al*, 1993 and1996). In their experiments mice were injected (i.v.) with Pluronic® F68 (15 mg/kg). An intravenous bolus of phagocyte resistant radioactive particles was administered four days after the Pluronic® injection. The clearance of the radioactive particles by the macrophages in the liver, spleen and lymph nodes was monitored which indicated that 60% were cleared in mice treated with Pluronic® F68 compared to 32% in control mice within 1 hour. The mechanism by which Pluronic® copolymers activate the macrophages has not as yet been fully understood but the involvement of complement products as mediators should not be ruled out (Hunter and Bennett, 1984, Cooper, 1993 and 1995). Pluronic® copolymers are amphipathic or surface-active in structure. This means that they possess distinct hydrophilic and hydrophobic groups in single molecules and that they will preferentially localise on hydrophobic surfaces in aqueous media and modulate interactions of other materials on the surface (Adamson, 1982). A feature which is shared with many adjuvant-active natural products such as lipopolysaccharides from Gram-negative bacteria, Quil-A, QS-21 saponins from the bark of the soapbark tree and trehalose dimycolate from mycobacteria (Allison and Byars, 1992a and 1992b, Waksman, 1979, Jolles and Paraf, 1973, Gall, 1967). One of the most useful methods in quantifying the properties of surface-active agents is the hydrophile-lipophile balance (HLB). This is a measure of the relative strength of the hydrophilic and hydrophobic activities of non-ionic surface-active agents and had been widely used to predict functional activities (Shick, 1967) (Becher, 1965). The first investigations into the adjuvanticity of Pluronic® copolymers were carried out on low-MW block polymers such as L101, L121, P103 and F108 (Hunter *et al.*, 1981). Hunter and co-

workers demonstrated that HLB values of 0.5 and 1 as those in L101 and L121 were indicative of strong adjuvanticity compared to higher HLB values, which presented weak adjuvant activity. However the HLB values proved to be only one aspect of the adjuvant activity. A minimal size of approximately 4000 daltons was also required, which suggests that the copolymers should be large enough and flexible enough to allow interactions with both the water and oil interfaces of the emulsions (Hunter *et al.*, 1995).

Copolymer	HLB	Molecular Weight	Percent POE	Antibody* Titre	Percent** Retention
L31	1.0	1100	10	100	0
L81	2.0	2750	10	2178	ND
L92	5.5	3650	20	1664	ND
L101	1.0	3800	10	84,338	70
L121	0.5	4400	10	67,814	99
L122	3.5	5000	20	184	ND
P103	9.0	4950	30	100	0
F108	24	14,600	80	100	0
None	—	—	—	100	0

Fig 3. 3. Effects of HLB, size and POE content of non ionic block copolymers on adjuvant activity (adapted from Newman et al., 1998). * = serum antibody levels 4-6 weeks after single, foot-pad immunisation of ICR mice with BSA (50 μg) in an oil-in-water emulsion supplemented with 2.5 mg/ dose of the selected polymer. **= Retention in footpads determined 7 days after immunisation with BSA (50 μg) in co-polymer supplemented oil-in-water emulsions. ND= not determined.

The identification and use of the adjuvant-active copolymers led to the development of a new research adjuvant termed TitreMax®. This adjuvant is a water-in-oil emulsion, which resembles CFA and IFA in structural format (Bennett *et al.,* 1992). However, TitreMax® differs in composition, as it contains squalene, instead of mineral oil. Squalene is a metabolisible oil and is derived from shark liver and certain plant oils (Vogel and Powell, 1995). The copolymer used is a new pharmaceutical-grade product termed CRL8941.

This copolymer has a MW of 6000, is 90% POP and 10% POE, more pure and has less polydispersity than the BASF Pluronic® copolymers. TitreMax® benefits from lack of toxicity associated with CFA and IFA. This is due to the lower oil content (10-20% squalene) compared to CFA and IFA emulsions (50% oil), which would decrease the potential for toxicity. This lack of toxicity was demonstrated in a study carried out by Bennett and co-workers in 1992. In their investigations the adjuvanticity of the above product was compared to a number of commercially available adjuvants. These included the Ribi Adjuvant system (Ribi ImmunoChem research, Inc., Hamilton, MO), Adjuvax (Alpha-Beta Technologies, Inc., Worcester, MA), Alhydrogel (Superfos Biosector, Vedbaek, Denmark), CFA/IFA (GIBCO, Grand Island, NY), and Lipovant (Accurate Scientific, Westbury, NY). The above study showed the TitreMax®, to be the most potent adjuvant, inducing greater antibody titres without the site-of-injection toxicity associated with other adjuvants such as FCA. The site of injection with FCA frequently develops hypersensitivity granulomas, which may ulcerate to form draining abscesses (Hunter *et al.*, 1995). The ability of TitreMax® to induce cytotoxic T-lymphocyte responses has also been demonstrated. Immunisation of mice with synthetic peptides containing known CTL epitopes in TitreMax® emulsions resulted in the induction of CTL responses, while, the formulations containing the peptide alone or peptide with alum failed to induce such responses (Kast *et al.*, 1993, Ke *et al.*, 1995, Rousset *et al.*, 1995). Thus the TitreMax® type product seems to be a suitable adjuvant, capable of inducing both high antibody titres and cellular immune responses in many animal species. Pluronic® copolymers can also be used in oil-in-water emulsions, where the oil content is usually significantly less than 10%. The initial work with these types of emulsions was carried out by Hunter and colleagues (Hunter and Bennett, 1984, 1986, Bennett *et al.*, 1992) but many other researchers have developed oil-in-water emulsions of the above adjuvants (Allison and Byars, 1992, Vogel

and Powell, 1995, Lidgate and Byars, 1995). These include the Synex products (developed by Syntex Research (Palo Alto, CA) and the antigen formulation (AF) or Provax developed by IDEC Pharmaceuticals Corp (San Diego, CA). The Syntex products termed Syntex Adjuvant formulations (SAF) are composed of 5% squalane, threonyl-MDP, polysorbate-80 and Pluronic® L121 copolymer (Allison and Byars, 1992, Vogel and Powell, 1995, Lidgate and Byars, 1995). The IDEC product, AF is similar and composed of 15% squalane with polysorbate-80 and Pluronic® L121. Both of these products are routinely microfluidised and consist of oil droplets with mean diameters of 150-175 nm. These emulsions are prepared without the antigen, which can be added immediately before administration. Thus, the antigenic proteins are not subjected to the shearing forces used in preparing emulsions and are assumed to be more likely to retain their native structure. The ease of formulating the above vaccines and the retention of the antigenic proteins make these oil-in-water emulsions advantageous over water-in-oil emulsions. Both SAF and AF have been tested in animal species, but not in humans. The focus of the investigations on the SAF product has been the evaluation of the antibody titres in multiple animal species. This product was administered in combination with a variety of vaccines or other antigens in mice, rats and guinea pigs. It was found that SAF enhanced antibody titres and induced antibodies specific for native structure of the proteins (supporting the fact that the emulsion had not caused denaturation) (Allison and Byars, 1986, Byars *et al.*, 1994). Administration of experimental vaccines based on viral proteins and SAF in ponies and rhesus macaques, have resulted in protective immune responses to equine infectious anaemia virus (EIAV) and simian immunodeficiency virus (SIV) (Issel *et al.*, 1992, Montelaro *et al.*, 1996, Murphey-Corb *et al.*, 1989). The AF product has proved to be a potent inducer of CTL responses in mice, which have included immune responses that were protective against tumour challenge (Raychaudhuri *et al.*, 1992, Fenton *et al.*, 1993).

3.1.3. Mechanism of action for NIBCs

The mechanism of action of copolymer adjuvants has been investigated in several studies (Hunter *et al.*, 1981, Hunter and Bennett, 1984, Hunter and Bennett, 1986). The block copolymer adjuvants such as L101 and L121 are insoluble and act as adhesive molecules. Thus, they bind antigens and host components to hydrophobic surfaces. These interactions involve a combination of hydrophobic and hydrogen-bonding reactions (Hunter *et al.*, 1994). Adhesive copolymer adjuvants are able to fold in such a way as to produce a hydrophilic surface which binds protein antigens to hydrophobic surfaces such as oil droplets (Hunter *et al.*, 1994). This appears to explain the lack of adjuvanticity of copolymers smaller than L101. The length of the hydrophobic and hydrophilic segments has a direct effect on the adjuvanticity of these molecules. Molecules with fewer than 56 polyoxypropylene moieties in their hydrophobic chains are unable to fold sufficiently to place all of the hydrophile on the surface (Hunter and Bennett, 1984). On the other hand excessive hydrophile (POE) chain length is correlated with a reduced ability to bind protein antigens (Hunter *et al.*, 1981). It has been suggested that hydrogen bonding to the POE chains is important for binding of antigens by these copolymers (Byars and Allison, 1987). It is also known that a high proportion of hydrophobic POP is required for binding proteins, which makes the hydrophobic bonding an important factor in the overall interactions between these copolymers and the antigens. In addition the POE chain by itself is unable to bind native proteins (Ceresa, 1976). The protein binds firmly to the surface of the copolymers but retain much more of its native conformation compared to when it is bound to plastic or other hydrophobic surfaces (Hunter and Bennett, 1984). This phenomenon has been interpreted in antigen presentation to the immune system as a condensed two-dimensional matrix (Hunter *et al.*, 1994). The adjuvant effects of the copolymers are further increased by their adhesion to the host components. This contact

with copolymer surface results in the activation of complement and other host mediator systems (Fig 3.4). Activation of complement is known to enhance localisation of antigen in germinal centres and augment immune responses in multiple ways (Hanna and Hunter, 1971). It has been demonstrated that all of the copolymers which display adjuvant activity, enhance the expression of class II major histocompatibility antigen by macrophages in vivo (Howerton *et al.*, 1990). The mechanism of this effect is not fully understood as yet, however the presentation of high concentrations of antigen in a condensed two-dimensional matrix in association with activated host mediators is essential for the activity of copolymer adjuvants (Hunter *et al.*, 1994). The block copolymers are also thought to exert their adjuvant activity through the maintenance of a vaccine depot, decreasing antigen clearance and activation of antigen presenting cells (Hunter *et al.*, 1984, 1994). The adjuvant activity is influenced by both size and POE content. Maximal activity is associated with low POE content 5-10%, low HLB value (1 ≤), and a low molecular size of 11-12 kDa of POP cores (Newman *et al.*, 1998). The type of immune response is dictated by the POE content: POE content of 10% in the copolymer induces type 2 helper T-lymphocytes whereas copolymers with lower POE contents increase both type 1 and 2 helper T-lymphocyte responses (Newman *et al.*, 1998).

Different microsphere formulationss were prepared using two nonionic block copolymers. The pluronic® NIBC surfactants, L101 and L121 were incorporated as stabilisers of the oil in water emulsion and also as adjuvants to prepare microspheres by a solvent displacement technique.

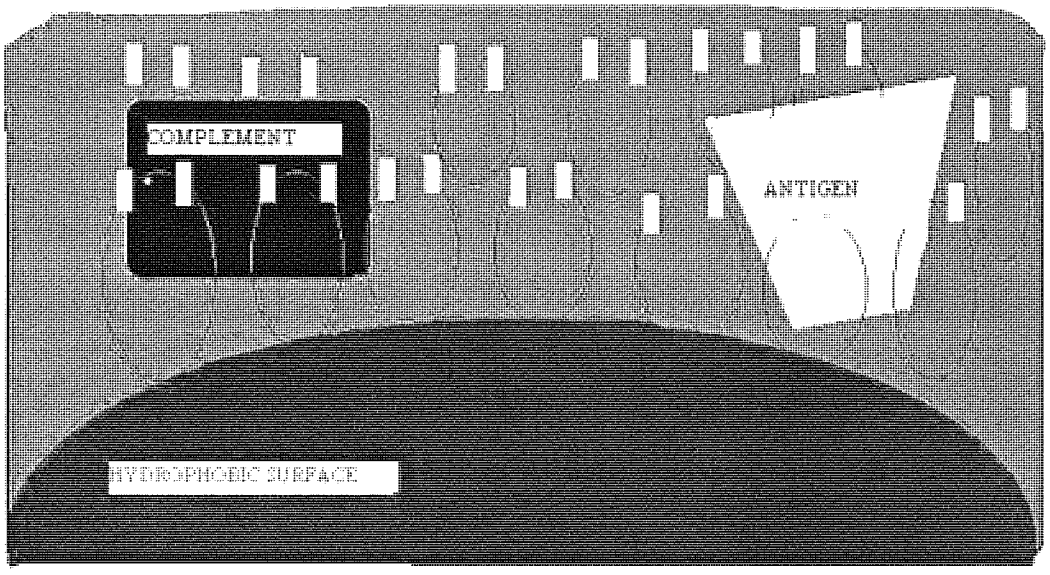


Figure 3.4. Model of the proposed interactions of the block copolymer with the antigen and the hydrorophobic surfaces, which represent the oil-in-water or water-in-oil emulsions. Block copolymers are thought to align at the interface of the oil and water phases and antigens to interact or bind with both the POP and POE blocks. Complement proteins may also interact with copolymers, which would activate the alternative complement cascade and augment the immune responses adapted from Newman *et al.,* 1998).

3.2 preparation of poly(DL-lactide) microspheres using nonionic block copolymers (NIBCs)

3.2.1. Materials

3.2.1.1. Block copolymers (Pluronic® L101 and L121)

Pluronic® polyol surfactants were kindly donated by ICI Chemicals and Polymers Ltd., (Cleveland, UK). The surfactants used were L121 and L101 pluronics which, are block copolymers of ethylene oxide and propylene oxide containing 10% polyoxyehylene in the total molecule. The surfactants were clear viscous liquids at room temperature. These surfactants were insoluble in water but were soluble in 95% ethanol and toluene solution. The Pluronic®compounds were stored at room temperature before use. Table 3.1. describes a list of their determined physical properties.

Pluronic® surfactant	Molecular weight (Da)	Viscosity (cP)	Density ($g\ cm^{-1}$)	Surface tension ($Dyn\ cm^{-1}$)	HLB
L101	3800	800	1.02	34.1	1.0
L121	4400	1600	1.02	33.2	0.5

Table 3.1. Physical properties of Pluronic® surfactants (adapted from Newman *et al.*, 1998).

3.2.1.2. Polymers

Poly (dl-lactide$_{124kDa}$, Resomer 206 and dl-lactide$_{283kDa}$, Resomer 208) were purchased from Boehringer Ingelheim KG (Ingelheim, Germany). Poly(vinyl alcohol)(PVA), Mw 13-23000 was obtained from Aldrich chemicals (Gillingham, UK).

3.2.1.3. Model proteins

Hen egg white lysozyme (HEWL)-fraction V (Mw 14.3 kDa) and ovalbumin (OVA)-fraction V (Mw 45 kDa) were purchased from Sigma Chemical Company (Poole, UK).

The diphtheria toxoid (Mw, 65 kDa, 4450 Lf/ml, 19.3 mg/ml) was kindly donated by Pasteur Merieux, France, Lyon. All proteins were stored desiccated at 2-8 C°.

3.2.1.4. Other chemicals

All chemicals and reagents were supplied by BDH Chemicals Ltd. (Poole, Dorset, UK), Sigma Chemical CO. (Poole, Dorset, UK) and Fisons (Loughborough, Leis, UK) unless otherwise stated and were of Analar grade or equivalent. Double distilled water was used in all experimental procedures unless otherwise stated.

3.2.2. Methods

3.2.2.1. Preparation of PDLA/ block copolymer microspheres

Microspheres were prepared by emulsification-diffusion method adapted from Fessi *et al* (1989). This method has been used successfully to prepare biodegradable nanospheres in an efficient and reproducible manner (Quintanar-Guerrero *et al.*, 1996; Berton *et al.*, 1999; Delie *et al.*, 2001; Kwon *et al.*, 2001). This method relies on the rapid diffusion of the solvent (usually acetone) from the internal into the external phase, thus provoking polymer aggregation in the form of solid colloidal particles. Then when an oil is incorporated in the internal phase, it is proposed that polymer aggregation could follow two probable paths. The first is termed an independent aggregation in which the dispersion obtained will contain oil globules and nanospheres or polymer particles and the other is the aggregation around the oil droplets forming capsules (Quintanar-Guerrero *et al.*, 1998). In the present studies the polymers (10.86 mg ml^{-1}) and non-ionic block copolymers L121 and L101 (in range of 1.5-4.3 mg ml^{-1}) were dissolved in acetone (20 ml). The model proteins (Chicken egg white lysozyme, OVA, DT in the range of 6-7% w/w) were dispersed in the organic phase (acetone) by sonication for 30 seconds. The acetone was then added to the aqueous

solution (40 ml), at low speed stirring (100 rpm) at room temperature. The acetone was allowed to evaporate overnight by gentle stirring. The particles were then, collected by ultracentrifugation, and lyophilised.

3.2.2.2. Microsphere characterisation

The morphology and size of the microspheres were evaluated using SEM (section 2.3.2). Protein entrapment efficiency (2.4.2) and particle size analysis (2.2.2) were determined as previously described.

3.2.2.3. *In vitro* release studies in phosphate buffered saline (PBS, pH 7.5)

Protein release from microspheres was determined in 1ml of PBS/5mM SDS and sodium azide (0.01 % w/v) at 37 °C. Microspheres were suspended in phosphate buffered saline (pH 7.5) in eppendorf tubes and shaken (~120 cycles min) in a thermostatted water bath at 37 °C. At increasing time points, aliquot samples were (200 µl) removed and centrifuged at (13,000 rpm, 10 min). This was replaced by fresh buffer (200 µl) in the original samples. Protein concentrations were determined by a BCA assay (section 2.4.2). Release studies were carried out in triplicates samples for each time point. The results are expressed as percent protein released against time point and are the mean of three individual samples for each time point, (± SD). Volume dilutions were corrected for throughout the experiment.

3.2.2.4. Protein quantification assay (BCA assay)

The entrapment efficiency of the above particles was determined using BCA assay (section 2.4.2).

3.2.2.5. Surface charge measurement (zeta potential)

The surface charge characteristics of NIBC particles were analysed in terms of zeta potential (section 2.6.2).

3.2.2.6. Scanning electron microscopy (SEM)

The morphology of microspheres was analysed using scanning electron microscopy. (See section 2.3.2).

3.2.2.7. Particle size analysis

The Microparticle diameters were determined by laser diffractometry (section 2.2.2).

3.2.2.8. Stability of encapsulated protein

The integrity of the entrapped antigenic proteins was determined by SDS-PAGE (section 2.5.2) using 10% polyacrylamide gels.

3.3. Results and Discussion

3.3.1. Microsphere preparation

The initial studies involved the use of poly (dl-lactide, Mw 124kDa) polymer and lysozyme as the model protein antigen. The choice of the polymer was governed by numerous data established confirming its biocompatability and complete biodegradability (Aftabrouchad and Doelker, 1992, Schade *et al.*, 1995, Couvreur *et al.*, 1995). The above polymer is soluble in acetone. The use of this solvent has two major advantages, i) it is more acceptable for the regulatory bodies such as FDA and MCA, and ii) due to its high water-miscibility improves hydrophilic drug loading in microspheres (Bodmeier and McGinity, 1988). The initial formulations investigated in this study suffered from poor loading efficiency and low yield (table 3.2.). The low yield was mainly due to the formation of much larger aggregates during emulsification process, which is usually associated with this method (Fessi *et al.*, 1987), but not to the extent that was observed with the earlier preparations. The size range of the particles was between 20-70 μm which were mainly due to the aggregation of smaller particles. The above formulations were

modified to achieve smaller size particles with better yields and entrapment efficiencies. Although both L121 and L101 were screened for the initial studies, the L101 formulations were chosen for further modifications, due to better dispersion properties and load reproducibility.

Table.3.2. Characterisation of NIBC/PLA microspheres prepared using a solvent displacement method. Yield = % of the total mass of microspheres prepared to the weight of initial drug plus polymer. Theoretical loading = % (w/w) of antigen loaded to polymer for encapsulation. Entrapment Efficiency = actual antigen content x 100/ theoretical loading

Water: Acetone ratio	PLA concentration (mg ml^{-1})	L101 concentration (mg ml^{-1})	Number mean (μm) (±SD)	Volume mean (μm) (±SD)	Theoretical Loading (% w/w)	Entrapment Efficiency (%) (±SD)	Yield (%)
*1: 0.41	10.86	1.52	4.69 (± 1.52)	58.2 (± 1.2)	6.0	13.2 (±5.6)	49.3 (±2.4)
1:0.41	10.86	1.52	1.49 (± 0.16)	33.5 (± 2.5)	6.0	12.7 (±0.7)	64.3 (±2.3)
*1: 0.41	10.86	4.34	1.45 (± 0.16)	41.9 (± 1.4)	7.2	22.9 (±2.2)	43 (±3.1)
1: 0.41	10.86	4.34	2.43 (± 1.42)	35.8 (± 2.1)	7.2	9.8 (±0.1)	45.9 (±2.6)
1:0.41	10.86	3.22	0.57 (± 0.02)	20.4 (± 3.8)	6.4	12.9 (±0.9)	53.4 (±6.3)
1: 0.5	10.86	1.52	1.44 (± 0.01)	39.1 (± 1.9)	6.0	15.1 (±5.4)	26.9 (±3.5)
1: 0.74	10.86	1.52	5.31 (±.0.37)	71.2 (± 4.1)	6.0	8.86 (±2.4)	63.4 (±5.4)

* Drop-wise addition of the organic phase unto the aqueous phase as opposed to pouring.

3.3.1.1. Influence of preparative variables on formulation optimisation

Different formulations were prepared by varying the ratios of the polymer to the NIBC component, the organic phase to the aqueous phase, by varying the aqueous phase components and by the inclusion of certain additional excipients (Tween 80) to minimise the aggregation of the particles. Different parameters were varied namely a) the methods of the addition of the organic phase to the aqueous phase, b) the removal of the organic solvent (overnight evaporation at room temperature while stirring, rotary evaporation at 30

°C) and c) the collection of the particles such as ultracentrifugation or suspension in PVA and d) polymer concentration. The following tables (tables 3.3a and 3.3b) shows the effect of varying different parameters on the characteristics of the microparticles. Investigations were then carried out on the effect of water: acetone ratio over the range of 1:0.1 to 1:0.64 (up to 40 ml). The polymer concentration varied in the range of 5.2-13.3 mg ml^{-1}.

Table.3.3a. PLA/NIBC empty (no antigens entrapped) Microparticle characteristics prepared by solvent displacement method using NIBC, n=3.

Aqueous phase: Acetone ratio	PLA concentration (mg/ml)	L101 concentration (mg/ml) (±exciepients)	Number mean (μm) (±SD)	Volume mean (μm) (±SD)	Yield (%) (±SD)
1: 0.64	13.33	2.11	2.56 (±1.33)	14.2 (±13.1)	25.8 (±6.2)
1: 0.64	13.33	2.78	0.82 (±0.93)	40.6 (±22.5)	28.8 (±4.1)
1: 0.25	12	1.92	3.08 (±SD)	10.3 (±6.1)	58.1 (±10.8)
1: 0.25	12	25	2.26 (±1.64)	21.4 (±5.9)	16.4 (±3.4)
1: 0.25	12	12	1.21 (±0.83)	12.7 (±6.2)	7.9 (±6.7)
1: 0.15 (4% PVA)	5.2	1.08	0.18 (±0.09)	1.36 (±3.12)	68 (±5.5)
1: 0.15 (4% PVP)	5.2	1.08	0.81 (±0.95)	39.3 (±22.8)	24 (±5.7)
1: 0.15	5.2	1.08	2.35 (±1.84)	33 (±23.6)	52 (±9.2)
1: 0.5	12	12+(6mg Glycerol)	0.19 (±0.11)	10.3 (±9.8)	41.6 (±10.2)
1: 0.14	12	12 +(120mg Glycerol)	0.15 (±0.14)	12.9 (±11.4)	20.3 (±8.1)
1: 0.14	12	10.0	0.2 (±0.12)	2.04 (±5.1)	37 (±6.3)
1: 0.33 (1% PVA)	12	4.5	0.19 (±0.9)	19.1 (±27.7.)	49.9 (±9.8)
1: 0.33 (2% PVA)	12	2.5	0.19 (±.10)	6.2 (±11..9)	68.9 (±11.2)
1: 0.1 (1% PVA)	12	2.5	0.18 (±0.08)	15.2 (±13.9)	57.5 (±8.2)

Table.3.3b.Physical characteristics of PLA/NIBC Microparticle encapsulating lysozyme prepared by solvent displacement method using NIBC, n=3.

Aqueous Phase: Acetone Ratio	PLA concentration (mg ml^{-1})	L101 concentration (mg ml^{-1})	Number mean (μm) (±SD)	Volume mean (μm) (±SD)	Actual Loading (% w/w)	Entrapment Efficiency (±SD)	Yield (%) (±SD)
1: 0.33 (4%PVA w/v)	12	2.5	0.19 (±0.10)	20.3 (±1.9)	0.45 (0.01)	8.9 (±1.2)	37.0 (±2.3)
1: 0.1 (4% PVA w/v)	12	2.5	0.19 (±0.08)	14.5 (±2.2)	0.34 (±0.003)	6.8 (±3.1)	20.1 (±8.3)
1: 0.64 (4% PVA w/v)	12	2.5	0.20 (±0.08)	5.4 (±1.0)	0.77 (±0.01)	15.44 (±5.2)	42.0 (±5.2)
1:0.1 (0.75% PVA w/v)	12	2.5	0.19 (±0.09)	11.0 (±1.8)	1.3 (±0.002)	26 (±0.9)	44.5 (±6.1)
1: 0.5 (0.75% PVA w/v)	12	2.5	0.19 (±0.08)	8.16 (±1.4)	1.2 (±0.03)	24 (±0.7)	81.3 (±8.2)
1: 0.5 (2%PVP w/v)	12	5.7	2.56 (±1.73)	31.1 (±3.9)	1.4 ±0.06)	28 (±0.6)	46.4 (±4.4)
1: 0.5 (2%PVP w/v)	12	5.7	1.76 (±0.2)	29.4(±3.9)	1.2 (±0.01)	24 (±0.7)	38.3 (±7.5)
1: 0.33 (4%PVP w/v)	12	2.5	0.18 (±0.05)	9.2 (±2.8)	0.45 (±0.01)	8.9 (±1.6)	37.0 (±4.2)
1: 0.1 (4%PVP w/v)	12	2.5	0.19 (±0.09)	1.8 (±0.01)	0.93 (±0.04)	18.6 (±0.4)	31.8 (±5.8)
1: 0.33 (4% PVP w/v)	12	2.5	0.22 (±0.14)	1.27(±0.5)	0.11 (±0.002)	2.11(±0.31)	26.4 (±3.6)
1: 0.5 (0.25% TWEEN 80)	12	2.5	0.18 (±0.08)	14.9 (±3.5)	0.17 (±0.01)	9.7 (±0.5)	62.7 (±4.6)
1: 0.1 (0.25% TWEEN 80)	12	2.5	0.18 (±0.08)	4.5 (±1.2)	1.90 (±0.01)	38 (±1.3)	84.1 (±10.9)
1: 0.5 (0.75% PVA w/v)	12	2.5	2.69 (1.87)	10.1 (±2.6)	0.63 (0.01	31.4 (±2.6)	27.1 (±5.6)

All formulations were carried out in triplicates. In general the results (table 3.3) show that, there is no relation between the aqueous to organic phase ratio and the entrapment efficiency of microarticles. There is also no significant relation between the concentrations of NIBC L101 and a favourable yield and a better entrapment efficiency. The formation of the microspheres, using the above method, is based on the rapid diffusion of the acetone from the organic phase to the aqueous phase. The resultant interfacial tension decrease and migration of the insoluble PLA towards the o/w interface, where it is deposited leads to the formation of the particles (Fessi *et al.*, 1989). Surfactans such as L101 and L121, are known to suppress interfacial flow (Berg, 1982). In preparations where the aqueous phase consisted of PVA, lower concentrations (0.75% w/v) of this emulsifier produced better yield and entrapment efficiency of microparticles. Among the important factors that contribute towards the variations of the interface are solute transfer out of the phase of higher viscosity and the steep concentration gradients near the interface (Fessi *et al.*, 1989). This might explain the effects of the low concentration of the PVA on the yield and entrapment efficiency of the microspheres. Different concentrations of PVA exerted an inconsistent effect on the size range of the particles. The use of PVP (Mw, 10 kDa) in different concentrations offered no advantage over the PVA-based preparations. The best preparations were two batches, with PVA (0.75% w/v) and Tween 80 (0.25% v/v) in the aqueous phase. These preparations offered small size range (4-8 μm), high yield of up to 84% and lysozyme loading efficiency of up to 38% in the microspheres (table 3.3b). The Tween-based formulations lacked the batch reproducibility which is required when used in immunisation studies and thus the formulation with 0.75% w/v PVA and the aqueous to organic phase ratio of 1:0.5 was chosen for further development with different antigens, polymers and *in vivo* immunisation studies.

3.3.1.2 The effect of different proteins on the characteristics of microspheres

The reproducibility of the above microsphere batches encapsulating lysozyme, lead into the investigation of the effects of other model proteins on the characteristics of these particles. Ovalbumin was chosen as a model antigen since it has been used in many studies of vaccine delivery systems (Uchida *et al.*, 1995, Lavelle *et al.*, 1999) and its encapsulation would present a comparative assessment to the findings present in the literature. Diphtheria toxoid is another suitable candidate and a clinically relevant antigen for vaccine delivery systems (Singh *et al.*, 1991 and 1992). A recent resurgence of diphtheria specially in the former Soviet Union has meant that a single dose combined vaccine against tetanus, diphtheria and pertussis has been given high priority by the WHO (Lambert, 1994). Diphtheria toxoid and OVA were encapsulated in two optimised formulations, which were previously tested for lysozyme entrapment.

Code PLA/NIBC formulation	Aqueous Phase: Acetone Ratio	PLA concentration (mg ml^{-1})	L101 concentration (mg ml^{-1})	Number mean (μm (±SD)	Volume mean (μm) (±SD)	Actual loading (% w/w)	Entrapment efficiency (±SD)	Yield (%) (±SD)
A	1: 0.5 (0.75% PVA w/v)	12	2.5	0.19 (±0.08)	8.16 (±1.4)	1.2 (±0.06)	24 (±0.03)	97.6 (±6.8)
B	1: 0.5 (4% PVA w/v)	12	2.5	0.19 (±0.08)	14.5 (±2.2)	0.34 (±0.02)	6.77 (±0.03)	20.1 (±2.6)

Table 3.4. PLA/NIBC microspheres encapsulating lysozyme with further modified formulation parameters, n=3.

Encapsulating lysozyme within formulation A generated much higher yield (97.6%) than formulation B. The encapsulation efficiency (24%) was also greater in formulation A. The microparticles prepared in formulation B resulted in larger mean volumes (table 3.4). These results are contrary to the findings of Quintanar-Guerrero and co-workers. The authors investigated the influence of stabilising agents and preparative variables on the formation of poly(D,L-lactic acid) nanoparticles by emulsification-diffusion method

(Quintanar-Guerrero *et al.*, 1996). They found that the mean nanoparticle size decreased sharply between 0.5 and 5% w/v of PVA, but that little change was observed above 5%. PVA concentration in the external water phase is thought to be a key factor to influence the size of microspheres (Jeffrey *et al.*, 1993, Carrio *et al.*, 1995, Yeh *et al.*, 1995). On the other hand Murakami and co-workers found the particle size not to be affected by varying the concentrations of PVA in the preparation of poly(D,L-lactode-co-glycolide) nanoparticles by emulsification-diffusion method (Murakami *et al.*, 1999). There are also contrary reports in the literature on the effect of PVA concentration on particle size prepared by w/o/w double emulsion. Some authors report that on increasing the PVA concentration, the particle size was decreased (Heya *et al.*, 1991; Benoit *et al.*, 1984; Yang *et al.*, 2001). Others present contrary reports (Bentia *et al.*, 1984;), while Julienne and co-workers observed that the PVA concentration did not affect microsphere particle size (Julienne *et al.*, 1992). Since PVA is a polymer with a high molecular weight (13-23,000), the presence of PVA in the external water phase may increase the viscosity of the emulsion, resulting in an increased difficulty to break up the emulsion to smaller droplets. This could explain the yield of bigger microspheres in formulation B. In comparison encapsulation of DT in the formulation with lower PVA concentration (0.75% w/v) in the external phase (formulation A), resulted in preparations which had lower loading (0.98 w/w as opposed to 1.2 w/w), and lower yield (54.6%). This formulation also generated larger size particles (14.2 μm) compared to the particles containing lysozyme.

It is known that molecular charge and hydrophobicity play a role in protein adsorption and deposition at interfaces. The adsorption of most proteins is largely governed by non-electrostatic interaction (Luey *et al.*, 1991). Protein adsorption at oil-water interfaces is governed by the same parameters as its adsorption at air-water interfaces (Graham and Philips, 1979) and parallel conclusions can be drawn for the processes involved during

encapsulation of a mixture of proteins and their subsequent release (Conway and Alpar, 1996). Proteins cause a decrease in water/dichloromethane interfacial tension, the extent of which is dependent on the degree of their hydrophobicity (ratio and distribution of the polar/ apolar amino acids) and their structural flexibility. The cross-interactions between the aqueous phase and the organic polymer phase are dependent on the chemical structure of the protein, and as such will influence the protein entrapment and release profile of the resultant microparticles. Lysozyme is a relatively smaller protein (14.6 kDa) compared with DT (65 kDa). It is a single polypeptide chain of 129 residues. This highly stable protein is cross-linked by four disulfide bridges. Lysozyme is a compact molecule, roughly ellipsoidal in shape. Hydrophobic interactions play an important role in the folding of lysozyme (Stryer, 1988). The compact structure of lysozyme may facilitate its increased exposure at the interfacial film, leading to more interactions with the polymer, thus increasing its overall encapsulation efficiency. On the other hand diphtheria toxin is a high molecular weight (65 kDa) and is composed of two domains, subunit A, and subunit B, which binds to a receptor molecule and is required to translocate subunit A into the cytoplasm (Zhao and London, 1986). The bulkier size of this protein could result in less efficient accommodation within the interface film, thus minimising its interactions with the polymer, leading to its lower encapsulation efficiency. Formulation B (4% w/v PVA in the external phase) resulted in smaller particles in terms of size (11.2 μm as compared to 14.5 μm for lysozyme), similar protein loading, but presented lower yield. Murakimi and co-workers investigated the effect of PVA concentration in the aqueous phase on the yield of microparticles, prepared by emulsification-diffusion method in a recent study. The authors found that at a concentration range of 0-20% (w/v) PVA, the yield of particles ranged between 30-58%. At low PVA concentrations (lower than 1% w/v), formulations only had 30% yield (Murakami *et al.*, 1999). It was suggested that at lower concentrations,

decreasing coacervation of PVA on the microparticle surface could be responsible for lower yields (Murakami *et al.*, 1999). In emulsification-diffusion method, the stabilisation of droplets is important to avoid coalescence and the formation of agglomerates. The adsorption of stabilisers such as PVA at the interface is the driving force to lower the coalescence of particles in this method (Kwon *et al.*, 2001). It is possible that at lower concentrations of PVA, the physical stability of particles is not optimal resulting in the loss of yield due to the formation of aggregates. The OVA-loaded particles (formulations A and B) showed similar characteristics to those encapsulating DT. Formulation A had a higher yield (51%), but similar protein loading to that of formulation B. In general formulation B resulted in poor microsphere yield for all three proteins. The particle size seemed to be similar for DT and OVA formulations but presented the opposite pattern for lysozyme (table 3.5.).

Formulation	Encapsulated protein	Number diameter (μm ± SD)	Volume diameter (μm ± SD)	Actual loading (%w/w) (± SD)	Entrapment efficiency (%) (± SD)	Yield (%) (± SD)
A	DT	2.69 (±1.8)	14.2 (±15.1)	0.98 (±0.02)	19.72 (±2.7)	54.6 (±9.8)
B	DT	2.2 (±1.3)	11.2 (±14.1)	0.97 (±0.02)	19.52 (±2.9)	21.9 (±2.5)
A	OVA	3.4 (±2.2)	17.8 (±17.7)	0.98 (±0.04)	19.60 (±3.1)	29.3 (±2.8)
B	OVA	2.1 (±1.4)	13.6 (±14.1)	1.0 (±0.08)	19.92 (±2.8)	51.0 (±8.4)

Table 3.5. PDLA/NIBC microspheres encapsulating OVA and DT (theoretical loading of 5% w/w) with modified formulation parameters. A=PDLA/NIBC preparations with aqueous to organic phase ratio of 1:0.5 and PVA (0.75% w/v) in the external phase. B= PDLA/NIBC preparations with aqueous to organic phase ratio of 1:0.5 and PVA (4% w/v) in the external phase, n=3.

3.3.1.3. The effect of polymer molecular weight on the characteristics of microspheres

Poly (dl-lactide$_{124kDa}$, Resomer 206 and dl-lactide$_{283kDa}$, Resomer 208) were used in the present study to investigate the effect of different molecular weight polymers on the

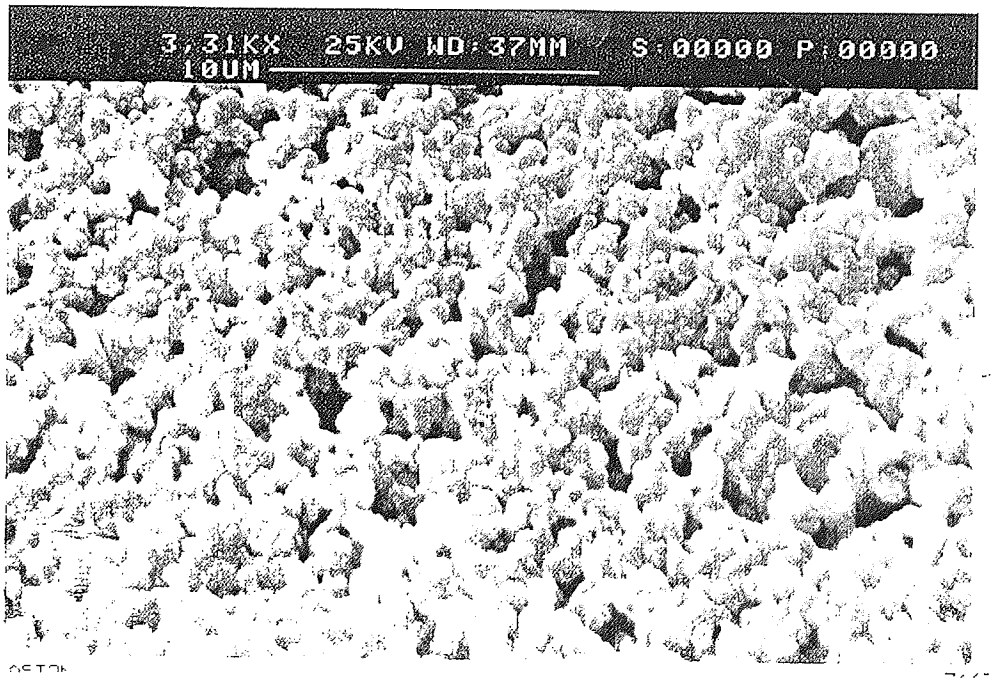
NIBC/PDLA microparticle characteristics. Comparisons were also made with microspheres lacking NIBC in their formulations. The results (table 3.6.) show that the higher molecular weight polymer did not affect the protein (DT) loading, but produced particles with slightly bigger diameters. The entrapment efficiency was the same for all the preparations (including that lacking the NIBC). These findings differ from the findings of other researchers investigating the effect of higher molecular weight of polymers and the loading efficiency of particles using a w/o/w double emulsion (Boury *et al.*, 1997, Cohen *et al.*, 1991). Their findings showed that the higher molecular weight polymer resulted in the highest yield of the particles. In a recent study Boury and co-workers investigated the effect of different molecular weight PLGA polymers on the surface properties and release of BSA from microspheres. In their findings they indicate that microspheres prepared with the lowest molecular weight PLGA exhibited both the highest BSA content and encapsulation efficiency (Boury *et al.*, 1997). It is thought that lower molecular weight polymers are more hydrophilic and thus, relative solubility of BSA in PLGA is enhanced, leading to higher protein contents (Cohen *et al.*, 1991). Another explanation, which was brought forward by the authors involved the possible ionic interactions between the BSA (positively charged amine groups), and the carboxylic polymer's end groups (negatively charged), these groups being more in the lower molecular weight polymer. It is also noted that higher hydrophilicity of the lower molecular weight polymer probably enhances the penetration of the protein in the PLGA monolayer at the dichloromethane water interface (Boury *et al.*, 1997). The presence of the NIBC in the formulations does not seem to have an apparent effect on the characteristics of the particles in terms of size, loading and yield. The scanning electron microscopy of all three different preparations showed spherical particles with smooth

surfaces figure (3.5). There were no apparent differences in the morphology of different formulations.

Formulation	Polymer	Number mean (μm±SD)	Volume mean (μm ±SD)	Actual loading (%w/w)	Entrapment efficiency (%)	Yield (%)
1	124 kDa	0.2 (±0.0)	3.0 (±0.5)	4.96 (±1.10)	99.2 (±3.1)	62.0 (±5.8)
2	283 kDa	0.2 (±0.0)	5.9 (±0.3)	4.90 (±0.81)	97.6 (±2.8)	81.8 (3.4)
3	124 kDa (-NIBC)	0.2 (±0.0)	2.7 (±0.8)	4.60 (±0.68)	92.0 (±2.4)	72.4 (±2.9)

Table 3.6. Physical characteristics of different PDLA/NIBC formulation encapsulating DT theoretical loading of 5% w/w, -NIBC= L101not included, n=3.

Figure 3.5. Scanning electron micrograph of NIBC/PLA microspheres encapsulating DT.



3.3.1.4 The effect of suspending particles in PVA on size and dispersion of microspheres

In all preparations a certain degree of particle aggregation had been observed and although in general the preparations were mostly free flowing powders, the presence of large cohesive particles could not be avoided. In order to address this matter, the particles were suspended in PVA (0.75% w/w) prior to freeze-drying, as it had been assumed that most aggregation would take place during the centrifugation process. This approach proved to be fruitful and produced fine, free flowing particles, which were easily dispersed in PBS. The following table (table 3.7.) lists the physical characteristics of all different (optimised) formulations including those suspended in PVA before freeze-drying. The suspension in PVA did not seem to affect the size of the particles, and these preparations resulted in microspheres with diameters of 3.9 μm. The entrapment efficiency and the yield of the above particles had not been affected either. In a recent study Murakaki and co-workers investigated the influence of different PVA concentrations on the preparation of PDLA nanoparticles. Their results showed that at PVA concentrations of 2% (w/v), small particles (~ 300nm) were formed which did not aggregate upon redispersion after freeze-drying (Murakami *et al.*, 1999). They suggested that the most likely reason for this observation was the adsorption of PVA to PLGA nanoparticles. Regarding the surface adsorption of PVA, Iler proposed a model to express the physical adsorption of PVA to colloidal silica particles, in which PVA molecules are fixed on the surface *via* hydrophilic bonding with the silanoyl groups (Si=O) of the colloidal silica (Iler, 1973, 1975). Another explanation for the strong adsorption of PVA on the surface of PLGA nanoparticles may be due to hydrogen bonding between the hydroxyl groups of PVA molecules and the acetyl groups of the PLGA (Murakami *et al.*, 1999). The authors suggest that the better rehydration of powdered nanoparticles *via* freeze-drying without the addition of any

cryoprotectant could be due to the hydrophilicity of PVA molecules, which were strongly bound to the surface of PLGA nanoparticles (Murakami *et al.*, 1997).

Formulation	Polymer	Number mean (μm±S)	volume mean (μm±S)	Actual loading (%w/w)	Entrapment efficiency (%)	Yield (%)
1	124 kDa	0.2 (±0.0)	3.0 (±0.5)	4.96 (±1.10)	99.2 (±3.1)	62.0 (±5.8)
2	283 kDa	0.2 (±0.0)	5.9 (±0.3)	4.90 (±0.81)	97.6 (±2.8)	81.8 (±3.4)
3	124 +283 kDa	———	———	———	———	———
4	124 kDa (s)	0.3 (±0.0)	3.9 (±0.3)	4.99 (±0.62)	99.8 (±4.3)	68.0 (±2.3)
5	124 kDa (-NIBC)	0.2 (±0.0)	2.7 (±0.8)	4.60 (±0.68)	92.0 (±2.4)	72.4 (±2.9)

Table 3.7. Physical characteristics of different PDLA/NIBC formulation encapsulating diphtheria toxoid with theoretical loading of 5% w/w, formulations prepared using aqueous phase: acetone ratio 1:0.5 and 0.75% w/v PVA in the external phase, (s)= suspension in PVA, (-NIBC)= L101not included, n=3.

3.3.2. *In vitro* protein release from NIBC/PDLA microspheres in PBS

The *in vitro* release profiles of lysozyme and DT were compared over a 28 day incubation time (Fig 3.6). The release of both proteins follows the same pattern, with a similar initial burst release (40% for lysozyme, and 37% for DT). After the initial burst release, the amount of proteins released increased over time (up to 80% for lysozyme and 50% for DT on day 14). The release profiles for both proteins showed a pattern in which neither protein was released completely on day 28 of the incubation period. Since the sampling for released proteins was carried out by removing a fraction of the release medium (200μl out of total 1 ml) the slight decrease in the release profile on day 7 may be explained due to the re-absorption of the released proteins on the polymer surface. The importance of the *in*

vitro test systems and conditions during prolonged drug released from biodegradable microspheres has been studied by Conti and co-workers (Conti *et al.*, 1995). In their investigations different dissolution methods and test conditions for the release of indomethacin from PLA microspheres were evaluated. They found that the drug release kinetics depended greatly on the test parameters. The release of peptides and proteins from PLA/PLGA microspheres is generally not solely diffusion controlled but follows a pulsatile pattern. This is thought to be due to strong interactive forces between peptides or protein compounds and polyesters. Thus this release behaviour is particularly sensitive to the actual in vitro test conditions (Johanssen *et al.*, 1998). The adsorption of the released protein onto the vials used for the release test and on the microspheres themselves affects the measured protein release at different sampling intervals which might explain the depression points on the release profile after the initial burst in the present study. This study also shows that there was a higher protein release in case of lysozyme than DT at all time points. The release profile of DT was also investigated from five different NIBC/PDLA formulations (table 3.7) in triplicate measurements at different time points up to 28 days of incubation. The initial release of DT ranged between 19% for formulation R206 (s) to 37% for the R206 preparation (Fig 3.7). All formulations show a peak release at day 14, before levelling off at day 28. In many cases, the release of proteins from microspheres displays an inconsistent profile with a significant burst and incomplete release even though the polymer degrades sufficiently (Crotts and Park, 1998). This effect could be attributed to protein stability problems such as aggregation and non-specific protein adsorption.

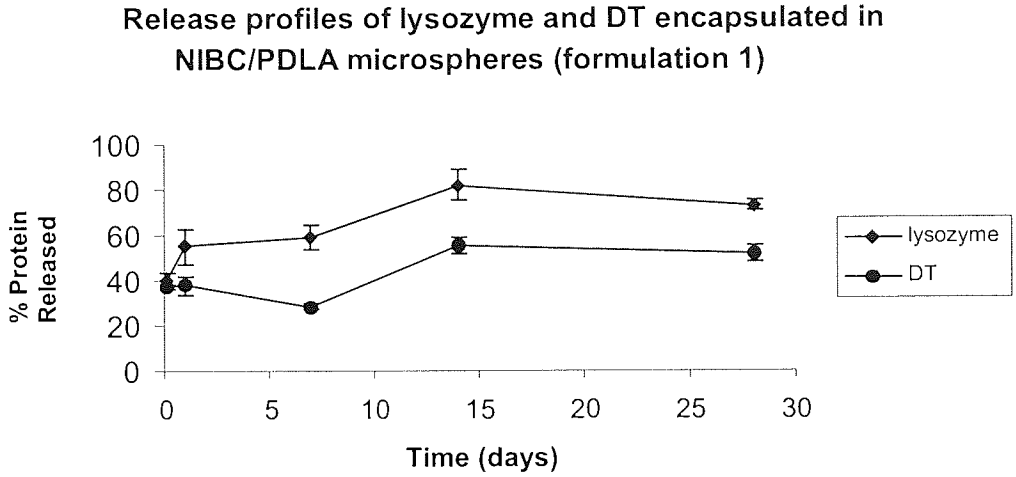


Figure 3.6. *In vitro* release profiles for entrapped DT and lysozyme from NIBC/PDLA microspheres, formulation 1= PDLA (124kDa, water to organic phase ratio:1: 0.5, PVA (0.75% w/v in the external phase). n=3 (±SD).

Crotts and co-workers found that even after the sufficient disintegration of PLGA 5050 microspheres, as assessed by molecular weight decrease and weight loss, cumulative release never reached 100% (Crotts and Park, 1998). The stability of poly(D,L-lactide) nanospheres in aqueous media was investigated in a study by Belbella and co-workers. In their study the influence of the molecular weight (25kDa and 95kDa), polydispersity and the pH of the medium was investigated. These authors found that the degradation rate of the poly(D,L-lactide) polymers (depended on molecular weight and polydispersity. The degradation of poly(D,L-lactide) polymers resulted from hydrolysis which is much more catalysed at acidic and alkaline pH than at neutral pH. The degradation of the higher molecular weight polymer (95kDa) was found to reach 20-30% at 24 days and to 50% at day 102 (Belbella *et al.*, 1996). This might indicate that there is sufficient degradation of the polymer within 28 days to release up to 50% of the entrapped protein (Fig 3.7).

However the incubation period in this release study is not within the time period required for the complete degradation of the polymers. Also the incomplete release of the protein might still be evident even after the complete degradation of the polymer (Crotts and Park, 1998). In a recent study by Park *et al*, 1998, the lysozyme was encapsulated in PLGA microspheres using a single o/w emulsion. They found that, the non-covalent aggregation contributed significantly to the diminished protein release and that an ionic interaction was crucial in the early stages of protein release but not in the later stages of the profile (Park *et al,* 1998). The burst effect has been shown to be dependent on the pH of the external phase (Rojas *et al.*, 1999). The dependence of the release on the pH is explained by the strong adsorption of the protein on the surface of the microspheres (Leo *et al.*, 1998). Release from biodegradable microspheres is dependent both on diffusion through the polymer matrix and on polymer degradation. There are additional factors contributing to the release profiles of the microspheres, which are entirely dependent on the specific nature of the encapsulated protein. These properties include the diffusivity in the core material, solubility, the size of the protein, its distribution in the microsphere matrix and specific interactions with the polymer (Wang *et al.*, 1991). Additional factors such as the increase in the concentration of the external phase and the aqueous phase to acetone ratio affect the entrapment efficiency of the microspheres and thus alter the release profile of proteins from these particles.

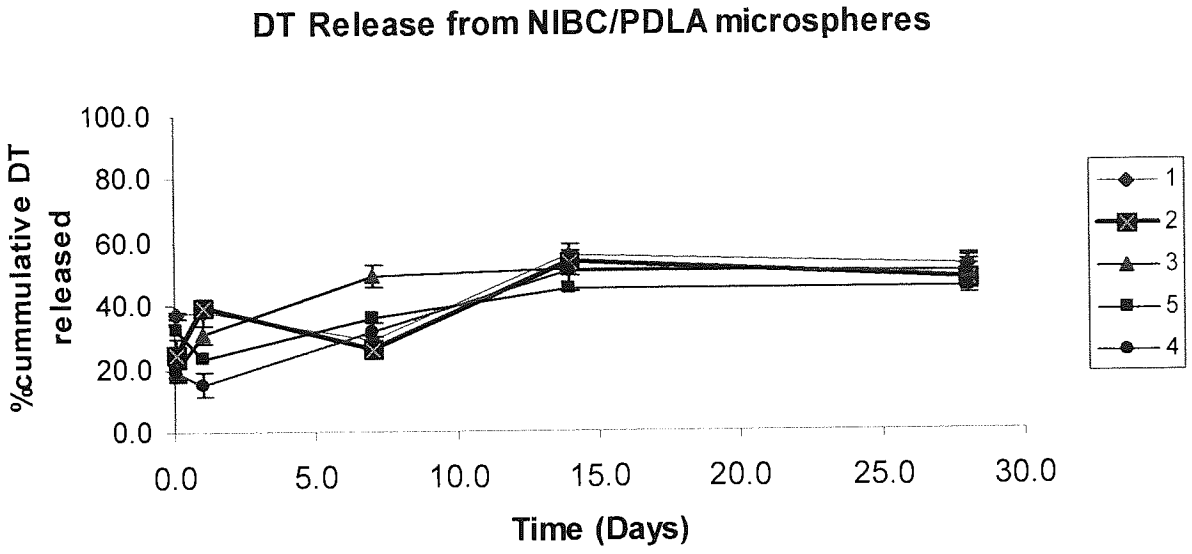


Fig 3.7. In vitro release profile of entrapped DT from NIBC/PDLA microsphere, 1=PDLA (124kDa), 2= PDLA (283 kDa), 3= PDLA (124+283 kDa), 4= PDLA (124kDa, particles suspended in PVA), 5= PDLA (124 kDa, NIBC not included in the formulation), n=3.

3.3.3. Stability of encapsulated antigens: the effects of entrapment within NIBC/PDLA microspheres on the structural integrity and immunogenicity of diphtheria toxoid

The integrity and immunogenicity of encapsulated DT was determined by SDS-PAGE, using 10% polyacrylamide gels immediately and six months after microsphere preparations. The protein was extracted from microspheres (freshly made and those stored at room temperature over a period of six months) and run immediately on SDS-PAGE gels. The appearance of the band corresponding to the DT toxoid is that of a diffuse band which is thought to be that of a toxoid with intramolecular cross-linking. This intramolecular cross-linking causes the appearance of a more compact conformation in SDS-PAGE

(Paliwal and London, 1996). The analysis shows that both preparations (freshly made and that stored for six months), preserves the integrity of the encapsulated DT, as evident in the SDS gels analysis (Fig 3.8.). Antigen preservation within our preparations could have been due to the very mild conditions, which were employed during their formulations. These particles were not in contact with dicholormethane. It has been shown that dichloromethane/ water interface serves as a hydrophobic adsorbent which plays a role in triggering protein denaturation and aggregation (Sah, 1999). Also the emulsification-diffusion method protected the antigen from the common destabilising factors involved in w/o emulsions. These include heat and shear during the microencapsulation process. The inclusion of non ionic block copolymers in our formulations was carried out to stabilise the formulations and more importantly to act as adjuvants. Their inclusion, in these formulations might have also played a stabilising effect on the entrapped antigen. Audran and co-workers investigated the co-encapsulation of L121, L101 and TT within PLA and PLGA microspheres. In evaluating the effect of stabilising agents on the antigenicity of TT, the antibody titres of microspheres with the additives (including L101 and L121) were compared with those lacking the additives and serving as controls. Their results showed that the same dose of TT, immunisation with MS-additive induced higher antibody responses than the MS without the stabilising additive (Audran *et al.*, 1998).

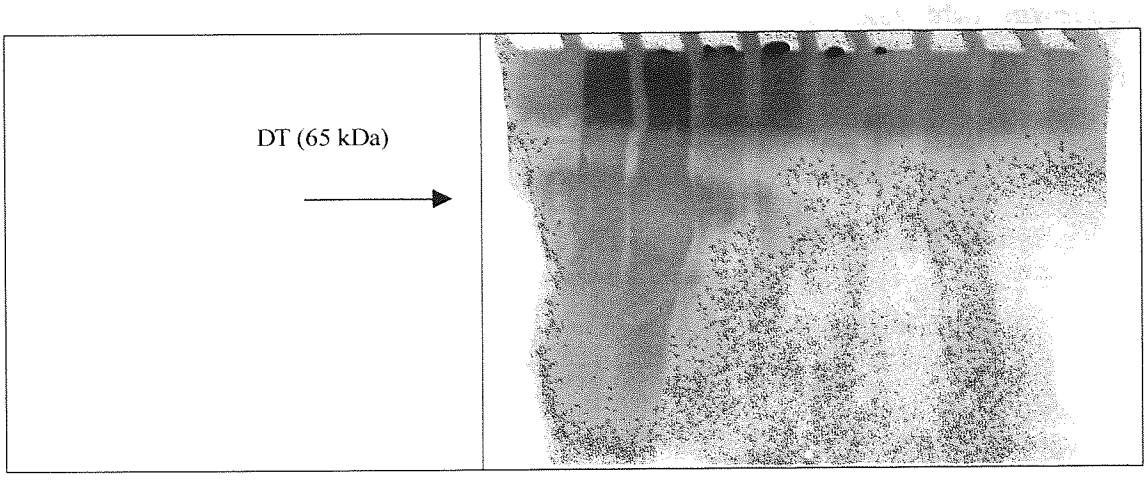


Figure 3.8. SDS-PAGE analysis of the diphtheria toxoid antigen encapsulated within NIBC/PDLA microspheres stored dry at room temperature for a period of 6 months. Lanes 2,3= DT in solution used as control, lane 4= R206 (Mw 124kDa) formulation. Lane5= R208 (Mw 283 kDa). lane 6= -NIBC formulation, lane8= R206 (Mw 124 kDa in PVA suspension)(s).

Formulation	Encapsulated protein	Size (number mean) (±SD)	Size (volume mean) (±SD)	Actual loading (%w/w)	Entrapment efficiency (%)	Yield (%)
A	DT	2.69 (±1.8)	14.2 (±15.1)	0.98	19.72	54.6
B	DT	2.2 (±1.3)	11.2 (±14.1)	0.97	19.52	21.9
A+B	DT	———	———	———	———	———
Free solution	DT	———	———	———	———	———
A	OVA	3.4 (±2.2)	17.8 (±17.7)	0.98	19.60	29.3
B	OVA	2.1 (±1.4)	13.6 (±14.1)	1.0	19.92	51.0
A+B	OVA	———	———	———	———	———
Free solution	OVA	———	———	———	———	———

Table 3.8. Different formulations used for the intramuscular dosing of antigens encapsulated within PDLA/NIBC microspheres. A=PDLA/NIBC preparations with aqueous to organic phase ratio of 1:0.5 and PVA (0.75% w/v) in the external phase. B= PDLA/NIBC preparations with aqueous to organic phase ratio of 1:0.5 and PVA (4% w/v) in the external phase, n=3.

The immunogenicity of the stored microsphere samples was also investigated, by intramuscular administration of these particles in mice and comparing the immune response to that of the freshly made preparations. The ELISA results shows that the encapsulated DT, retains its immunogenicity after long period of storage at room temperature.

4.0. Immunisation with nonionic block copolymer/poly (DL-lactide) microspheres encapsulating model antigens

4.1. Introduction

The adjuvant activity of low molecular weight non-ionic block copolymers have been investigated in several studies by Hunter and co-workers (Hunter *el al.*, 1981, 1991, 1995). In one model system an oil-in-water emulsion of the copolymers and BSA as model antigen were prepared. The authors found that the copolymer L101 promoted the stability of emulsions *in vitro*, and antigen retention in the injected footpad. L101 was also found to be an effective adjuvant for both antibody and cell-mediated immunity. The results of this study also demonstrated that although both L101 and L121 were powerful adjuvants, they displayed important differences. They both enhanced the retention of much more antigen in the footpad than could be accounted for by the stability of the emulsions *in vitro*. The copolymer L121 was found to be a better adjuvant for antibody production, whereas L101 stimulated the production of cell-mediated immunity more effectively. The authors observed that the block copolymer adjuvants profoundly altered the distribution of antigen in tissue. The intravenous injections of copolymer L101 by itself (with no antigen) induced proliferation of cells in periarteriolar sheath or T cell area of the spleen while similar injection of L121 induced greater germinal centre hyperplasia. These copolymers which display adjuvant activity have been found to not only bind antigen but also activate complement, activate macrophages and antigen, and induce expression of class II (Ia) (Romussi *et al.*, 1980; Takechi *et al.*, 1992). The above adjuvant activity of these copolymers is thought to be due to their ability to modulate hydrophobic adhesive interactions (Hunter *et al.*, 1995). Hunter and co-workers found that the adjuvant particles bound protein tightly so that it could not float away, but loosely, so that its native

conformation was preserved. In other words, the antigen appeared to be attached to non-denaturing hydrophilic surfaces of copolymer rather than pressed and distorted against denaturing hydrophobic surfaces such as plastic or mineral oil. Varying the length of the hydrophobic POP and hydrophilic POE chains proved to be effective in determining the type of antibody isotype induced. Preparations with POP chains less than 5000 Da (e.g. L101) induced more IgG1 antibody than any other subclass. Larger copolymers induced higher IgG2 titres specially IgG2b responses. These adjuvants were found to be less soluble and formed more stable structures at oil-water interfaces than the smaller preparations. The copolymers, which are effective adjuvants were found to be adhesive molecules which bind the protein antigens to the surface of oil drops. These also bind and activate host mediators including complement. The adhesive copolymers with adjuvant activity also induce increased expression of class II (Ia) molecules on macrophages and increase their ability to present antigen to T cells. Thus, the antigen is presented in a highly concentrated form to cells of the immune system in conjunction with a variety of activated mediators. The copolymer L121 formulated in an oil-in-water emulsion conjugated with a BSA-S. *pneumoniae* type 3 hexasaccharide was shown to result in significant protection against virulent challenge in young mice which was not achieved by vaccination with the conjugate alone (Zigterman *et al.*, 1988). In the following studies it was suggested that protection could be correlated to elevated levels of more of avid IgG2a antibody against the type 3 capsule (Van Dam *et al.*, 1989).

The majority of the commercial vaccines are administered in an injectable form. This route of immunisation does not usually stimulate responses in the external secretions covering the mucosal membranes, which is the most common site of entry of infectious agents (Mestecky, 1987). A more effective route for vaccine administration is *via* ingestion or inhalation of antigens, which induces a generalised immune response resulting

in the appearance of specific antibodies in several external secretions (saliva, milk, tears, Mestecky, 1987, McGhee *et al.*, 1990). The nasal route has come to represent a great potential as a mucosal site for drug and vaccine delivery. Nasal absorption avoids first-pass effects and conditions in the nasal cavity are less aggressive, than those present in the GI tract due to its lower enzymatic activity, and less extremes of pH. The existence of the nasal-associated lymphoid tissue is of great importance since it has a role, which is analogous to that of the GALT and constitutes part of the common mucosal immune system (CMIS). The potential of intranasal studies has been investigated in several studies. Many studies have evaluated the potential of PLG-based microparticles as vaccine delivery system for intranasal administration. Intranasal immunisation with the above microparticles has been shown to induce protection in mice against aerosol challenge with ricin toxin (Yan, 1995) and parainfluenza-type-3 virus (Ray *et al.*, 1993). Moldoveanu and co-workers investigated the effect of different administration routes on the immune response to influenza virus vaccines in healthy human subjects. Their study demonstrated that the immunisation route affects the magnitude and the type of antibodies produced. The authors found that intramuscular immunisation induced the highest antibody titres with intranasal administration producing similar serum antibody and antibody secreting cells, but at a much lower magnitude. The oral route was shown to be the poorest stimulator of the immune response. In general, their findings showed that the specific antibody levels induced by mucosal immunisation did not reach the magnitude of those induced by systemic injection (Moldoveanu *et al.*, 1995). However, it has been shown that lower titres of circulating antibodies do not necessarily correlate with lower levels of protection (Boudreault *et al.*, 1976). Moldoveanu and co-workers found that after oral or intranasal immunisation the antibodies were mainly of the IgA class, whereas intramuscular injection produced IgG and IgM antibodies. Their study has demonstrated

that secretory antibody responses to viral antigens can be induced following oral as well as intranasal immunisation. Almeida and colleagues have also demonstrated that intranasal delivery of tetanus toxoid in PLG microspheres induced higher systemic and local immune responses than those obtained after oral immunisation. (Almeida *et al.*, 1993). Other studies on the intranasal administration of the influenza virus in humans have also shown the effectiveness of this route in the induction of both serum and nasal antibodies (Kaji *et al.*, 1992; Martin, 1997).

In a recent study, the adjuvant effects of M59 (a stable oil in water emulsion) for the systemic and intranasal delivery of subunit influenza vaccine was investigated (Barchfel *et al.*, 1999). The authors found that in direct comparison of the intramuscular and intranasal routes of immunisation in naive mice, the intranasal route could only enhance serum antibody response of the adjuvanted influenza subunit vaccine, but only to the same level as that of the unadjuvanted vaccine administered *via* the intramuscular route. The results of this study suggest that intranasal vaccination of humans with subunit influenza vaccine administered together with an adjuvant, may provide superior protection than i.m. immunisation for both initial infection and cross-protection. In the present studies the adjuvant activity of non ionic block copolymers within biodegradable microspheres, encapsulating diphtheria toxoid and OVA as model antigens was investigated. The immunogenicity of PLA/PLGA-based diphtheria vaccines was evaluated in a recent study by Johansen and co-workers (Johansen *et al.*, 2000). This study investigated the feasibility of a single-dose delivery system for DT using biodegradable polymeric microspheres to provide controlled antigen release, thus mimicking priming and booster doses (Johansen *et al.*, 2000). The authors examined the potential adjuvant effects of different types of PLA and PLGA microspheres encapsulating diphtheria toxoid. Their results presented high and persisting titres of protective antibodies in guinea pigs, comparable to those induced by

diphtheria toxoid adsorbed on alum. Their data have shown that the relatively hydrophilic PLGA 50:50 microspheres elicited very strong antibody responses 4-8 weeks after administration whereas the end-group stearylated PLA particles induced weak responses. One of the interesting findings of this work, has been the observation that the antibody levels at week 4 were slightly higher upon subcutaneous immunisation with the larger microspheres (15-60 μm) than the small ones (1-5 μm). This observation might suggest that full particle uptake by APCs might not be necessary for antigen processing. Several studies have examined the potential of nasal delivery of diphtheria vaccines (Aggerbeck *et al.*, 1997; Schroder and Svenson, 1999; Isaka *et al.*, 2000; McNeela *et al.*, 2001). In this chapter we investigated the immunogenicity of the optimised NIBC/PDLA formulations encapsulating two model antigens OVA and DT, using intramuscular and intranasal administration routes. We set out to examine the effect of different administration routes on the magnitude and duration of the immune response, for both antigens and followed the immunisation studies for a prolonged period of time.

4.2. Materials

4.2.1. Block copolymers

See section 3.2.1.1.

4.2.2. Antigens

See section 3.2.1.3.

4.2.3. Polymers

See section 3.2.1.2.

4.2.4. Chemicals

See section 3.2.1.4.

4.2.5. Animals

Outbred female BALB/c mice (6 to 8 weeks old; ~25 g) bred at Aston University (Birmingham) were used in these experiments. During the experiments and at all other times, animals were allowed food and water *ad libitum*.

4.2.6. Anaesthetics

A Boyle's veterinary anaesthetic apparatus (British Oxygen Company, Crawley) was used to administer a gaseous mixture of 3% halothane (RMB, Animal Health Ltd, Dagenham) in oxygen (300 cm^3 min^{-1}) nitrous oxide (1000 cm^3 min^{-1}) by inhalation.

4.3. Methods

4.3.1. Intramuscular immunisation with single dose of OVA-loaded PDLA/NIBC microspheres

Five groups of adult female BALB/c mice (5 animals *per* group) were primed with either different formulations encapsulating OVA (15 μg/Ms) or free solution of the above protein. Each dose of vaccine was suspended in isotonic PBS (pH 7.4), and each animal received 50 μl injection into the right quadriceps muscle. Tail vein blood samples were collected on days 14 and 28 post-immunisation. Serum was assayed by ELISA for the presence of anti-OVA IgG. The following table (4.1.) describes the different formulations, which were used in the intramuscular dosing of OVA-encapsulated formulations. No booster doses were administered in this study, since our objective in developing these formulations was to design single-dose vaccines.

Code PLA/NIBC formulation	Aqueous Phase: Acetone Ratio	PDLA (124kDa) concentration (mg ml^{-1})	L101 concentration (mg ml^{-1})	Number mean (μm (±SD)	Volume mean (μm) (±SD)	Actual loading (% w/w) (±SD)	Entrapment efficiency (±SD)	Yield (%) (±SD)
A	*1: 0.5 (0.75% PVA)	12	2.5	0.19 (±0.08)	8.16 (±14.7)	1.2 (±0.06)	24 (±0.03)	97.6 (±6.8)
B	*1: 0.5 (4% PVA)	12	2.5	0.19 (±0.08)	14.5 (±14.2)	0.34 (±0.02)	6.77 (±0.03)	20.1 (±2.6)
A+B Ratio (1:1)	—	—	—	—	—	—	—	—
Free solution	—	—	—	—	—	—	—	—

Table 4.1. PLA/NIBC microspheres encapsulating OVA (theoretical loading of 5% w/w) with modified formulation parameters, n=3.

4.3.2. Intranasal immunisation with OVA-loaded microspheres

Five groups of animals of adult female BALB/c mice (5 animals *per* group) were primed with either formulations (described in table 4.1.) encapsulating OVA (15 μg/Ms) or free solution of OVA. Each dose of vaccine was suspended in isotonic PBS buffer (pH 7.4), and each animal received 25 μl injection into each nostrils. Tail vein blood samples were collected on days 14, 28, 55 and 75 post-immunisation. Serum was assayed by ELISA (section 2.7.2) for the presence of anti-OVA.

4.3.3. Intramuscular immunisation with DT-loaded microspheres

Five groups of animals of adult female BALB/c mice (5 animals *per* group) were primed with either formulations (A, B and combinations of these as described in table 4.1.), encapsulating DT (5 Lf/Ms) or free solutions of DT. Each dose of vaccine was suspended

in isotonic PBS pH 7.4, and each animal received 50 µl injection into the right quadriceps muscle. Tail vein blood samples were collected on days 14, 28, 55 and 75 post-immunisation. In a follow up study six groups of animals were immunised with formulations which were further modified (table 4.2.). The immune response for this study was investigated on days 7,14, 35, 60, 105 and 286 post-immunisation. The effect of co-administration of the adjuvant QuilA (QS21) with the NIBC/ microspheres was also investigated in a study with four groups of animals (n=5 *per* group) (see table 4.3.). Serum was assayed by ELISA for the presence of anti-DT IgG, IgG1, IgG2a and IGg2b.

Table 4.2. Physical characteristics of different PDLA/NIBC formulation, (s)= suspension in PVA, (-NIBC)= L101not included

Formulation	Polymer	Size (number mean) (±SD)	Size (volume mean) (±SD)	Actual loading (%w/w)	Entrapment efficiency (%)	Yield (%)
1	124 kDa	0.2 (±0.0)	3.0 (±0.5)	4.96	99.2	62.0
2	283 kDa	0.2 (±0.0)	5.9 (±0.3)	4.90	97.6	81.8
3	Free DT	_____	_____	_____	_____	_____
4	124 kDa (s)	0.3 (±0.0)	3.9 (±0.3)	4.99	99.8	68.0
5	124 kDa (-NIBC)	0.2 (±0.0)	2.7 (±0.8)	4.60	92.0	72.4
6	124 kDa +283kDa	0.2 (±0.0)	9.5 (±0.1)	4.80	94.2	96.7

Table 4.3. Physical characteristics of different NIBC/PDLA microspheres administered with Quil A (QS21) (15µg) via intramuscular route.

Formulation	Polymer	Size (number mean) (±SD)	Size (volume mean) (±SD)	Actual loading (%w/w)	Entrapment efficiency (%)	Yield (%)
1	124 kDa	0.2 (±0.0)	3.0 (±0.5)	4.96	99.2	62.0
2	283 kDa	0.2 (±0.0)	5.9 (±0.3)	4.90	97.6	81.8
3	124 K +283K	0.2 (±0.0)	9.5 (±0.1)	4.80	94.2	96.7
4	Free DT	_____	_____	_____	_____	_____

4.3.4. Intranasal immunisation with DT-loaded microspheres

Similar groups of animals of adult female BALB/c mice (5 animals *per* group) were primed with either formulations (described in table 4.1. 4.2) encapsulating DT (5 Lf/Ms) or free solution of the above protein. Each dose of vaccine was suspended in an isotonic PBS (pH 7.4), and each animal received 25 µl injection into each nostril. Tail vein blood samples were collected as described for the intramuscular study on days 14 and 28 post-immunisation. Serum was assayed by ELISA for the presence of anti-DT IgG, IgG1, IgG2a and IgG2b.

4.4. Results and Discussion

4.4.1. Intramuscular administration of PDLA/NIBC microspheres encapsulating OVA

Serum anti-OVA IgG antibody titres were measured for all formulations on days 14, 28, 55 and 75 post immunisation. Titres for Formulations (A) and (A+B) were significantly higher than the free antigen on day 14, ($p<0.05$). The only disappointing result was that of formulation (B), which induced titre production lower than the free antigen for day 14 only, but unlike the free solution of the antigen maintained its adjuvanticity until day 28 post-immunisation. On day 28 all formulations administered *via* the inramuscular route, induced significantly higher IgG antibody titres than the free antigen ($p<0.05$) (Fig. 4.1). Formulation (A+B), which produced the highest antibody titres (7920) on day 14, contained two formulations with different release characteristics. The first one (A) had an initial burst release of 40% whereas formulation (B) had a burst release of 60%. The two on their own failed to induce a significant antibody titres but their combination managed to stimulate antibody response which was more than five times higher than the free antigen solution. The immune responses produced by OVA-loaded PLGA microspheres have been frequently linked to an adjuvant effect rather than to slow release of the encapsulated

protein, since there is evidence, that antigens adsorbed onto microparticles induce potent immune responses after subcutaneous (Eldrige *et al.*, 1988; O'Hagan *et al.*, 1993) and nasal administration (Alpar and Almeida, 1994). However, there are contradictory reports from the literature on the induction of immune response by a mixture of OVA and blank PLGA microspheres. Uchida *et al.*, (1994), showed that the administration of a mixture of OVA and blank PLGA failed to induce an immune response. In contrast O'Hagan and co-workers (1993), reported similar IgG antibody responses over 6 weeks following boosting, when 100 μg of OVA was administered either encapsulated or adsorbed onto blank PLGA. Uchida *et al.*, (1994), have also shown a dose-dependent immune response following subcutaneous administration of OVA-loaded microspheres, with no need for additional boosting at week three after the first inoculation. The same group also reported an inverse relation between the load of OVA in PLGA microspheres and the immune response. The present study has demonstrated that a single intramuscular administration of microencapsulated OVA, induces much higher, continuous immune responses which are significantly higher than the free antigen. On days 55 and up to day 75 (when experiment was terminated), all three formulations administered, produced higher antibody titres than a solution of free OVA ($p<0.05$).

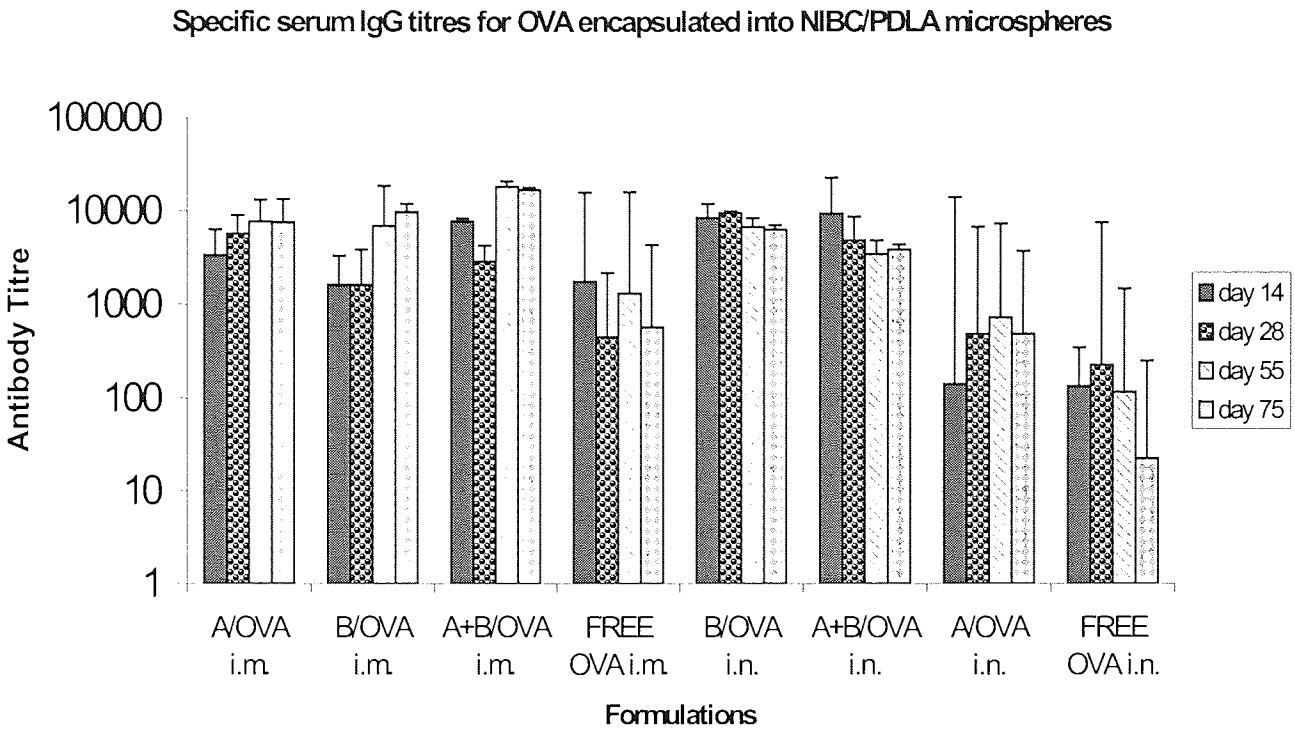


Fig 4.1. Specific serum IgG response to a single intramuscular and intranasal dose of OVA (15μg) encapsulated within NIBC/PDLA microspheres. A= formulation with aqueous to organic phase ratio of 1:0.5 and 0.75% w/v PVA in the external phase, B= formulation with aqueous to organic phase ratio of 1:0.5 and 4% w/v PVA in the external phase. A+B= combination of formulations A and B in 1:1 ratio.

The sustained immune response observed above could be due to a combination of different factors. The NIBC/PDLA particles releasing an immunogenic OVA over a prolonged period may act as an effective controlled delivery system. The particles may also provide a stable antigen source, presenting an intact antigen over a long period of time. The latter mechanism has been reported to explain the sustained immune responses (> 1year) to antigen, associated with PLGA particles (Coombes *et al.*, 1996).

4.4.2. Intranasal administration of block copolymer microspheres encpsulating OVA

The antibody titres produced at day 14 post-intranasal immunisation, showed very promising results. As with intramuscular results, the highest antibody titres corresponded to the formulation (A+B). This formulation produced up to 9427 antibody titre compared with measurements of 135 for the free antigen. Formulation B also stimulated antibody responses (8474), which were significantly higher than the free antigen (Fig.4.1). The intranasal responses were surprisingly high compared with the intramunscular antibody titres. Although at day 28 post-immunisation, the pattern of responses had slightly changed. Formulation (A+B) was not the highest antibody titres inducing formulation, and formulation (B) had peaked to reach nearly twice as much as that of the formulation (A+B). The immune response was sustained at levels, which were significantly higher than those for the free antigen for formulations B and A+B up to day 55 post-immunisation. On day 75 however, the antibody titres produced with both these formulations were no longer significantly higher than those induced by the free antigen.

4.4.3. Intramuscular administration of block copolymer microspheres encpsulating DT

Specific serum antibody (IgG) responses to microspheres loaded with DT were quite promising following i.m. delivery. All formulations induced IgG antibody titres, which were higher than free antigen administered through this route (Fig. 4.2.). Formulation (B) produced IgG titres which were more than ten fold higher than the free antigen. The serum antibody response was generally higher following intramuscular than that of the intranasal administration. The DT-loaded microspheres produced lower antibody titres compared with OVA-loaded microspheres on the whole.

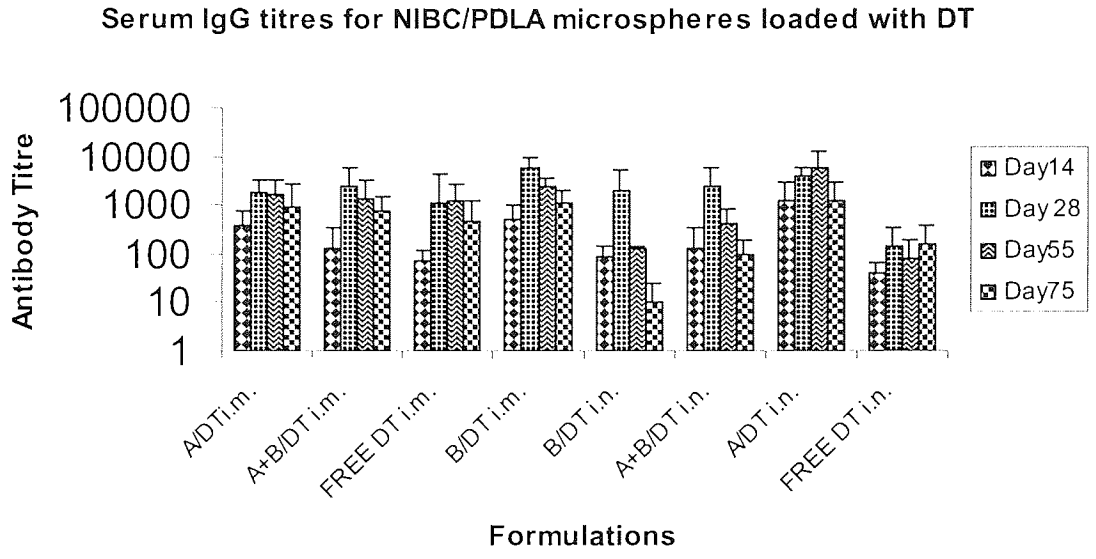


Figure 4.2. Serum IgG response to a single intramuscular and intranasal dose of DT (5Lf) encapsulated within NIBC/PDLA microspheres, n=5. A= formulation with aqueous to organic phase ratio of 1:0.5 and 0.75% w/v PVA in the external phase, B= formulation with aqueous to organic phase ratio of 1:0.5 and 4% w/v PVA in the external phase. A+B= combination of formulations A and B in 1:1 ratio.

4.4.4. Intranasal administration of block copolymer microspheres encapsulating DT

Intranasal administration of DT-loaded microspheres produced lower IgG titres compared with OVA-loaded particles. Formulation (A) induced the highest antibody titres (1261) compared with free antigen (17) on day 14 post-immunisation ($p<0.05$). Formulation (B) also, produced high titres (131), but the formulation (A+ B) containing equal amounts of the (B) and (A) failed to induce higher response than the titres associated with the individual formulations on this time point (Fig. 4.2.). The same formulation encapsulating OVA had resulted in the highest antibody titres (9427) amongst the different formulations on day 14. Formulation A continued to induce significantly higher immune response than

the free antigen ($p<0.05$) on days 28 and 55 and 75 post-immunisation. Formulations B and A+B also produced high immune response on day 28 post-immunisation. The latter formulation sustained a significantly higher immune response than the soluble antigen on day 55, $p<0.005$. This profile was not observed for formulation B on day 55. These results may be explained in terms of the effect of particle size on the extent of immune response through intranasal administration. It is thought that intranasal administration of particles might result in smaller particles (between 1 and 2 μm) being absorbed at the nasal mucosa or at the NALT. However, these particles may be translocated to regional lymph nodes, inducing a systemic response (Rebelatto *et al.*, 2001). Larger particles (<3 μm) have been shown in humans to be retained in the nasal cavity when inhaled (Stuart, 1984) thus inducing a mucosal immune response. Although the most appropriate size for nasal administration is yet to be determined, it may be that the better performance of formulation A compared to the other two formulations is due to the larger size of these particles (volume mean of 14 μm, with 20% of the particles being <5 μm) compared to the size of formulation B (volume mean of 11 μm, with 50% of the particles being <5 μm). The Formulation A particles may have been retained at the NALT to a larger extent than formulation B due to the higher percentage of particles bigger than 5 μm, thus inducing a mucosal immune response. The smaller particles of formulation B having a higher percentage of particles below 5 μm may have been partly absorbed by the nasal mucosa and partly translocated to the regional lymph nodes. This might explain the immune response induced by formulation A+B, which was not as strong as formulation A but was better and longer-lasting than that of formulation B. Singh and co-workers were the first group to describe the use of PLG polymers for the microencapsulation of DT. They demonstrated that a single intramuscular dose of DT entrapped in microparticles induced comparable serum IgG titres in mice to three divided dose of DT adsorbed to calcium

phosphate adjuvant (Singh *et al.*, 1991). The same group also reported that optimal immune responses were obtained with combinations of PLG microparticles and alum (Singh *et al.*, 1998). These studies have shown that PLG microspheres have significant potential for the development of single-dose vaccines for DT. Diphtheria is a bacterial disease, which is caused by the toxin *Corynebacterium diphtheriae*. Diphtheria toxoid vaccines have been administered through the intramuscular or subcutaneous injections for a long time, in order to stimulate toxin neutralising antibodies, which is extremely important in achieving protection. Oral administration of toxoid incorporated lozenges and inhalation of aerosolised toxoid had been tried a long time ago, with limitations such as the need for high amounts of antigen or adverse allergic reactions (Bousfield and King-Brown, 1938). The intranasal route of delivery has also been reported for DT vaccines recently. These have included the intranasal administration of fluid DT with non-ionic excipients in mice (Gizurason *et al.*, 1995) and with an enhancer mixture of caprylic/capric glycerides and polysorbate in humans (Aggerbeck *et al.*, 1997). In a recent study the effect of intranasal administration of DT together with recombinant cholera toxin B subunit on the induction of systemic and mucosal immunity in mice was investigated (Isaka *et al.*, 2000). This study appears to yield promising results on the potential of intranasal delivery of DT vaccine. The investigators found that intranasal immunisation of aluminium-non-adsorbed DT in the presence of rCTB not only induced high levels of DT-specific serum IgG antibody, but also provided diphtheria antitoxin titres above a protective level of 0.1 IU ml^{-1}. The major serum IgG subclass induced was IgG1 followed by IgG2b antibody titres, although significant IgG2a responses were observed too. There is evidence that, the complement binding subclasses IgG2a and IgG2b are more important in mice than IgG1 or IgG3 for combating certain infectious agents (Allison and Byers, 1986; Katz *et al.*, 1991). However, the IgG1 and IgG2b seem to be just as important in neutralisation of exotoxins

produced by *C. diphtheriae* according to the patterns of IgG antibody-subclasses (Isaka *et al.*, 1998).

4.4.5. Effect of varying formulation parameters on the immune response to intramuscular administration of single dose (5Lf) DT-loaded particles

The immune response to different formulations (listed in table 4.2), in mice was followed up to day 286 following the intramuscular administration of one single dose of diphtheria toxoid (5Lf) given either as free or encapsulated within microspheres. The aim of this study was to achieve two objectives, the first was to evaluate the extent of the adjuvanticity of the block copolymer (in comparison to formulations without the NIBC), and the second to investigate the effect of varying parameters such as molecular weight and the surface coating of particles with PVA, on their immune response. The serum IgG titres were measured on days 7, 14, 35, 60, 105 and 286 post-immunisation. On day 7, all formulations induced serum IgG titres, which were significantly higher than the free antigen ($p<0.05$) (Fig. 4.3). The order of the immunogenicity on day 7 is PVA-suspended particles > microsphere blend > high molecular weight polymer-particles > lower molecular weight particles. The immune response continued to increase on days, 14 and day 35 (showing the highest antibody titres). On day 35 all formulations induced high antibody titres with no significant differences between different formulation. This point was of particular interest as it highlighted the importance of the block copolymer in the induction of immune response at the early stages of immunisation. The serum IgG titres showed a slight decrease on day 60 for all formulations, a trend, which continued on day 105 and day 286 post-immunisation. The exception to the above was the immune response elicited by the blend formulation on day 286, which showed an increase in the antibody titre compared to day 105. All formulations sustained an immune response, which was significantly higher ($p<0.05$) than the free antigen from day 7 to day 286 after one single

intramuscular immunisation. The difference between the formulations seemed to manifest itself only in the early stages of the immunisation (days 7 and 14). By day 35 and from then onwards no significant difference was observed between the immune response elicited by different formulations.

The IgG sub-classes (IgG1, IgG2a, and IgG2b) were measured for the above particles at day 35 post-immunisation (Fig. 4.4). The IgG1 subclass dominated the immune response elicited by all different formulations. In all formulations, the amount of IgG2a and IgG2b seemed to be similar and no significant difference was observed between formulations. These findings are in agreement with those of Hunter and co-workers (Hunter *et al.*, 1991), in that the block copolymer L101, induced a predominant IgG1 response with lesser amounts of IgG2a and IgG2b. The ratio of IgG1 to IgG2b antibody showed no significant difference between different formulations. The stimulation of specific isotype of antibody and/or type of cell mediated immunity is necessary for protection against certain infectious diseases. Therefore, in developing novel vaccines, an understanding of the properties of immunogens which influence the induction of different types of immune response, would be extremely valuable (Kenny *et al.,* 1989, Kaminski *et al.*, 1986; Brodskin, *et al.*, 1988; McKendall *et al.*, 1988).

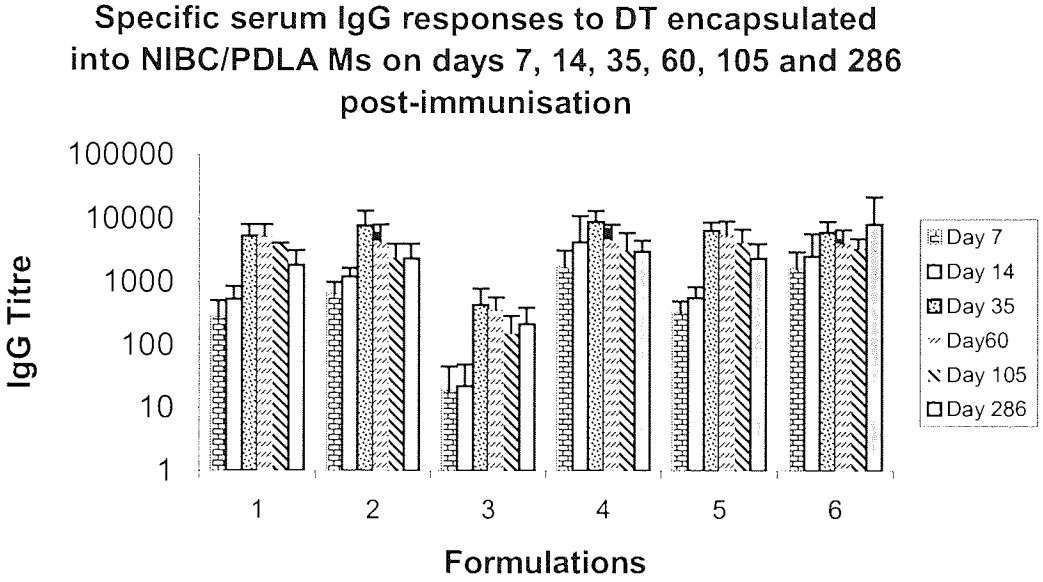


Figure 4.3. Serum IgG response to a single intramuscular dose of DT (5Lf) encapsulated within NIBC/PDLA microspheres (aqueous phase: acetone ratio 1:0.5 and 0.75% w/v PVA) in the external phase. 1=PDLA 124kDa, 2= PDLA 283 kDa, 3=free DT, 4=PDLA 124kDa with microspheres suspended in PVA.,5 =PDLA 124kDa, without NIBC, 6= PDLA 124+283 kDa, n=5.

Switching of the immunoglobulin (Ig) class is a process in which B cells that initially express either IgM and/or IgG switch on immunisation, to the expression of IgE, IgA, or one of four IgG subclasses (Snapper and Mond, 1993). T cells can play an important role in regulating the above switch, due to their ability to secrete cytokines. Three cytokines, interleukin 4 (IL-4), gamma-interferon (IFN-γ), and transforming growth factor beta (TGF-β), play pivotal roles in regulating Ig isotype switching in the mouse. It has been shown that *in vitro,* IL-4 selectively stimulates the production of murine IgG1 and IgE, whereas IFN-γ, induces IgG2a (Mosmann and Coffman, 1989). Infection with intracellular

pathogens, such as viruses, typically induces Th1 responses, and as would be expected IFN-γ would be a critical effector component. The Th2 responses dominate, when antibody responses are the critical effector mechanism and the activation of these responses is influenced by IL-4. In the present study the effect of the inclusion of the block copolymer L101 in microsphere formulations on the IgG subtypes, has been evaluated as an indirect evaluation of the type of immune response induced. The results show that although, all formulations produced lower IgG2a titres, there was a significant difference between formulations 1 (low molecular weight polymer, 124kDa) and 2 (high molecular weight polymer, 283kDa) and the formulation which lacked L101 at day 35 post-immunisation (Fig 4.4a). The influence of the block copolymers on the induction of specific antibody sub-types has been evaluated mostly in the works of Hunter and co-workers (Hunter *et al.*, 1995) with recent evidence presented by Newman and his colleagues (Newman *et al.*, 1999). Preparations with POP chains less than 5000Da (such as L101) induce more IgG1 antibody than any other subclass, whereas larger copolymers stimulate higher titres of IgG2a and IgG2b.

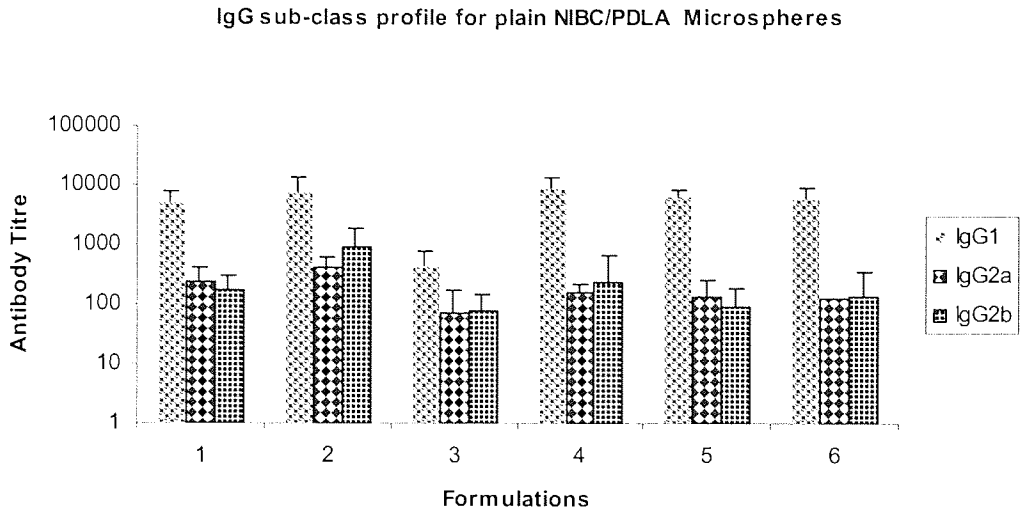


Figure 4.4a. Subclass IgG responses in mice to a single intramuscular dose of DT (5Lf) encapsulated within NIBC/PDLA microspheres microspheres at day 35, values are mean ± SD, n=5. Formulations contain (aqueous phase: acetone ratio 1:0.5) and 0.75% w/v PVA in the external phase. 1=PDLA 124kDa, 2= PDLA 283 kDa, 3=free DT, 4=PDLA 124kDa with microspheres suspended in PVA.,5 =PDLA 124kDa, without NIBC, 6= PDLA 124+283 kDa,

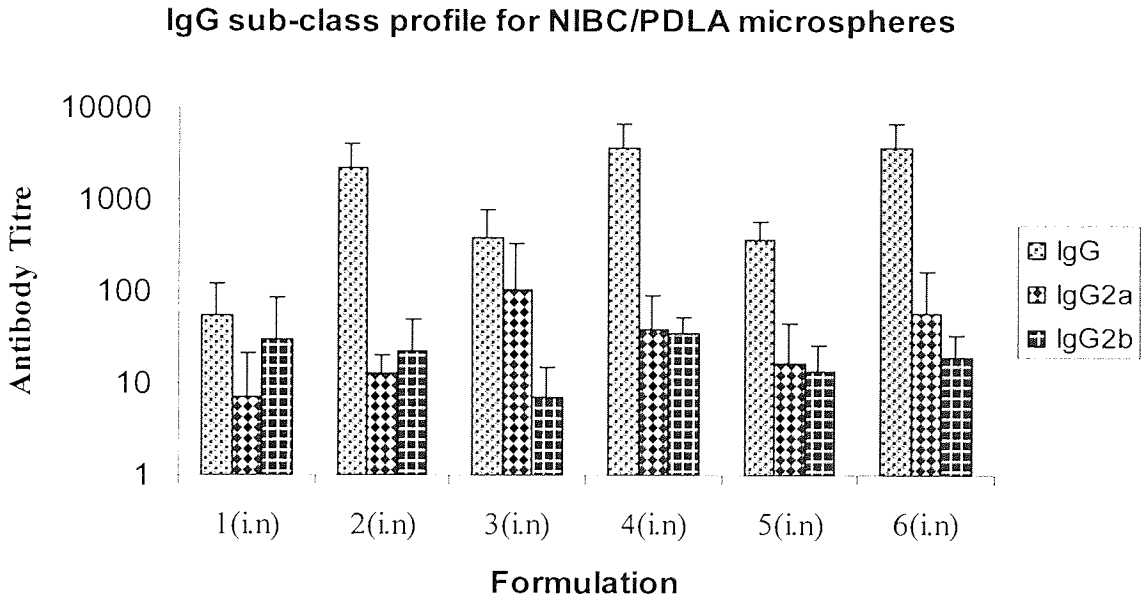


Figure 4.4b. Subclass IgG responses in mice to a single intranasal dose of DT (5Lf) encapsulated within NIBC/PDLA microspheres microspheres at day 35, values are mean ± SD, n=5. Formulations contain (aqueous phase: acetone ratio 1:0.5) and 0.75% w/v PVA in the external phase. 1=PDLA 124kDa, 2= PDLA 283 kDa, 3=free DT, 4=PDLA 124kDa with microspheres suspended in PVA.,5 =PDLA 124kDa, without NIBC, 6= PDLA 124+283 kDa,

The data presented by Hunter and colleagues highlights the importance of particular surface-active properties of the copolymers which facilitates their adhesion to the surfaces of the antigen and the antigen presenting cells, thus inducing their adjuvant activity. The present work emphasises the potential of the NIBC-based polymeric delivery systems as vaccine formulations. In this work, data has been presented, which highlights the early and sustained immune response elicited by single-dose administration of such delivery systems.

4.4.6. Effects of combination dosing of DT (5Lf) within NIBC/PDLA microspheres and saponin (Quil A, QS-21) through intramuscular administration

Saponins have been successfully used in combination therapies, complementing the adjuvanticity of existing vaccines (James and Pearce, 1988). Encouraged by previous, a study was carried out in which to examine the effect of combination dosing of the two adjuvants (L101 and Quil A, QS-21) on the induction of immune response in mice. Four groups of animals (n=5 /group) were immunised through intramuscular route with formulations listed in table 4.3. Serum IgG subfractions (IgG1, IgG2a and IgG2b) were collected and measured on days 14 and 35 post-immunisation. Direct comparison between the combined formulations and those of NIBC/PDLA particles presents a clear shift towards the enhancement of IgG2a subclasses (Fig. 4.5.).

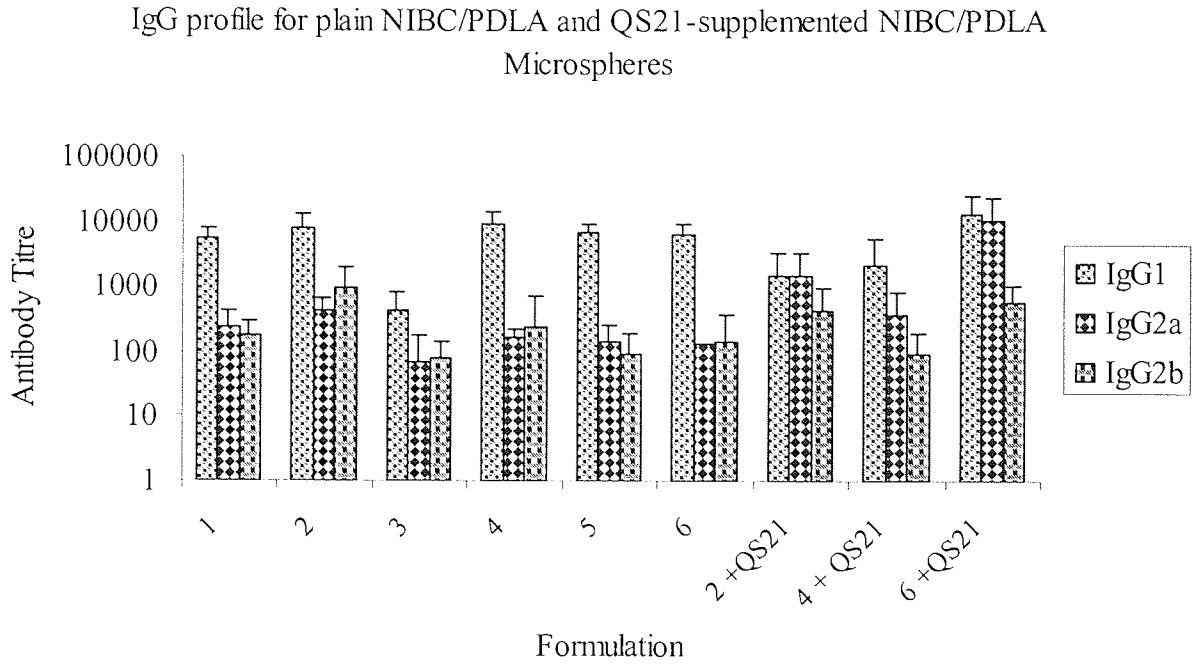


Figure 4.5. Comparison of the subclass IgG responses in mice to a single intramuscular combination dose of DT (5Lf) encapsulated within NIBC/PDLA microspheres and QS 21 (15μg) and NIBC/PDLA particles alone, at day 35, values are mean ± SD, n=5. Formulations contain (aqueous phase: acetone ratio 1:0.5) and 0.75% w/v PVA in the external phase. 1=PDLA 124kDa, 2= PDLA 283 kDa, 3=free DT, 4=PDLA 124kDa with microspheres suspended in PVA.,5 =PDLA 124kDa, without NIBC, 6= PDLA 124+283 kDa.

The biggest shift was observed in the formulation, which was a mixture of the two high and low molecular weight polymers with NIBC. The formulation with the higher molecular weight polymer in combination with the QS-21 also induced high IgG2a titres, which was significantly higher than that induced by the NIBC/PDLA formulation ($p<0.05$). These findings present a promising possibility of a vaccine formulation, which can be modulated to induce a specific immune response (antibody isotype), with much ease. Newman and co-workers have presented evidence, which suggests that using appropriate adjuvant-active copolymers, it may be possible to split the activities of the immune system (Newman *et al.*, 1998). These authors also investigated the type of

immune response, induced between alum, Quil A saponin and high-molecular weight block copolymers. They found that based on the cytokines produced *in vitro,* alum induced primarily a Th2 response characterised by high levels of IL-10 and IL-5 with low levels of γ-IFN and no detectable IL-2. Quil A saponin induced higher levels of γ-IFN and IL-2 but comparable levels of IL-5 and IL-10. On the other hand the more hydrophobic copolymer (5% POE), induced a mixture of Th1 and Th2 immune responses characterised by high levels of γ-IFN, IL-2, IL-5 and IL-10. The less-hydrophobic copolymer resulted in lower levels of γ-IFN, undetectable levels of IL-2, and high levels of IL-5 and IL10. Bomford has characterised saponin as a potent adjuvant for strong but not weak antigens, and that it is a particularly effective adjuvant for antigens in cell membranes (Bomford, 1980). The administration of QS-21 intradermally in a 15 μg dose, stimulated antibody titres against *Escheichia coli* 055:B5 polysaccharide by more than 10-fold (White *et al.,* 1991). QS-21 has been shown to increase antibody response to T-independent antigens (Flebble and Braley-Mullen, 1986a, 1986b). In addition to potentiating the humoral immune response, saponin is also an efficient inducer of cell-mediated immunity in combination with killed vaccine preparations or soluble antigens, when given parentally (Newman *et al.*, 1992). These researchers found that, in the presence of QS-21, a specific $CD8^+$ cytotoxic T lymphocyte (CTL) response is induced in mice against OVA given subcutaneously. This finding is of particular interest, since CTL response are more commonly induced against cells infected with replicating virus, in which the antigens are processed through the endogenous MHC class I-restricted pathway. On the other hand, the non-particulate antigens normally stimulate a class II MHC response. Therefore, it appears that saponins can alter the intracellular processing of OVA from the class II to the class I MHC antigen pathway. The same phenomenon has been observed with ISCOMs (Heeg *et al.*, 1991, Takahashi *et al.*, 1990). The combination of saponins and non ionic block

copolymers as evident from our own data presents a promising approach for the safe and efficient delivery of microencapsulated antigens.

4.4.7. Splenic cell responses to DT-loaded NIBC/PDLA microspheres

4.4.7.1. Splenic-cell proliferation assay

In order to evaluate the type of immune response stimulated by the DT-loaded microspheres, the antigen-induced splenocyte T cell proliferation assays and cytokine release assays were performed. The T cell assay was carried out to complement the analysis of the antibody isotypes induced in response to DT-loaded microspheres. The results have been presented graphically in figures 4.6. and 4.7. Results show that significant T cell proliferative responses were obtained in all formulations administered both via i.m and i.n routes. The proliferation order was as follows, Free DT< R206 i.m.< R208 i.m< -NIBC< R206i.n. Formulation R206 (low-molecular weight polymer) delivered intranasally produced the strongest T cell proliferation, whereas the same formulation delivered via the intramuscular route was amongst the least effective preparations. This suggests that other factors than the physical characteristics of the microspheres have influenced the rate T cell proliferation induced through the intranasal administration. The induction of both mucosal and systemic immunity through nasal administration could be one explanation for the above observation.

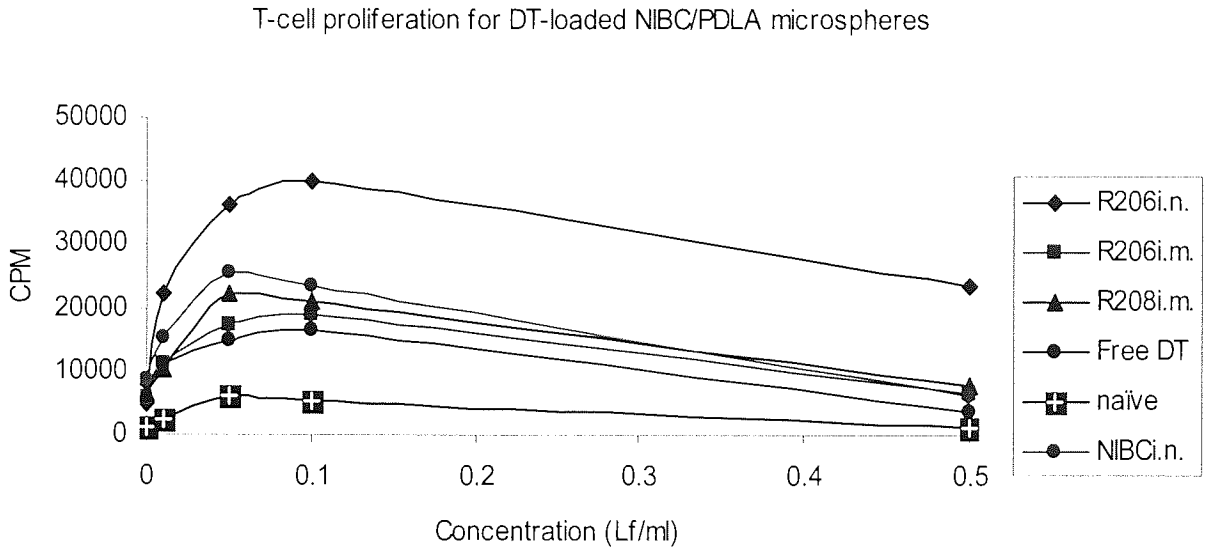


Figure. 4.6. Proliferative responses in BALB/c mice following administration of one single dose of DT-loaded formulations (5Lf/dose). At four months post-immunisation 3-5 mice were sacrificed for performing proliferation assays. The values represent mean c.p.m. (difference between the testing and background) for triplicate wells from cultures cells and mean c.p.m for each immunization group. Formulations contain (aqueous phase: acetone ratio 1:0.5) and 0.75% w/v PVA in the external phase. R206=PDLA 124kDa, R208= PDLA 283 kDa, DT75=PDLA 124kDa with microspheres suspended in PVA, Blend= PDLA 124+283 kDa. NIBC= PDLA 124kDa with no NIBC included. I.n= intranasal delivery, i.m= intramuscular delivery.

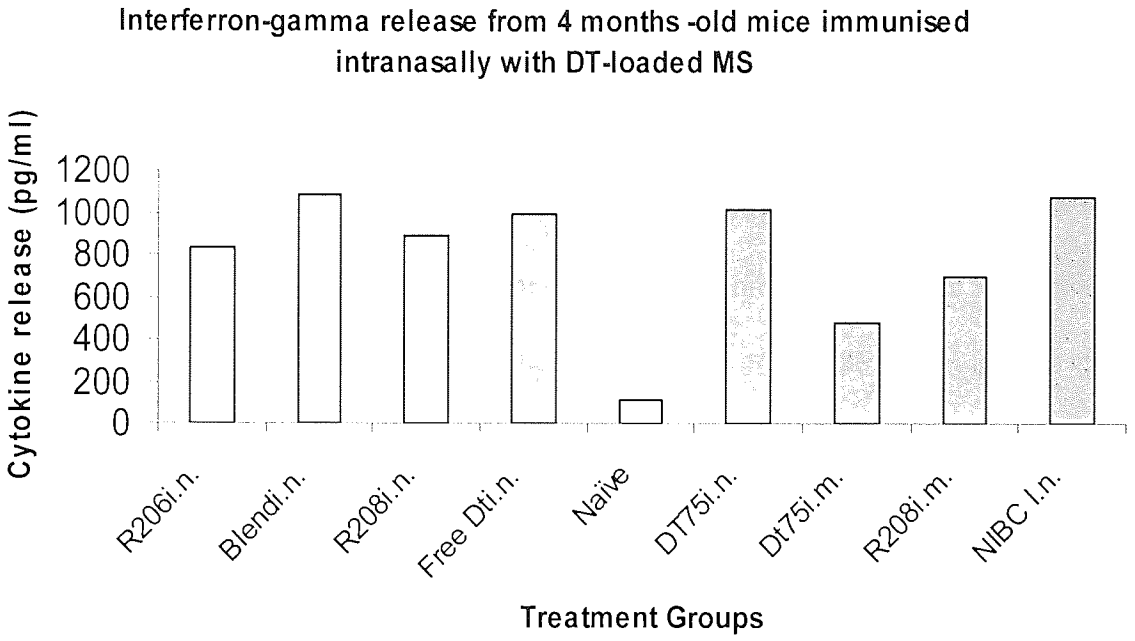


Figure. 4.7. IFN-γ in BALB/c mice following administration of one single dose of DT-loaded formulations (5Lf/dose). At four months post-immunisation 3-5 mice were sacrificed for performing cytokine release assays. . Formulations contain (aqueous phase: acetone ratio 1:0.5) and 0.75% w/v PVA in the external phase. R206=PDLA 124kDa, R208= PDLA 283 kDa, DT75=PDLA 124kDa with microspheres suspended in PVA, Blend= PDLA 124+283 kDa. NIBC= PDLA 124kDa with no NIBC included. I.n= intranasal delivery, i.m= intramuscular delivery.

The stimulation of T cell responses four months after a single dose of DT-loaded particles, suggests that as well as an induction of an early immune response, these particles are capable of sustaining a more durable effect on the activation state of specific lymphocytes.

4.4.7.2. Cytokine release analysis

The cytokine release profile showed high levels of IFN-γ released four months after immunisation with one single dose of DT encapsulated within NIBC/PDLA microspheres (Fig.4.6). The high cytokine responses observed in spleen cells DT specific, thus as well as the microsphere formulation, the DT alone also stimulated elevated IFN-γ titres. The release of this cytokine is an indication of the involvement of Th1 responses to the administered antigen. IFN-γ is an important effector function of T cells, due to its ability to activate macrophages and reduce viral replication (Ahmed *et al.*, 1987, Byrne *et al.*, 1984). The IL-4 release was marginal compared to the IFN-γ release. The results (Fig.4.8.) are consistent with the findings of Kim and co-workers (Kim *et al.*, 2000), who found that the adjuvant TitreMax (containing block copolymers) was totally ineffective in the IL-4 release assay. Over all the present study seems to point towards a stronger Th1 stimulation than a Th2 response based on the cytokine release findings. However, it is possible that the time of sampling might not have been optimal for a stronger IL-4 release. These results together with the antibody responses to the above formulations present a promising view of a safe and effective delivery vehicle for DT antigen, capable of stimulating both humoral and cell-mediated immunity a long time after administration of a single dose.

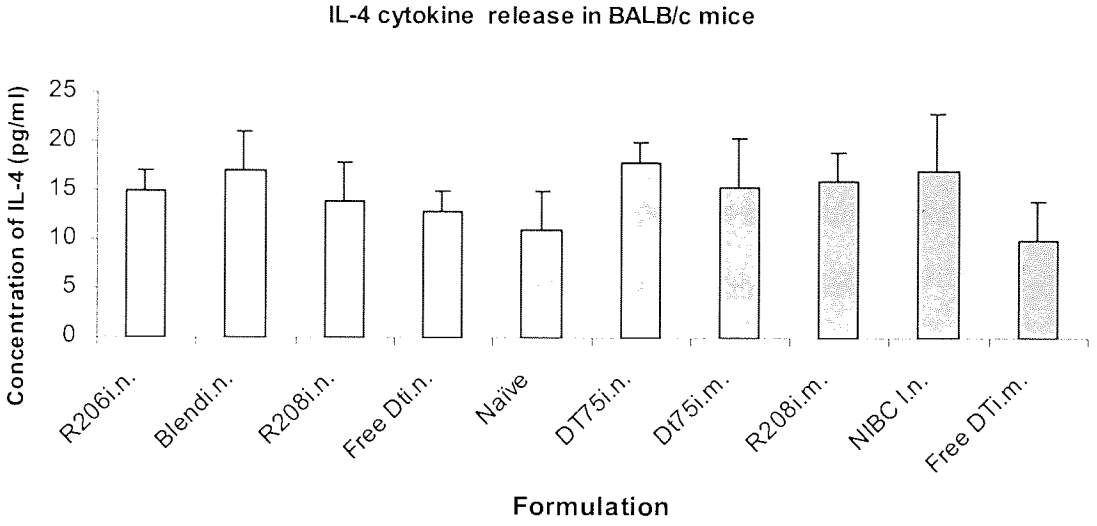


Fig. 4.8. IL-4 in BALB/c mice following administration of one single dose of DT-loaded formulations (5Lf/dose). At four months post-immunisation 3-5 mice were sacrificed for performing cytokine release assays. Formulations contain (aqueous phase: acetone ratio 1:0.5) and 0.75% w/v PVA in the external phase. R206=PDLA 124kDa, R208= PDLA 283 kDa, DT75=PDLA 124kDa with microspheres suspended in PVA, Blend= PDLA 124+283 kDa. NIBC= PDLA 124kDa with no NIBC included. I.n= intranasal delivery, i.m= intramuscular delivery.

4.4.8. Effect of long-term storage on the immunogenicity of NIBC/PDLA microspheres encapsulating DT

Development of protein therapeutics has been for long a point of focus for biotechnology industry. To be successful as a therapeutic device, a protein must be highly purified and concentrated, must be lyophilised and sustain a long shelf life (at least two years). Proteins being large molecules with complex secondary, tertiary and in some cases, quaternary structure are liable to denaturation, aggregation or complete loss of activity when processed under biotechnological conditions. However, the development of drug delivery vehicles consisting of biodegradable polymers in which the proteins can be protected from the harsh conditions of the environment has presented an important route into utilising

proteins in therapeutic areas. The difficulty in developing microencapsulated protein preparations has been due to the harsh conditions to which the proteins are exposed in the process of formulation. These include the use of organic solvents, high shear forces and also the lyophylisation of protein preparations. Additional problems also arise from the need to sustain a biologically active protein within the microspheres. Particulate delivery systems such as microspheres have been shown to possess adjuvant activity, Silvestri *et al* (1990). The stability of the entrapped antigen within these delivery vehicles and the induction of rapid immune response which provides protection immediately before or after exposure to pathogens remain the utmost important considerations in developing novel drug delivery vehicles. The use of organic solvents and the high-speed homogenisation usually employed during (W/O)/ W emulsions may compromise the integrity of the antigen and its subsequent release, which may cause a delay in the induction of immune response. In the formulations using the NIBC/PDLA system, the development of microsphere formulations using mild manufacturing conditions and the induction of rapid immune response by such delivery systems have been evaluated. The immunogenicity of the Diphtheria toxoid was investigated by administering the microspheres six months following their preparation (kept at room temperature in a desiccated vessel). The intranasal administration of the above preparations have resulted in immune responses which are comparable (in some cases higher) than the intramuscular administration of newly prepared microspheres. The stability of PDLA microspheres has been investigated by Delgado *et al* 1998, which found that the microspheres size, shape and the drug (methadone) loading were unchanged throughout three years of storage at room temperature in the dark in glass vessels containing silica gel. The NIBC/PDLA formulations presented in this study administered both intranasally and through

intramuscular route seem to produce high antibody responses early on in the course of vaccination.

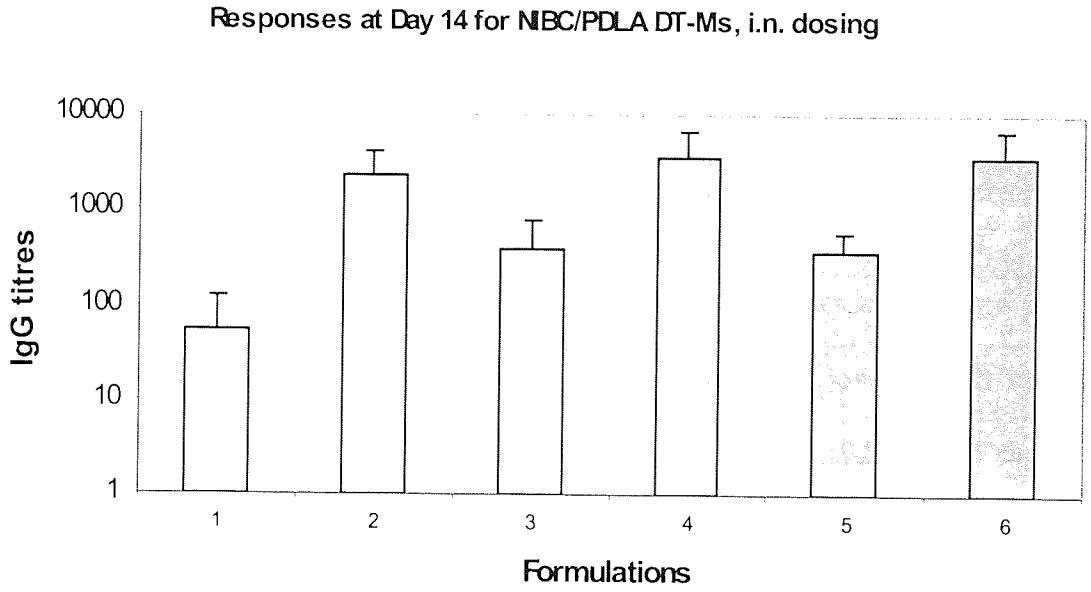


Figure 4.9. Total antibody titres in mice to Diphtheria Toxoid (5Lf) in NIBC/PDLA microspheres and in free solution administered via intranasal route six months after the date of microsphere preparation. . Formulations contain (aqueous phase: acetone ratio 1:0.5) and 0.75% w/v PVA in the external phase. 1=PDLA 124kDa, 2= PDLA 283 kDa, 3=free DT, 4=PDLA 124kDa with microspheres suspended in PVA.,5 =PDLA 124kDa, without NIBC, 6= PDLA 124+283 kDa. I.n= intranasal delivery, i.m= intramuscular delivery.

5.0. Encapsulation of alum-associated antigens within PLA and PLGA microspheres

5.1. Introduction

Aluminium compounds, including aluminium phosphate ($AlPO_4$), aluminium hydroxide [$Al(OH)_3$], and other alum-precipitated vaccines are currently widely used with human vaccines (Gupta *et al.*, 1995). They have often been referred to as 'Alum' in the literature. This is a misleading concept because aluminium phosphate and aluminium hydroxide have different physical characteristics and differ in their adjuvant properties. Aluminium adjuvants have a long history of use with routine childhood vaccines since it was first discovered that a suspension of alum-precipitated diphtheria toxoid (DT) had a much higher immunogenicity than the soluble toxoid (Glenny *et al*, 1926). The superiority of alum-precipitated DT and tetanus toxoid (TT) in humans has been well established since the 1930s and they have been commonly used ever since. Aluminium compounds therefore have become the benchmark or reference preparations for evaluating new adjuvants. To avoid batch-to-batch variations and non-reproducibility due to the use of different preparations of aluminium compounds, a specific preparation (Alhydrogel®, aluminium hydroxide, from superfoss Biosector, Denmark) has been recommended as a scientific standard for evaluation of new adjuvant formulations (Stewart-Tull, 1989). This product has been identified as poorly crystalline aluminium oxyhydroxide. It has a high surface area and high pI (11), which provides the adjuvant with a high adsorptive capacity for negatively charged proteins (in physiological pH range around 7.4). Two methods have been commonly used to prepare vaccines and toxoids with the above compounds: *in situ* precipitation of aluminium compounds in the presence of antigen, and adsorption of antigen onto preformed aluminium gel (Aprile and Wardlaw, 1966; Edelman, 1980). The

first method has been described by World Health Organisation report, to produce a compound that could not be defined either quantitatively or qualitatively (World Health Organisation, 1976) and therefore is no longer in common use. The adsorption of the antigens onto preformed aluminium phosphate or aluminium hydroxide gels under controlled conditions (Bomford, 1989) is the method most currently used resulting in preparations, usually referred to as aluminium phosphate- or aluminium hydroxide-adsorbed or- adjuvanted vaccines. Electrostatic forces between the antigen and the adjuvant are the main determinants of antigen adsorption, with the secondary contributions of hydrophobic, van der Waals and hydrogen bonding interactions. These secondary contributors however, may not exert an effect if the same charge (electrostatic repulsive force) is present on adjuvant and the antigen. At neutral pH, aluminium hydroxide is positively charged whereas aluminium phosphate is negatively charged, so the choice of the right adjuvant is crucial in relation to the charge of the antigen at neutral pH. Antigens having a pI below 7.4 will be most strongly absorbed by aluminium hydroxide adjuvant, while antigens whose pI is above 7.4 will be best adsorbed by aluminium phosphate adjuvant. Pre-treating aluminium hydroxide with phosphate anions will lower the pI, allowing for adsorption of antigens having pIs above 7.4. The main physical conditions affecting adsorption are pH, temperature and ionic strength of the reaction mixture. The optimal pH for adsorption of DT and TT onto aluminium phosphate is 6.0-6.3. The adsorption of DT is influenced by the presence of excess phosphate ions in the reaction mixture, (therefore need to avoid phosphate-buffered saline). Adsorption of TT and DT onto Alhydrogel has been found not to be sensitive to the conditions of pH and excess phosphate ions. In contrast, a low ionic strength and absence of excess phosphate ions and impurities are recommended for optimal adsorption of antigens on aluminium phosphate gels. In general the adsorption of antigens [DT, TT, human serum albumin (HSA)] is

higher on aluminium hydroxide than aluminium phosphate (at room temperature, overnight, pH of 6.0). There has been some discussion on desorption of antigens from adjuvant after injection into the body where a physiologically neutral pH and presence of body fluids containing proteins and anions might desorb the antigen from the gel. Studies on desorption of antigens from aluminium hydroxide have shown that the phosphate and sulfate anions cause desorption of OVA, while nitrate anions had no effect (Rinella *et al*, 1995). In one study the amount of the antigen exceeded the adsorptive capacity of the aluminium hydroxide adjuvant (2.6mg/mgAl). As a result 45% and 10% of the antigen was desorbed in the presence of 4mM phosphate and 4mM sulfate anions respectively, on the same day of adsorption. This effect decreased with the age of the protein-adjuvant complex (down to 23% on day 17). When the protein concentration was approximately 50% of the adsorptive capacity of the gel, the behaviour was similar except much less OVA was desorbed (23 and 2% for phosphate and sulfate anions respectively). The addition of 4mM phosphate anions results in the change of the surface charge of the aluminium hydroxide (becomes negative at pH 7.4). The resultant repulsive force desorbs the negatively charged OVA from the alum.

5.1.1. Mechanism of action

The mechanism of action of the aluminium adjuvants is not fully understood as yet and is thought to involve a number of different mechanisms. These compounds have been shown to exert their effect through depot formation at the site of injection, allowing slow release of antigen, and thus prolonging the time for interaction between antigen and antigen-presenting cells and lymphocytes. Their mechanism of action also involves the non-specific activation of immune system (Gupta *et al*, 1998). The particle diameters are 3.07 and 4.26 μm for aluminium hydroxide (Alhydrogel®) and aluminium phosphate (Adju-

their adjuvanticity (Eldridge *et al*, 1991). They may activate the complement system, which may lead to a local inflammatory response, thus enhancing the antibody response. Aluminium compounds have an extensive record of safety and offer many advantages in their preferred use for human vaccines. These advantages can be summarised as follows:

a) Development of earlier, higher, and longer lasting immunity after primary immunisation compared to soluble vaccines.
b) Immunogenicity dependent on the degree of adsorption of antigen and the dose of adjuvant.

Adsorption of 80% or more of TT and DT onto aluminium adjuvants is recommended by world health organisation (WHO, 1977). As the amount of adjuvant increases the effect is increased up to a certain concentration after which the effect declines with further increases of adjuvant. Reasons for this could be that excessive amounts might suppress immunity by covering the antigen completely with minerals or by being toxic to macrophages. The recommended dose of aluminium used for human vaccines is around 0.5 mg. The upper acceptable limits of aluminium adjuvants for human injection is 1.25 mg aluminium as *per* (WHO) regulations and 0.85-1.25 mg aluminium as *per* FDA guidelines (Gupta *et al*, 1998). Despite their safety record, the above adjuvants suffer from a number of limitations. The following lists the disadvantages associated with the use of these compounds (Gupta *et al*, 1995):

a) Inconsistent antibody production
b) Increase IgE-mediated allergic reactions
c) Ineffectiveness for certain antigens and induction of mainly humoral immunity by eliciting primarily Th2-type responses
d) Non-biodegradable

e) Difficulties in their freezing and lyophilisation.

Aluminium compounds have been used in combination with liposomes and mono-phosphoryl lipid A (MPL) (Alving *et al.*, 1993) and QS-21 (Kensil *et al*, 1991) and biodegradable polymers (Diwan *et al*, 1998). The combination of these compounds with the biodegradable polymers has the potential of maximising the immune response using smaller doses of the alum than is routinely used. This project was set out to encapsulate the alum (Alhydrogel®) within PLLA microspheres with a view to enhancing the immune response to formulations encapsulating clinically relevant antigens such as tetanus toxoid, over a long period of time without the need for boosting experiments. The encapsulation of alum within polymeric microspheres facilitates the long-term storage of the above mentioned tetanus toxoid vaccines in dry solid state, without the need for refrigeration.

5.2. Materials

5.2.1. Aluminium compounds

Commercial preparations of aluminium hydroxide, $Al(OH)_3$, aluminium phosphate ($AlPO_4$), and calcium phosphate, were purchased from Superfoss Biosector, Denmark.

5.2.2. Antigens

TT (Mw of 150kDa, 3150 Lf/ml, 27.5 mg/ml) was kindly donated by Pasteur Merieux, France, Lyon.

5.2.3. Polymers

The polymers PLLA (100kDa, 2kDa) were purchased from Boehringer Ingelheim, Germany, PLGA (75:25, 113.2kDa, 50:50, 57kDa and 14 kDa), were purchased from Medisorb Products, USA, CA.

5.2.4. Chemicals

All chemicals and reagents were supplied by BDH Chemicals Ltd. (Poole, Dorset, UK), Sigma Chemical CO. (Poole, Dorset, UK) and Fisons (Loughborough, Leis, UK) unless otherwise stated and were of Analar grade or equivalent. Double distilled water was used in all experimental procedures unless otherwise stated.

5.3. Methods

5.3.1. Preparation of PLLA microspheres encapsulating aluminium hydroxide-adsorbed TT using commercial vaccines

The first batches of formulations were prepared using commercial tetanus toxoid vaccines. These preparations were composed of PLLA (100kDa and 2kDa) microparticles and were made using a modified double emulsion method. In this method the internal phase consisted of 1.5ml of aqueous solution containing, either 27Lf or 216Lf of (commercially available) alum-adsorbed TT and 2.5% (w/v) PVA (Mw, 13-23,000, 88% hydrolysed). The secondary phase consisted of 5ml of dichloromethane containing 255mg of PLLA (100kDa or 2kDa). Both phases were emulsified using the small probe Silverson homogeniser for 2 minutes at 16000 rpm. The primary emulsion formed was added drop-wise to the external phase (75ml aqueous solution of 5 % w/v PVA). The secondary emulsion was homogenised for 6 minutes with a large probe at maximum speed (~16,000 r.p.m.). The final emulsion was stirred and the organic solvent was allowed to evaporate overnight. The preparation was washed in water three times with the aid of centrifugation at 30,000 rpm for 25 minutes. The microparticles were then freeze-dried using a VIRTIS ADVANTAGE, Biopharma process system at a shelf temperature of –20°C for 48 hours. The same method was followed for the preparation of the corresponding PLLA microspheres encapsulating (27Lf or 216Lf) of soluble TT.

5.3.2. Preparation of PLLA microspheres encapsulating aluminium hydroxide-adsorbed TT using in-house vaccines

The next batches of formulations included the use of in-house alum-adsorbed TT. In the following experiments attempts were made to maximise the adsorption efficiency of the antigen onto alum in preparing in-house vaccines. The effect of different buffers on the adsorption efficiency of TT onto alum was also investigated. TT (100Lf) was adsorbed onto either 0.5 or 1ml of neat alum of in the presence of 0.5 ml of different loading media. These included distilled water, PBS (10mM) and saline (0.9% w/v, 0.15M NaCl) in 1.25% w/v and 2.5% w/v concentrations. The solutions were mixed thoroughly and stored at 4°C overnight. These were then centrifuged (IEC, Micromax) at 10,000 rpm for 15 minutes. Supernatants were then removed and assayed for non-adsorbed proteins using BCA. The remaining pellets were suspended in the appropriate buffers (as for the adsorption study). The above in-house alum-adsorbed tetanus toxoid vaccines were then used for the preparation of microparticles composed of PLLA (2kDa and 100kDa). The following w/o/w double emulsion method was used in the preparation of microspheres. The internal phase consisted of 0.5 ml of aqueous solution containing alum-adsorbed TT in 2.5% (w/v) PVA solution. To this was added the secondary phase which consisted of 5ml of dichloromethane containing 200mg of PLLA (2kDa or 100kDa). Both phases were emulsified using the small probe Silverson homogeniser for 2 minutes at ~16000 rpm. The primary emulsion formed was added dropwise to the external phase (75ml aqueous solution of 1.5 % w/v PVA). The secondary emulsion was homogenised for 6 minutes with a large probe at maximum speed (~16,000 r.p.m.). The final emulsion was stirred and the organic solvent was allowed to evaporate overnight. The preparation was washed three times with the aid of centrifugation at 100g for 25 minutes. The microparticles were then

freeze-dried at a shelf temperature of –20°C. The same method was followed for the preparation of the corresponding microspheres encapsulating unbound TT (100Lf).

5.3.3. Preparation of PLLA microspheres co-encapsulating aluminium hydroxide and soluble TT without prior incubation

The first set of alum-based formulations were prepared using commercial tetanus toxoid vaccines which yielded a promising immunological response after one single administration. The next batches of formulations involved the adsorption of tetanus toxoid onto alum in different buffers and the encapsulating complex within biodegradable polymer. In the recent batches of microsphere preparations, free soluble TT and the alum have been mixed directly into the emulsion without prior incubation for adsorption. In this part of the study the effects of sonication on the particle size of the alum has also been investigated (table 5.2), and appropriate sonication time resulting in the smallest size was chosen on the basis of our findings. Here the effect of different quantities of aluminium hydroxide on the microsphere characteristics was also investigated. The following describes the method employed to prepare these microparticles. The internal phase consisted of 1.5 ml of aqueous solution containing, 200Lf (TT), alum in three different quantities (4.4, 2.2 and 1.1 mg) and 2.5% (w/v) PVA (Mw, 13-23000, 88% hydrolysed). The secondary phase included 5ml of dichloromethane containing 250mg of different polymers (PLLA 100kDa, PLGA 75:25 113.2 kDa, PLGA 50:50 0.4 dL/g 57kDa and dL/g 0.16, 14 kDa). Both phases were emulsified using the small probe Silverson homogeniser for 2 minutes at full speed. The primary emulsion formed was added drop-wise to the external phase (75ml aqueous solution of 5 % w/v PVA). The secondary emulsion was homogenised for 6 minutes with a large probe at maximum speed (~16,000 r.p.m.). The final emulsion was stirred and the organic solvent was allowed to evaporate overnight. The preparation was washed three times with the aid of centrifugation at 30,000 rpm for 25

minutes. The microparticles were then freeze-dried at a shelf temperature of –20°C for 48 hours.

5.3.4. Microsphere characterisation

The morphology and size of the microspheres were evaluated using SEM (section 2.3.2). Protein entrapment efficiency (2.4.2) and particle size analysis (2.2.2) were determined as previously described.

5.3.4.1. *In vitro* release studies in phosphate buffer saline (pH 7.5)

See section 2.10.

5.3.4.2. Protein quantification assay (BCA)

See section 2.4.2.

5.4. Results and discussion

5.4.1. Characteristics of PLLA microsphere formulations prepared with commercial vaccines

The microsphere formulations encapsulating the commercial vaccine (Pasteur Meriex, Lyon, France) formed spherical particles with smooth surfaces (fig. 5.1.). The higher molecular weight polymer (100kDa) was used in this experiment. These particles were compared with those encapsulating soluble TT in terms of physical appearance (fig 5.1.) and characteristics (table 5.1.).

Table 5.1. Physical characteristics of alum-adsorbed TT/ PLLA microspheres, Al216 and Al27 = preparations containing 216 and 27 Lf alum-adsorbed TT, F216 and F27= preparations containing 216 and 27 Lf soluble TT

Formulation	Number mean (μm± SD)	Volume mean (μm ±SD)	Protein loading (%w/w) ±SD	Yield (%)
Al 216	3.8 ± 2.9	15.8 ± 11.5	1.40 ± 0.18	77.7
Al 27	2.1 ± 1.1	12.8 ± 16.9	0.76 ± 0.20	84
F 216	3.6 ± 3.4	30.1 ± 20.5	0.51 ± 0.13	68.1
F 27	2.5 ± 1.5	12.9 ± 15.6	0.66 ± .20	77.7

All preparations seemed to produce good microsphere yield with alum containing preparations Al27 (27Lf TT) having the highest yield. The antigen loading was highest for Al 216. The size of particles ranged between 12-15 μm for three of the formulations (Al 216, Al 27 and F27). The Preparations, containing the highest amount of soluble TT resulted in largest particles (30 μm). This formulation also had the lowest protein entrapment efficiency (68%). Many investigators have found that on increasing the initial amount of protein in the primary emulsion the entrapment efficiency is increased (Jeffery *et al.*, 1993, Uchida *et al.*, 1994). The results seem to show the opposite. One possible explanation could be that we had exceeded the optimal amount for microsphere loading, which may have resulted in the destabilisation of the emulsion by creating large liquid droplets in the primary emulsion leading to larger particles with lower loading. It is thought that emulsion droplet size and stability are the most important parameters determining the state of the primary liquid emulsion (Maa and Hsu, 1997). This

determining the state of the primary liquid emulsion (Maa and Hsu, 1997). This phenomenon was investigated by Maa and Hsu in a recent study (Maa and Hsu, 1997). The authors found that the size of liquid droplet of BSA (measured by laser-based particle size analyser in the primary emulsion) within the polymer solution and further emulsification of this into o/w had an effect on the emulsion droplets. Their study demonstrated that protein loading decreased with an increase in w/o emulsion droplet size or in protein powder size (if powder BSA was used). These phenomena were attributed to two possible mechanisms: fragmentation along the weak routes in the w/o/w system and particle redistribution as the result of terminal velocity in the double emulsion system (Maa and Hsu, 1997). The mechanism of microsphere formation in w/o/w explains the above observation. When a liquid droplet is dispersed in an immiscible, continuous medium (protein solution in dichloromethane in primary emulsion), The droplet is subjected to dynamic pressures resulting from high turbulent flow and also the interfacial tension (Maa and Hsu, 1997). When dynamic pressures exceed the interfacial tension and other cohesive forces, the liquid droplet fragments, and this fragmentation continues until once again equilibrium is reached between the dispersive and the cohesive forces. Increasing the droplet size has the effect of increased fragmentation with the subsequent result of exposing more of the internal aqueous droplet during secondary emulsification. Thus causing the leakage of protein into the medium, and resulting in reduced entrapment efficiency (Maa and Hsu, 1997). In a more recent study the effect of BSA loading was found to have no significant effect on the size of microspheres, confirming the results of other investigators (Yang *et al.*, 2001, O'Hagan *et al.*, 1994, Pradhan and Vasavada, 1994). The same study showed that an increase in BSA loading reduced the encapsulation efficiency. This was attributed to the high concentration of the protein in the emulsion, resulting in increased amount of BSA dissolving into the continuous water phase (Yang *et*

shows that Al 27 formulation, which contained the lowest antigen loading (27 Lf alum-adsorbed TT) had the least initial burst release (22% in 2 hours) amongst all the formulations. Its release remained low on the subsequent time points (up to 42 days). The formulation F216 (containing 216Lf unbound TT) showed the highest burst release (83% in 2 hours), and very slow release was observed for the remainder of the incubation period. Formulation Al216 started with a high initial burst release (69%). The release profile for this formulation peaked again on days 28. The release profiles and the initial bursts seem to be also related to the actual protein loading of the microspheres. The lower the actual TT loading the lower the initial bursts. These findings are in agreement with the results of other investigators (Yang *et al.*, 2001, Uchida *et al.*, 1996). The high initial burst for high-loaded microspheres is thought to be due to the existence of a large TT concentration gradient between the microspheres and the outer water phase.

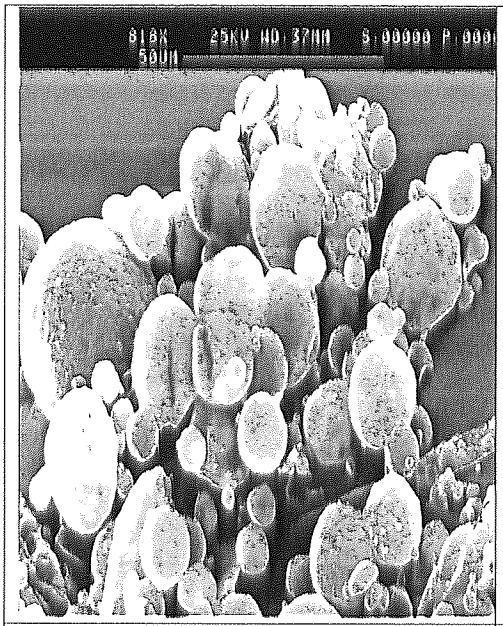


Fig 5.1a. Alum adsorbed/PLLA microspheres encapsulating TT(216Lf)

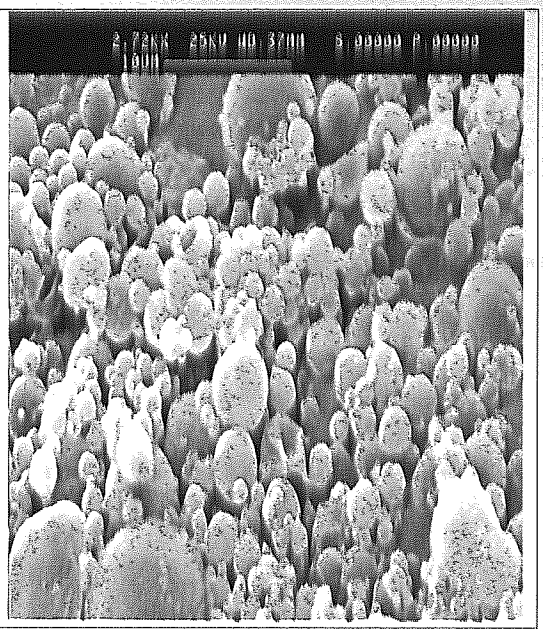


Fig 5.1b. Alum adsorbed/PLLA microspheres encapsulating TT (27Lf)

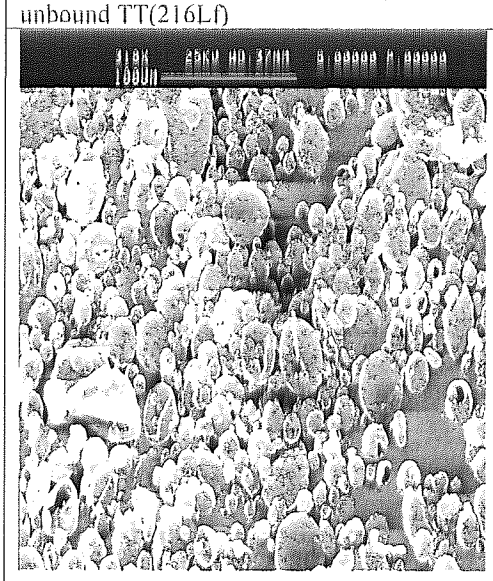


Fig 5.1c. PLLA Microspheres encapsulating unbound TT(216Lf)

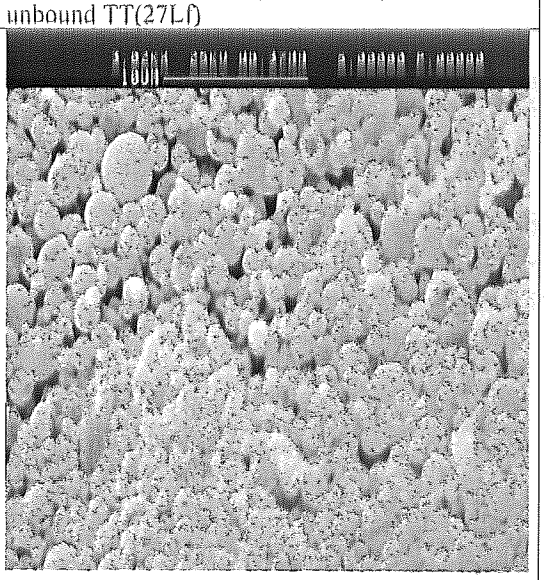


Fig 5.1d. PLLA Microspheres encapsulating unbound TT(27Lf)

Figure 5.1. Scanning electron microscopy of microspheres encapsulating commercial vaccine (Alhydrogel®).

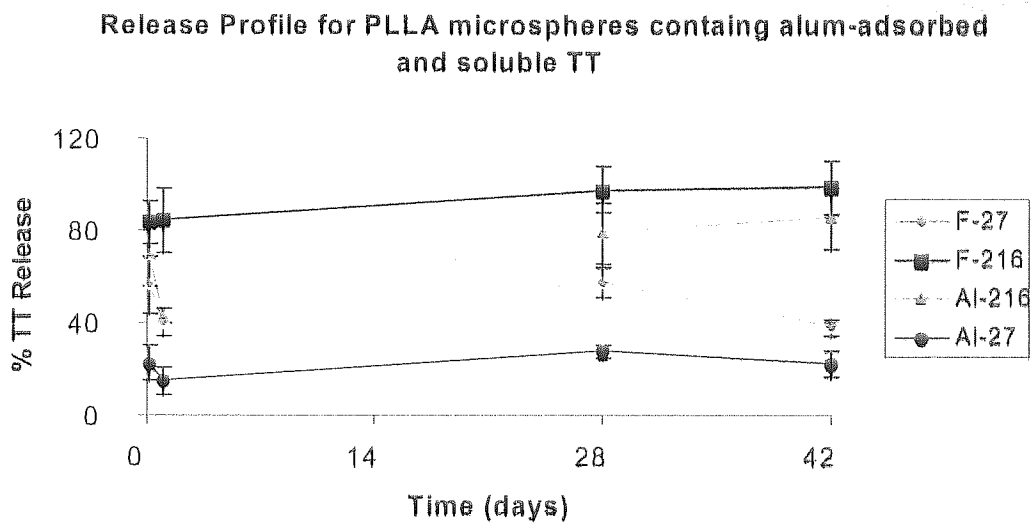


Figure 5.2. Release profile for PLLA (100kDa) microspheres containing 27 and 216 Lf alum-adsorbed and soluble TT over a period of 42 days. Al216 and Al27 = PLLA preparations containing 216 and 27 Lf alum-adsorbed TT, F216 and F27= PLLA preparations containing 216 and 27 Lf soluble TT. N=3

Since the gradient is the driving force in the protein diffusion within the polymeric matrix, the high loading of TT leads to a higher initial burst and a more rapid release rate. The burst release of protein is normally considered to be due to the surface-located protein (Wang *et al.*, 1991). At a high actual loading of protein, there may be more TT distributed near the surface of microspheres, leading to the greater initial release. With progressive

release of the protein more pores and interconnecting channels are formed for the release of the remaining TT. However, the protein has to travel a longer path under a lower concentration gradient, which leads to a slower rate of release at the later stage. At a lower loading, there seem to be no significant change in the concentration gradient of the protein, leading to a fairly constant but slower release after the initial burst (Yang *et al.*, 2001). In the present study, there was a reduction in the amount of released protein after the initial burst release. This may be due to the interaction of the released tetanus toxoid with the in vitro test vials and the polymeric microspheres. Johansen and co-workers evaluated the release of tetanus toxoid from adjuvants and PLGA microspheres in different release media. They reported that for in vitro release experiments, protein adsorption on test vials is of high importance and that the measured amounts of released protein may be greatly falsified by concomitant surface adsorption (Johansen *et al.*, 1998). These authors also relate the incomplete release of the tetanus toxoid from PLGA microspheres to such interactions between the released protein and the surface of the microspheres.

The release profile for microspheres in the present study presented a biphasic release pattern consisting of an initial release phase, and dormant period in which only a small amount of the protein was released. The burst release in this release profile is thought to be associated with initial diffusion of the antigen (Rafati *et al.*, 1997). Antigen is initially released from the polylactide microspheres by diffusion. Antigen which, is at or near the surface of the microparticles diffuses into the surrounding media within minutes to hours after its contact with the aqueous medium. After this a lag phase occurs until the polymer achieves bulk erosion resulting in a significant increase in the number of pores and channels for the diffusion of the protein. The microspheres with higher antigen loading show a near constant release rate after a relatively high initial burst (83% for F216 and 69% for Al216) for the duration of incubation period. The microspheres with lower

antigen loading show a similar profile, but the protein release is only about 30% of the total antigen loading on day 28. The low initial burst and the subsequent low rate of release may be explained due to the lower antigen loading and the incomplete release of the antigen may be due to the interaction of the entrapped TT with the polymeric matrix. It is thought that that some type of interactions between the negatively charged polymeric carboxyl groups and the protein must stop its complete release. This has specifically been related to an ion exchange mechanism (Bodmer *et al.,* 1992). The entrapped TT has a large size (150 kDa) and must exhibit a high density of ammonium groups able to interact with the polymeric carboxyl groups. The formation of an ionic complex between the carboxyl groups and the protonated protein is thought to occur within the approximate pH range of 3.5-5.5, close to or below the p*I* of the protein (p*I* of TT= 5.1) and above the pK_a of the glycolic and lactic acids (Thomasin *et al.*, 1996). It is believed that proteins of a smaller size or of a lower p*I* will dissolve at an earlier stage from the ionic carboxyl-protein complex than the larger proteins (Thomasin *et al.*, 1996).

5.4.2. Characteristics of microspheres prepared with in-house alum-adsorbed TT vaccines

Following successful encapsulation of alum-adsorbed commercial TT vaccine into biodegradable polymers, a study was set out to investigate the entrapment of in-house alum-adsorbed TT vaccines. For this, the adsorption of TT antigen onto alum was examined in several different adsorption media (Table 5.2.). The results indicate that the maximum antigen adsorption onto alum can be achieved in the presence of solutions with low ionic concentrations. The least amount of TT was adsorbed in the PBS medium, which may be due to the presence of negatively charged phosphate ions competing with the antigen for the adsorption sites onto the alum. The pH and the ionic strength affect the adsorption by way of changing the charge on the gel and the antigens (Santos *et al.*, 1957). Excess anions, particularly phosphate ions and impurities such as amino acids, peptides

and polysaccharides, have been found to reduce protein adsorption (Lindblad and Sparck, 1987).

Sample	Abbreviations	(%W/W) TT adsorption
*Whole alum	WALI	93.2 (±0.01)
*Whole alum	WALI	94.2 (±0.01)
alum in dH20	H-AL	94.7 (±0.01)
alum in saline (2.5% v/v)	S-AL 2.5%	94.2 (±0.02)
alum in saline (1.25% v/v)	S-AL 1.25%	93.9 (±0.02)
alum in PBS (2.5% v/v)	PB 2.5%	86.2 (±0.1)

Table 5.2. Effect of different buffers on the adsorption efficiency of aluminium hydroxide. n= 3 ± SD. *= alum used in the supplied buffer.

In general a low ionic strength and absence of excess phosphate ions and impurities are recommended for optimal adsorption of antigens onto aluminium gels (Bomford, 1989). Two different molecular weight PLLA polymers (100 and 2 kDa) were chosen for these studies. As a comparative measure, soluble TT was also entrapped within the above polymers. The physical characteristics of the microparticle preparations have been summarised in tables 5.3, 5.4 and 5.5. In general the 2kDa PLLA microparticles showed a smaller mean volume diameter and also a much narrow size distribution than the corresponding 100kDa microparticles. The particles appeared spherical with smooth surfaces (Fig. 5.2). The protein loading in the alum-adsorbed TT microspheres seemed to be similar for both 2kDa and 100kDa polymers. This loading was higher than achieved when soluble TT was entrapped within low molecular weight 2kDa and high molecular weight 100kDa polymers. It may be possible that the alum association would reduce the amount of TT leaching out in the outer aqueous phase. The particle size seemed to be

similar (3-4 μm) in the microspheres composed of 2kDa polymer except for one formulation (S-Al 2.5%). The higher molecular weight polymer resulted in the higher yield of particles for both soluble and alum-adsorbed TT. The immune responses to one single dose of the above formulations were measured over a period of several months (see chapter 6). Two routes of immunisation (intranasal as well as intramuscular, 1Lf TT) were chosen for the administration of the above compounds.

Table 5.3. Physical characteristics of 100kDa PLLA microspheres encapsulating alum-adsorbed TT. n=3 ±SD. * calculates the entrapment efficiency in relation to the yield of the particles.

Sample 100k TT-Al	Protein loading (Lf/ mg)	Entrapment Efficiency (%)	*Modified Entrapment Efficiency (%)(±SD)	Volume mean (±SD) (μm)	Number mean) (±SD) (μm)	% Yield
W-AL	1.2	127.6± 7.3	74.1	8.98 (±0.48)	3.24 (±0.09)	58.5 ±3.8
H-AL	1.0	106.0± 5.4	71.2	8.80 (±0.51)	2.90 (±0.02)	61.7 ±4.3
S-AL 2.5%	1.5	159.6± 3.2	121.3	6.66 (±0.50)	2.15 (±0.04)	76.2 ±6.3
S-AL1.25%	0.9	96.0± 6.1	73.1	8.33 (±0.89)	2.44 (±0.44)	76.0 ±5.2

Table 5.4. Physical characteristics of 2kDa PLLA microspheres encapsulating alum-adsorbed TT

Sample 2k TT-Al	Protein l oading Lf/ mg	Entrapment Efficiency (%) (±SD)	Modified Entrapment Efficiency (%)	Volume mean (±SD) (μm)	Number mean (±SD) (μm)	% Yield (±SD)
WAL	1.5	159.7± 4.4	65.4	2.86 (±0.23)	0.24 (±0.02)	40.7± 1.3
HAL	1.0	106.3± 8.1	35.1	3.18 (±1.51)	0.2 (±0.02)	33.0± 2.4
SAL2.5%	1.3	139.8± 6.7	51.8	7.74 (±0.45)	1.04 (±0.03)	36.7± 3.1
SAL1.25%	1.0	106.4± 5.2	40.2	3.22 (±0.10)	2.44 (±0.11)	37.1± 1.6

Table 5.5. Physical characteristics of 2kDa and 100kDa PLLA microspheres encapsulating unbound TT

Sample	Protein loading Lf/ mg	% Entrapment Efficiency (±SD)	Modified Entrapment Efficiency	Volume mean (±SDμm	Number mean) (±SDμm)	% Yield (±SD)
2kDa unbound	0.70	136.7(±6.4)	75.8	3.6(±0.4)	1.8(±0.0)	55.4 (±2.1)
100kDa unbound	0.71	139.5(±8.6)	89.4	12.5(±2.8)	2.5(±0.3)	64.1(±1.3)

The scanning electron microscopy (SEM) investigations of the above microspheres were carried out and the following figures (5.3 a-h) present the morphology of these particles as viewed by SEM.

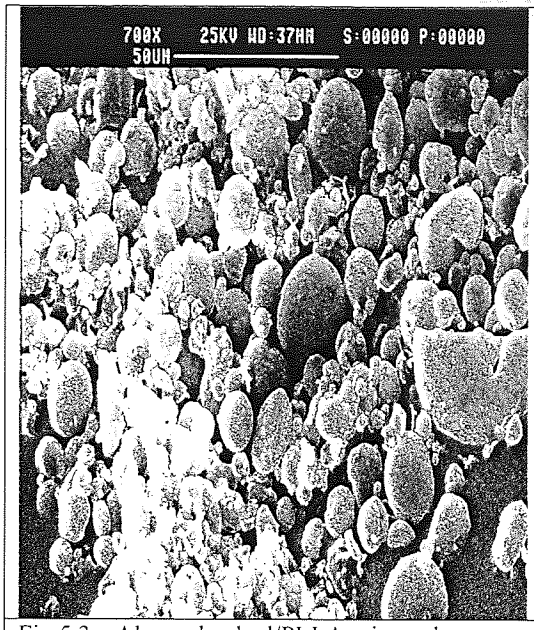


Fig 5.3a. Alum adsorbed/PLLA microspheres (100kDa, S-Al 2.5%)

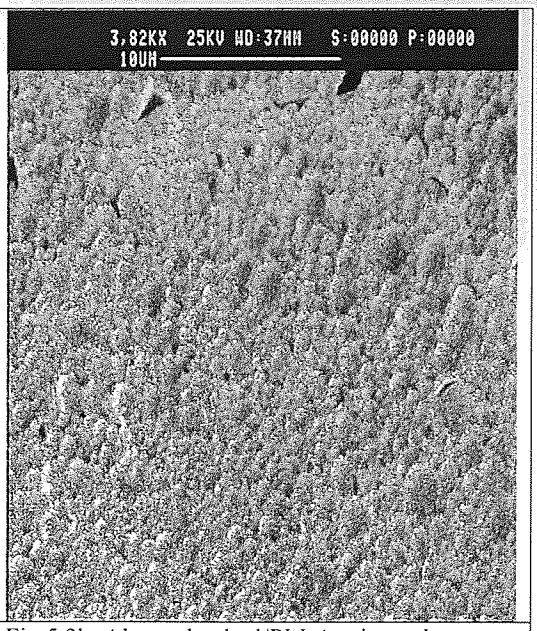


Fig 5.3b. Alum adsorbed/PLLA microspheres (2kDa S-Al 2.5%)

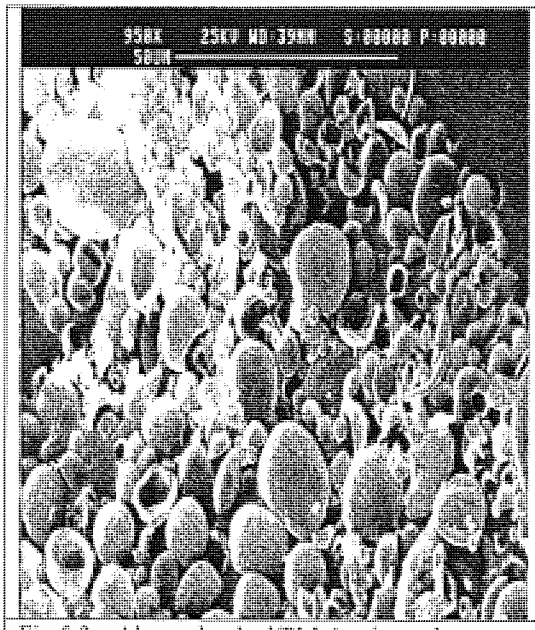


Fig 5.3c. Alum adsorbed/PLLA microspheres (100kDa, W-Al)

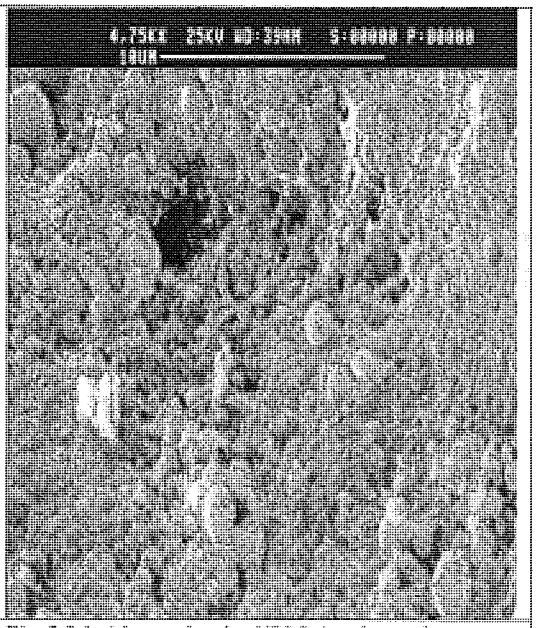


Fig 5.3d. Alum adsorbed/PLLA microspheres (2kDa, W-Al)

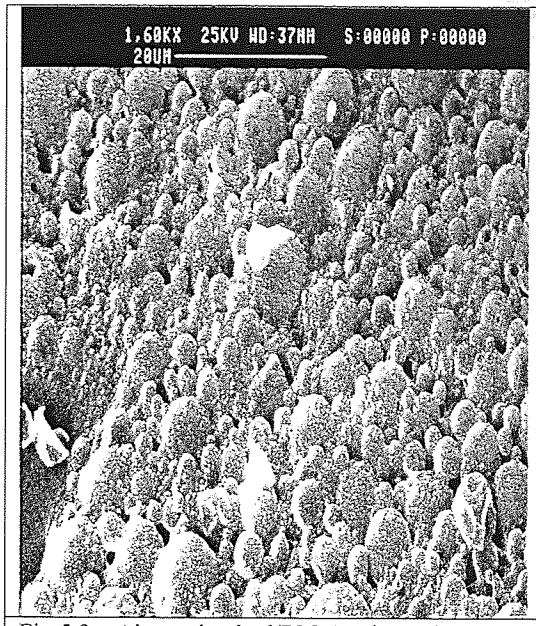


Fig 5.3e. Alum adsorbed/PLLA microspheres (100kDa, S-Al 1.25%)

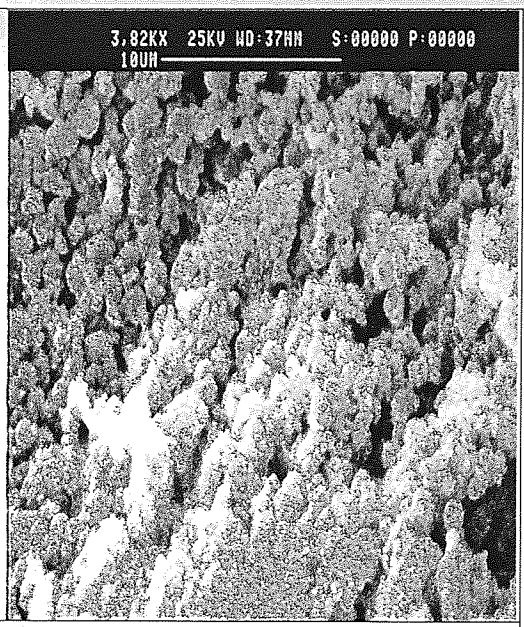


Fig 5.3f. Alum adsorbed/PLLA microspheres (2k Da, S-Al 1.25%)

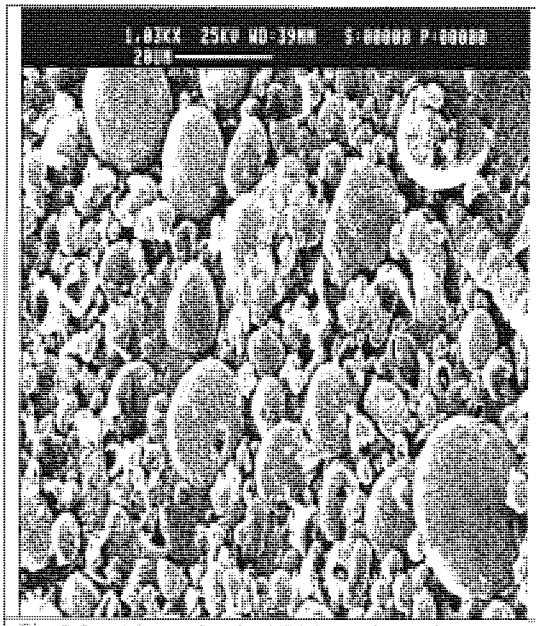


Fig 5.3g. Alum adsorbed/PLLA microspheres (100kDa H-Al)

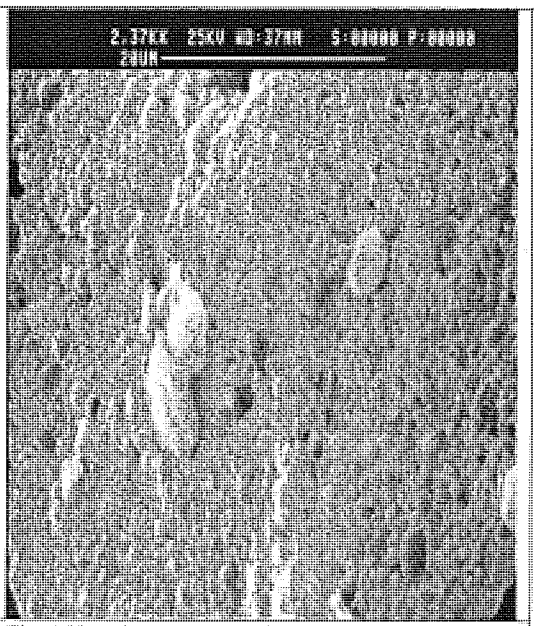


Fig 5.3h. Alum adsorbed/PLLA microspheres (2kDa H-Al)

5.4.3. Effect of sonication on the size of alum particles

It has been thought that the adjuvanticity of the alum particles may be due to their ability in converting the soluble antigens into particulate matters. In this form, the antigens are readily recognised and phagocytosed (Gupta *et al.*, 1995). The particle size of commercially available aluminium adjuvant gels is reported to be 3.07 μm and 4.26 μm for aluminium hydroxide, Alhydrogel® and aluminium phosphate, Adju-phos® respectively (Gupta *et al.*, 1995). There is evidence to suggest that poly lactide glycolide microspheres less than 10 μm are taken up by antigen presenting cells leading to strong adjuvant effects (Eldridge *et al.*, 1991). In attempts to formulate microspheres encapsulating alum particles, which would fall within that size, the alum particles were sonicated prior to encapsulation. The results are summarised in table 5.6. The size of alum particles on vigorous shaking (vortex mixing) without the use of sonication was reduced to 624 nm. The soncation time was varied between 2-10 minutes for sonication rate of 15 Watts and 2-3 minutes on sonication rate of 20 Watts. The most suitable sonication time was that of three minutes on 15 Watts current which produced alum particles of 468 nm. In all subsequent formulation this intensity of sonication was used prior to entrapment of alum particles.

Sample	Sonication Time (min)	Intensity of Sonication (W)	Z Average Mean (nm) (polydispersity)
1	_____	Mixing using whirly mixer	623.9 (0.684)
2	2	15	656.2 (0.635)
3	3	15	467.7 (0.431)
4	10	15	504.0 (0.457)
5	2	20	510.0 (0.498)
6	3	20	490.5 (0.389)

Table 5.6. Effects of sonication on the alum particle size, n=3.

5.4.4. Characteristics of microspheres prepared by direct mixing of alum and TT

Combination dosing of microspheres containing TT and alum-adsorbed TT have been examined *in vivo* in the past (Men *et al.*, 1995, Diwan *et al.*, 2000). The microencapsulation of alum-adsorbed TT has also been looked at briefly in order to improve the immunity and stabilise the entrapped antigen (Esparza and Kissel, 1992, Johanson *et al.*, 1998). The effect of direct mixing of alum and TT and further encapsulation of this mixture within biodegradable microspheres has not been presented in the literature. Therefore, experiments were designed in order to investigate this aspect of TT microencapsulation whilst evaluating the corresponding immune response to different quantities of entrapped alum. Three different quantities of alum were incorporated in these formulations (1.1, 2.2 and 4.4 mg of Alhydrogel®). Different polymers with varying molecular weight and composition (PLLA 100kDa, PLGA 50:50 dL/g 0.15 ≈14kDa, PLGA 50:50 dL/g 0.4 ≈57.4 kDa and PLGA 75:25, 113.2kDa) were used for the formulations of microspheres. These polymers were used in order to evaluate the best delivery vehicle in terms of high yield, loading, and efficient and sustained release for alum/TT microspheres. The physical characteristics of our microsphere preparations are summarised in table 5.3. The morphological analysis of the alum/TT based microspheres presents spherical particles with smooth outer surfaces (Fig5.3). The physical characteristics of particles show no significant difference in terms of yield amongst different polymers (Table5.7). The tetanus toxoid encapsulation effeicency was generally higher for the PLLA and PLGA 50:50 polymers compared to the 75:25 co-polymer of comparable molecular weight. These findings present comparable similarities to the results of other investigators (Johansen *et al.*, 1999). The authors investigated the encapsulation efficiency of TT and DT into different molecular weight PLA and PLGA polymers. In their examinations of different physical properties of microspheres composed

of different molecular weight polymers, they found that the encapsulation efficiency of diphtheria toxoid was 27-64%, 15-43%, and 5-18% for PLGA 50:50 12 kDa, PLGA 75:25, 16 kDa, and PLLA 14 kDa respectively. The TT encapsulation efficiency was found to follow a comparable pattern to DT.

Table 5.7. Physical characteristics of PLLA and PLGA microspheres encapsulating alum-TT mixture without prior incubation.* formulations with 4.4 mg alum, ** formulations with 2.2 mg alum, *** formulations with 1.1 mg alum. n=3 ± SD.

Formulation CODE	Size (number mean ±SD) (μm)	Size (volume mean ±SD) (μm)	Actual load (Lf TT/mg)	(%) Modified Loading Efficiency	Yield (%) (±SD)
*Al-TT PLGA50:50 dL/g 0.4 ≈57.4kDa	1.05 ± 0.08	5.01 ± 0.42	0.95 ± 0.01	120.3	81.7 ±2.8
**Al-TT PLGA50:50 dL/g 0.4 ≈57.4kDa	1.2 ± 0.03	6.43 ± 0.20	0.78 ± 0.01	87.6	79.9 ±2.6
*Al-TT PLGA50:50 dL/g 0.15 ≈14kDa	0.95 ± 0.01	12.95 ± 0.60	0.76 ± 0.01	93.4	71.6 ±1.7
**Al-TT PLGA50:50 dL/g 0.15≈14kDa	1.1 ± 0.01	10.35 ± 0.54	0.78 ± 0.03	95.0	85.1 ±3.8
**Al-TT PLLA 100kDa	0.88 ± 0.00	7.8 ± 0.27	0.99 ± 0.04	90.15	91.05±2.6
***Al-TT PLLA 100kDa	0.98 ± 0.02	7.26 ± 0.15	0.96 ± 0.01	88.15	84.1 ±1.4
*Al-TT PLGA 75:25 113.2kDa	1.00 ± 0.01	6.50 ± 0.11	0.77 ± 0.15	69.6	95.1±1.8
**Al-TT PLGA 75:25 113.2kDa	1.1 ± 0.01	6.78 ± 0.21	0.89 ± 0.01	73.6	91.5 ±4.9
*Al-TT PLLA 100kDa	1.25 ± 0.15	7.40 ± 0.20	0.78 ± 0.01	71.4	84.0 ±3.6

These findings demonstrate that polymer hydrophobicity plays an essential part in microencapsulation of diphtheria and tetanus toxoids, with more hydrophilic polymers encapsulating higher amounts of antigens (Johansen *et al.*, 1998 and 1999). These authors had found that end-group uncapped PLGA 50:50 (with free carboxylate end-groups) gave higher loading efficiencies for both diphtheria and tetanus toxoids, as compared with the standard PLGA 50:50 (esterified end-groups). Since the inherent viscosity's of the PLGA 50:50 and 75:25 are comparable, Johansen and co-workers brought forward the hypothesis that hydrogen bonds (H-bonds) and polar interactions play a crucial role in the encapsulation of drugs and antigens in PLA/PLGA. They support their hypothesis by indicating that with decreasing polymer hydrophilicity, lower H-bonding and polar interactions between the polymer and the hydrophilic protein can be expected. As proteins generally form a hydrophobic core and expose their hydrophilic domains into the aqueous environment, which could facilitate hydrogen and polar interactions with the polymer (Johansen *et al.*, 1999). The α-methyl group of the lactate may sterically hinder the H-bond interactions between the cabonyl oxygen and protein residues. In addition the authors found that polymer-solvent interaction energy increases in more hydrophilic polymer compared to the less hydrophilic. There is evidence that interactions between drug and polymer should become more important through weakening their individual interactions with polymer solvent (Gander *et al.*, 1996). The size distribution of particles ranged between 4 and 17 μm. Most particles had an average diameter in the range of 6-8 μm. The TT release from the above preparations ranged between 15-49% for the first two hours. The initial burst release profile of most formulations shows an increase and then a decrease in the amount of protein released between 24 hours and seven days after incubation. This profile is characteristics of the protein release from PLLA (Crotts and

Park, 1997) and PLGA (Bodmer *et al*, 1992) microspheres. In this profile the initial burst release is thought to correspond to the diffusion of the protein located near the surface of the polymer matrix. The second phase of rapid protein release is thought to be due to the hydrolysis of the polymer and the formation of channels from the microsphere core to the surface. In the third phase the protein diffusion is controlled by the degradation of the polymer matrix.

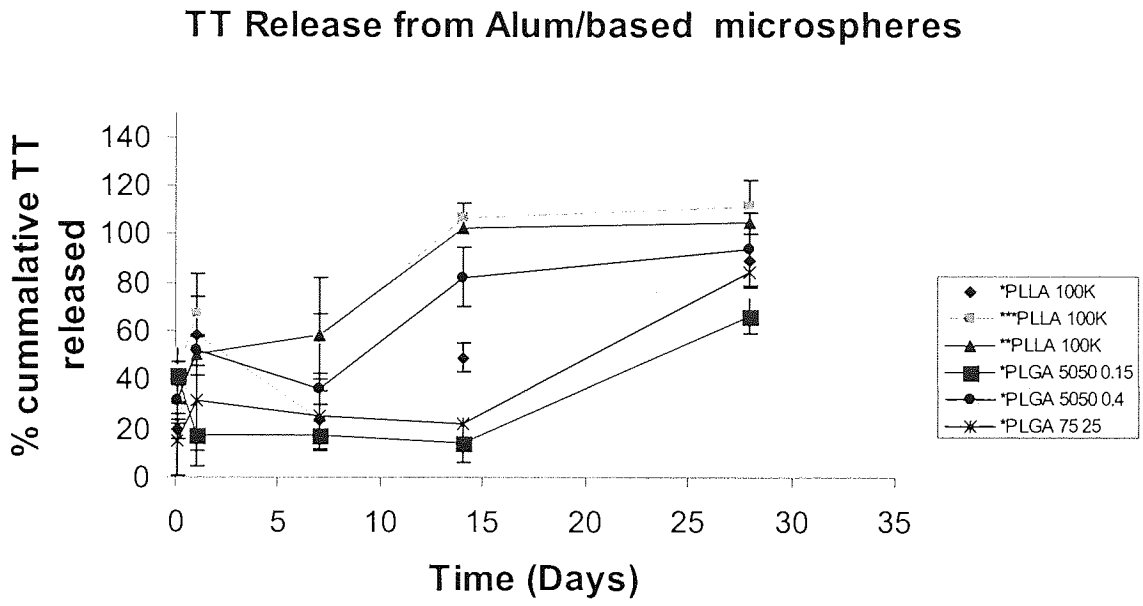
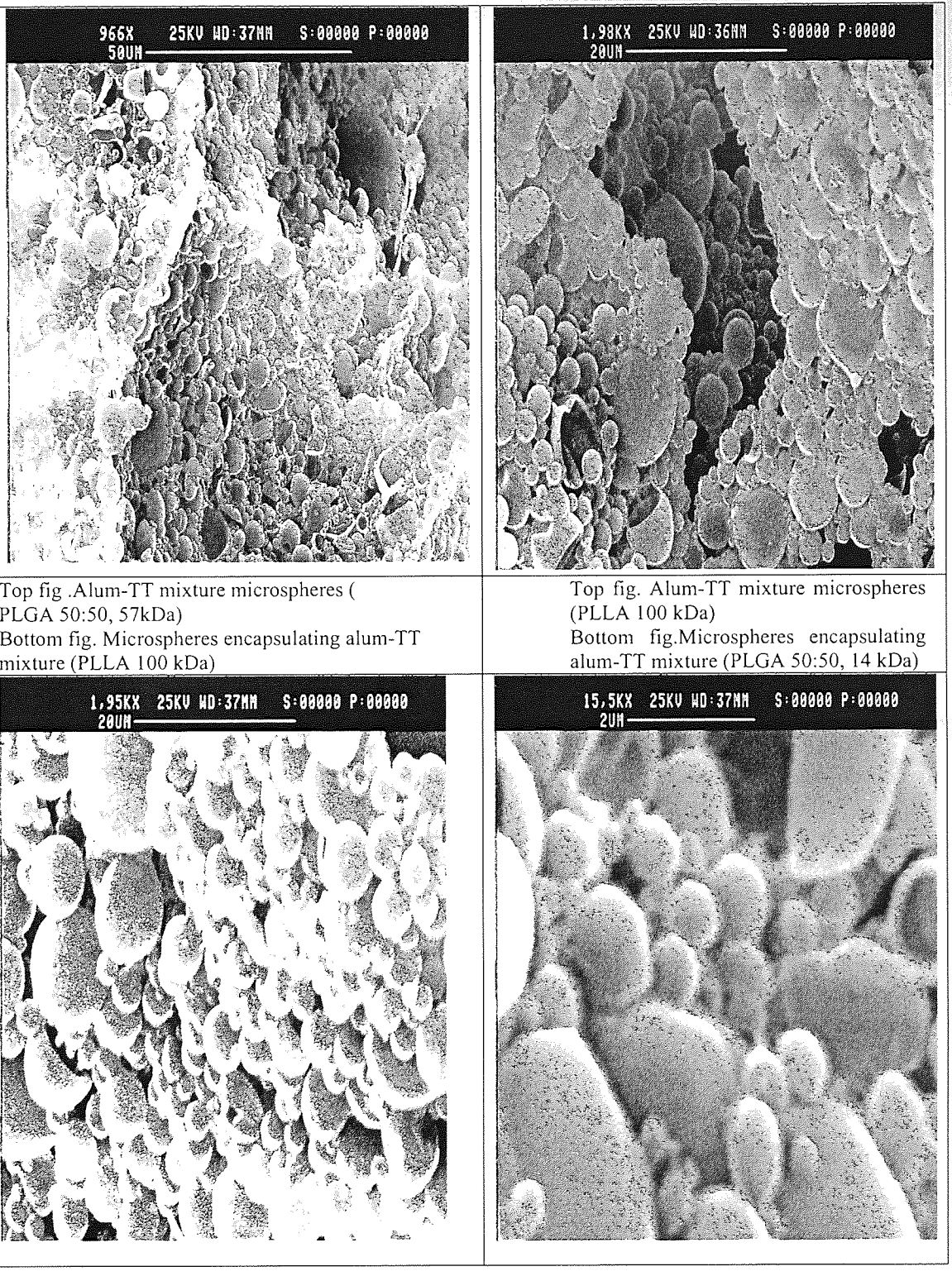


Figure 5.4. % Cumulative release of tetanus toxoid from PLLA and PLGA microspheres encapsulating a mixture of tetanus and aluminium hydroxide adjuvant, * formulations with 4.4 mg alum, ** formulations with 2.2 mg alum, *** formulations with 1.1 mg alum

Alonso and co-workers also found a triphasic release profile when evaluating the release profiles of microspheres composed of PLLA and PLGA polymers (100 kDa). Their result however indicated a small burst (3% release over the first day) as compared to 15-49% for the alum-based formulations. The small burst release was followed by a lag time of 10

days with very slow release and then by a faster constant release (Alonso *et al.*, 1994). Johansen and co-workers found that the *in vitro* release of tetanus toxoid from various PLGA microspheres depended strongly on the co-encapsulated additive and polymer used (Johansen *et al.*, 1998). The co-encapsulation of poloxamer L101 resulted in a burst release of 35-50% of the total dose after 24 hours. They found this release behaviour to be representative of microspheres containing poloxamer L121, trehalose and γ-hydroxypropylcyclodextrin (γ-HPCD). Conversely microspheres without additive or with calcium carbonate yielded a lower burst, followed by no or only a weak additional release. Similar profiles was also obtained when co-encapsulating calcium phosphate and ethyl stearate (Johansen *et al.*, 1998). In general their observations indicated an increased initial burst, when additives were used. In a more recent study they confirmed their previous findings, and observed that a burst release of 2% for the PLGA 50:50(12 kDa), and a protein release of about 20% after 30 days when no additives were used. The same authors found that co-encapsulation of BSA and trehalose caused a burst release of nearly 40% and an increased protein release at day 30, compared to the microspheres without any additives (Johansen *et al.*, 1999). The high burst release observed in our formulations could be due to the presence of aluminium hydroxide, which was co-encapsulated with the antigen. In addition we found that for PLGA 75:25 and PLGA 50:50 after a slow release up to day 21, between 84 to 61% release of tetanus was achieved on day 30. The incomplete release of antigen from PLLA and PLGA microspheres may be caused by antigen instability, which could be due to the acidic micro-environment developed during polymer degradation, or physico-chemical interactions between protein and polymer (Xing *et al.*, 1996). The release of high total tetanus toxoid from our formulations may be an indication of the increased stability of the antigen within our formulations.



Top fig .Alum-TT mixture microspheres (PLGA 50:50, 57kDa)
Bottom fig. Microspheres encapsulating alum-TT mixture (PLLA 100 kDa)

Top fig. Alum-TT mixture microspheres (PLLA 100 kDa)
Bottom fig.Microspheres encapsulating alum-TT mixture (PLGA 50:50, 14 kDa)

Figure 5.5. Scanning electron microscopy of alum/TT microspheres composed of PLLA and PLGA polymers

6.0. Immunisation studies with alum-loaded microspheres

6.1. Introduction

The adjuvant effects of aluminium compounds for human vaccines were clearly demonstrated particularly in relation to diphtheria and tetanus toxoids, during the 1930's (White and Schlageter, 1934; Jones and Moss, 1936; Volk and Bunny, 1939). Many studies have established the superiority of aluminium–adsorbed tetanus and diphtheria toxoids over their soluble forms. This effect is more pronounced after the first dose (Bomford, 1989, Aprile and Wardlaw, 1966, Jensen and Koch, 1988). The adjuvant effect of aluminium compounds was not prominent when used for booster or secondary responses (Jensen and Koch, 1988; Mark and Granstrom, 1994). There is evidence in the literature that soluble or calcium phosphate-adsorbed toxoids are more immunogenic in humans than toxoids adsorbed onto aluminium compounds in booster injections (Mark and Granstrom, 1994; Relyveld *et al.*, 1991). However, co-administration of tetanus toxoid adsorbed onto alum with an injection of tetanus antitoxin has been found to be more immunogenic than soluble tetanus (Levine *et al.*, 1966; Suri and Rubbo; 1961, Fulthorpe, 1965). This is because the soluble toxoid and antitoxin would be cleared faster through their own interaction which would also reduce their effectiveness. Aluminium-adsorbed diphtheria, tetanus and pertussis (DTP) vaccines are used universally, although the adjuvant effect on whole cell pertussis component is not clear (Gupta, 1998). Aluminium compounds have also been used with other vaccines such as inactivated polio vaccine (Butler *et al.*, 1962) human diploid cell strain rabies vaccine (Kuwert *et al.*, 1978) Hepatitis B vaccine (Murray *et al.*, 1984) and Hepatitis A vaccine (Andre *et al.*, 1990; Peetermans, 1992). There is also evidence that aluminium hydroxide adsorbed cholera vaccine provides better protection than the unadsorbed cholera vaccine (Saroso *et al.*, 1978). Aluminium adjuvants have produced variable results when given with Haemophilus influenzae type b (Hib) conjugate

vaccines. Although one study has shown the ineffectiveness of aluminium adjuvants administered with one Hib polysaccharide-protein conjugate vaccine, (Claesson, *et al.*, 1988), there is evidence in other studies that, these adjuvants can be used successfully with another Hib conjugate vaccine (Einhorn *et al.*, 1986), as well as Hib conjugates vaccines which are administered in combination with DTP vaccines (Center for Disease Control, 1993, 1996). Recent studies have demonstrated the immunogenicity of Hib conjugate and Meningococcus type C conjugate vaccines in infant baboons when given with aluminium hydroxide or another emulsion-based experimental adjuvant MF59 (Granoff *et al.*, 1997). The infant baboon model has been described as a reliable predictor of immunogenicity of Hib and meningococcus type C conjugate vaccines in infants (Granoff *et al.*, 1997). Aluminium vaccines have also been widely used with a variety of veterinary vaccines such as those against avian infectious bronchitis (McDougall, *et al.*, 1969), canine hepatitis (Wilson *et al.*, 1977), foot and mouth disease (Sellers *et al.*, 1974; Hyslop *et al.*, 1969), Newcastle disease (Pini *et al.*, 1965). The effectiveness of aluminium adjuvans seems to be related to the degree of antigen adsorption and the dose of the adjuvant (Gupta and Siber, 1995; Gupta *et al.*, 1995). The optimal degree of adsorption and its role on the adjuvanticity of aluminium adjuvans is still a point for debate, since there is hardly any study in humans carried out to elucidate this point. The degree of adsorption is still considered to be a very important factor in the adjuvanticity of aluminium compounds despite all the controversies and uncertainties about their precise mechanism of action (Gupta *et al.*, 1995). Thus in measuring the degree of adsorption, the consistency of the adjuvant formulation can be achieved. Although vaccines with less antigen adsorption (~ 50% or even less) onto aluminium adjuvants, have proved to be effective in the field and meet all the national Control Authorities, adsorption of 80% or more of tetanus and diphtheria toxoids is recommended by WHO (World Health Organisation, 1997). The

United States Minimum Requirements (United States Minimum Requirements, 1956) for adult tetanus and diphtheria toxoid is at least 75% adsorption of diphtheria component on the aluminium adjuvants. The other important factor determining the overall immunogenicity of the vaccines is the dose of aluminium adjuvants (Bomford, 1989). Although a small amount of aluminium adjuvant may be required for the complete adsorption of the antigen, low doses may not provide an optimal adjuvant effect, and an excess amount of free adjuvant seems to be necessary for an optimal adjuvant effect (Jensen and Koch, 1988; Cooper *et al.*, 1991). There is evidence in animal studies that as the amount of aluminium adjuvant increases, the adjuvant effect is increased too, but only to a certain concentration after which the adjuvant effect declines (Bomford, 1989; Lindblad and Sparck, Hennessen, 1965; Holt, 1955). The exact mechanism for this optimal dose activity is not known, but it is speculated that a certain minimum amount of aluminium compound is necessary for depot formation at the site of injection or for the optimal stimulation of macrophages (Gupta *et al.*, 1995). It is also thought that excessive amounts of aluminium compounds may suppress immunity by covering the antigen completely with mineral compounds (Haas, *et al.*, 1961 and 1955) or through toxicity to macrophages (Munder *et al.*, 1969). The usual dose of aluminium used in humans is around 0.5 mg. The upper allowable limits of aluminium adjuvants are 1.25 mg aluminium for injection in humans as *per* World Health Organisation regulations (World Health Organisation, 1990), and 0.85 to 1.25 mg aluminium as *per* United States Food and Drug Administration guidelines (May *et al.*, 1984). Doses of vaccines used in animal studies are usually several-fold (sometimes 100 fold) lower than the human dose in order to determine the response on the logarithmic part of the dose-response curve. Dilution of the administered aluminium dose in saline has been shown to lower the levels of immunogenicity/ potency of the vaccine formulations in mice and guinea pigs compared to

aluminium adjuvant (Hennessen, 1965, 1967, Relyveld, 1985). This has been related to the disruption of the vaccine composition when diluted for testing in animals (Hennessen, 1965; Gupta and Siber, 1995b; Huet *et al.*, 1992). These studies have shown that the effect of dilution of vaccines on the immunogenicity depended upon the antigen, adjuvant and animal species. In using the aluminium phosphate adsorbed tetanus preparations, dilution in saline had no effect on the immunogenicity of these preparations in mice whereas it lowered the levels of IgG antibody levels in guinea pigs (Gupta and Siber, 1994). Dilution of aluminium adsorbed diphtheria preparations in saline caused a decrease in the immunogenicity of these preparations in mice (Gupta *et al.*, 1996). Similar results were obtained using calcium phosphate adsorbed tetanus toxoid (Relyveld, 1985), which may explain the lower potency of calcium phosphate adsorbed tetanus and diphtheria toxoids than the toxoids adsorbed onto aluminium adjuvants (Aggerbeck and Heron, 1995), as the World Health Organisation potency test requires dilution of vaccines in saline (WHO, 1990). Since these calcium phosphate adsorbed vaccines have been found to be more immunogenic in human than the corresponding aluminium adsorbed preparations (Relyveld, 1991, Aggerbeck, 1995), it follows that the potency tests in animals based on dilution of vaccines do not provide a realistic view of the immunogenicity of the final formulation. Therefore, it has been recommended that formulations intended for human use should be injected undiluted or with a minimum amount of dilution, when used in animal immunogenicity of adjuvanted vaccines. The mechanism of action of aluminium adjuvants is not yet fully understood, and is thought to involve several different mechanisms. The most simplified mechanism is the generation of ''depot'' at the injection site, which allows the slow release of antigen, thus prolonging its interaction with APCs and lymphocytes (Glenny *et al.*, 1931). Although considered to be one important mechanism of action of aluminium adjuvants, the above concept has been challenged by

Holt, when he demonstrated that the excision of the injection site and removal of the aluminium-antigen depot, did not impair the immune response (Holt, 1950). In a recent study it was found, that ~ 90% of radio-labelled aluminium adsorbed tetanus toxoid disappeared from the site of injection within 24 hours of subcutaneous injection, whereas the same antigen, encapsulated within biodegradable polymer microspheres, stayed at the site of injection for extended time periods (Gupta *et al*, 1996). However, amount of aluminium phosphate adsorbed tetanus toxoid at the site of injection was higher than its soluble form for four weeks, thus exhibiting some localised depot formation. There is direct evidence of local depot effect from experiments in which the local granulomas formed after injection of aluminium adsorbed vaccines were able to induce immune responses when excised from the site of injection several weeks later, macerated and injected into other animals (Harrison, 1935). The other possible mechanism of action of aluminium adjuvants is thought to be through targeting to antigen presenting cells. Soluble antigens may upon adsorption be presented to the immunocompetent cells in a particulate manner, which could facilitate antigen targeting through increased uptake by APCs (Manhalter *et al.*, 1985). There is evidence that aluminium hydroxide activates complement and improves trapping in the lymph nodes and also the retention of lymphocytes (Ramanathan *et al.*, 1979). It has been demonstrated that aluminium hydroxide in mice, attracts eosinophils to the injection site in the absence of antigenic stimulus (Walls, 1997). This reaction indicates the involvement of T cells. Studies carried out by Sakata and co-workers in guinea pigs suggest that aluminium induces the release of soluble potentiating factors by macrophages, which in turn stimulate the production of eosinophil chemotactic factor/lymphokine (EFC). Aluminium adjuvants stimulate mainly the humoral immunity, particularly IgG1 and IgE antibody responses, through IL-4 (Grun and Maurer, 1989) by activating Th2 type cells (Cooper, 1994, Cox and Coulter, 1997).

Although there is no evidence that aluminium adjuvants should be able to generate cytotoxic T cells and they were inefficient inducers of delayed-type hypersensitivity (DTH) response in guinea pigs (Bomford, 1980), their overall adjuvant effect seem to involve stimulation of T cells. In a study by Mannhalter and co-workers, it was found that aluminium adsorbed tetanus toxoid led to an increase in antigen-induced T cell proliferation, apparently due to increased release of IL-1. Gurn and Maurer have demonstrated that anti-IL-1α or anti-IL-4 was able to inhibit an antigen-specific T cell proliferative response when the mice had been immunised with antigen and aluminium adjuvant (Grun and Maurer, 1989). This however, was not the case when FCA was used as the adjuvant. It was observed that anti-CD4 antibody inhibited the proliferative response regardless of the adjuvant used. This observation indicates that the proliferating CD4 Tcells from mice immunised with antigen and aluminium adjuvant were of the Th2 subset. As indicated before, aluminium adjuvants stimulate the production of IgE and IgG1, which is in good agreement with the reaction profile of Th2 lymphocytes in mice (Mossmann and Coffman, 1989). Aluminium adjuvants have an extensive safety record over many years. Billions of doses of aluminium-adjuvanted vaccines have been used in human and veterinary vaccination for more than 50 years. Occasionally, these vaccines have been associated with severe local reactions such as erythema (Collier *et al.*, 1979), subcutaneous nodules (frost *et al.*, 1985), contact hypersensitivity (Clemmenson and Knudsen, 1980), and granulomatous inflammation (White *et al.*, 1955, Erodohazi and Newman, 1971). But there have been cases when aluminium adsorbed DTP vaccine produced fewer reactions than unadsorbed vaccine (Hilton and Wurland, 1970, Cameron, 1980). As mentioned before, aluminium adjuvants increase the levels of antigen specific and total IgE antibodies, which may promote IgE mediated allergic reactions (Aggerbeck *et al.*, 1995). Many studies have demonstrated that children who had primary

immunisation with aluminium-adsorbed vaccines are more likely to develop antigen specific IgE and a higher frequency of local reactions on subsequent booster injections with both soluble or aluminium-adsorbed vaccines than children who had primary immunisation with unadsorbed vaccines (Collier *et al.*, 1979, Mark *et al.*, 1995, Blennow *et al.*, 1990). Based on this evidence, there has been suggestion for re-evaluating aluminium compounds as vaccine adjuvants (Mark *et al.*, 1995). Systemic accumulation of aluminium, which has been associated with nervous system disorders and bone diseases (Gupta and Relyveld, 1991) is another point of concern in the use of these adjuvants for vaccination. Other limitations of aluminium adjuvants include their ineffectiveness when used with certain antigens such as typhoid vaccine (Cvjetanovic and Umera, 1965), and influenza haemagglutinin antigen (Davenport *et al.*, 1968) and Hib capsular polysaccharide tetanus toxoid conjugate (Claesson *et al.*, 1988). However, aluminium hydroxide has been successfully used with another conjugate vaccine composed of Hib capsular polysaccharide linked to outer membrane proteins of *Neisseria meningitidis* (Einhorn *et al.*, 1986). Aluminium adjuvants also suffer from the inability to stimulate cell-mediated immune response, particularly cytotoxic T cell responses (Cox and Coulter, 1997), which limits their use for vaccines against intracellular and some viruses, such as human immunodeficiency virus. Aluminium adjuvants are not easily frozen or lyophilised (Warren *et al.*, 1986, Alving *et al.*, 1993), since both of these processes result in the collapse of the gel structure, causing a gross aggregation and precipitation. Although immunogenic activity was achieved with tetanus toxoid-collapsed gel vaccine (Menon *et al.*, 1976), such a vaccine is not acceptable for clinical use. Aluminium adjuvants have also been lyophilised successfully, but these lyophilised aluminium-adjuvanted vaccines are not available commercially. There are reports of the use of a lyophilised DTP vaccine with acellular pertussis components adsorbed onto aluminium adjuvants and stabilised with

Haemocoel and sucrose (Rethy *et al.*, 1996). All of the above limitations of the aluminium adjuvants, highlights the need for the development of novel vaccine formulations which overcome the shortcomings of the aluminium compounds, yet be as effective in vaccine formulations. The development of novel vaccines using biodegradable polymers is one approach that has drawn a great deal of effort and attention in the recent years. Microspheres of PLGA polymer have been shown to form a long-term depot at the site of injection, with the added advantage of biodegradability and biocompataibility (Gupta *et al.*, 1996). Although the biodegradability of aluminium compounds may not be of clinical significance, the development of vaccine formulations using biodegradable polymers, which display similar or even better immunogenicity profile, may be the choice for the future. In the present studies attempts have been made to address a number of limitations of aluminium compounds by incorporating them within biodegradable microsphere systems, which allow smaller amount of aluminium compounds to be used as *per* preparation compared to the commercial vaccines. These microsphere preparations can also be lyophilised easily and stored at room temperature over long periods of time. The immune responses to the above formulations are found to be just as good if not better than commercially available vaccines when tested in mice.

6.2. Materials

6.2.1. Animals

Outbred female BALB/c mice (6 to 8 weeks old; ~25 g) bred at Aston University (Birmingham) were used in these experiments. During the experiments and at all other times, animals were allowed food and water *ad libitum*.

6.3. Methods

6.3.1. Intramuscular immunisation

Groups of animals of adult female BALB/c mice (5 animals *per* group) were primed with different formulations of alum-TT microspheres (1 Lf), commercial alum-adsorbed TT (1 Lf) or free TT in solution vaccine (1 Lf). Each dose of vaccine was suspended in an isotonic PBS pH 7.4, and each animal received 50 µl injection into the left hind leg muscle. Tail vein blood samples were collected as early as 7 days and serum IgG titres were monitored up to 14 months after the initial immunisation. Serum was assayed by ELISA (section 2.7.2) for the presence of anti-TT IgG.

6.3.2. Intranasal immunisation

Groups of animals of adult female BALB/c mice (5 animals *per* group) were primed with different formulations of alum-TT microspheres (1 Lf), commercial alum-adsorbed TT (1 Lf) or free TT in solution vaccine (1 Lf. Each dose of vaccine was suspended in an isotonic PBS pH 7.4, and each animal received 25 µl injection into the nostrils. Tail vein blood samples were collected as early as 7 days and serum IgG titres were monitored up to 215 days after the initial immunisation. Serum was assayed by ELISA (section 2.7.2) for the presence of anti-TT IgG.

6.3.3. Splenic cell proliferation studies

See sections 2.8, 2.8.1 and 2.8.2.

6.4. Results and Discussion

6.4.1. Intramuscular immunisation with microspheres encapsulating commercial vaccines

The total IgG antibody responses to microspheres incorporating commercial aluminium-adsorbed tetanus toxoid vaccine, was measured over a period of 14 months after a single

dose (1Lf) administration. The immune responses of these formulations were compared with soluble tetanus toxoid and commercial aluminium-adsorbed tetanus toxoid on its own. The immune response to all formulation measured on day 14, were significantly higher than the soluble antigen ($p<0.05$). Microspheres (encapsulating both alum-adsorbed and unbound TT) performed well and there were no significant differences between the immune response to the commercial vaccine and conventional microsphere formulations. The same trend was observed on day 35, 56 and 91 post-immunisation. On day 156 post-immunisation the immune response to the commercial vaccine and alum-based formulations (Al216, Al 27+ TT Vac, Al 27 and F216) was comparable (Fig 6.1). The total IgG response was sustained up to the last time point (14 months after the first immunisation) and displayed significant difference between the above formulations and soluble tetanus toxoid . Several studies have shown the ability of microspheres encapsulating tetanus toxoid to induce a primary response in mice and guinea pigs (Alonso *et al.*, 1994, Esparza and Kissel, 1992). However, the ability of the above microspheres to provide a booster response several months after the original vaccination, or to maintain a high neutralising antibody response over time equivalent to that induced by conventional vaccines is not clearly demonstrated (Alonso *et al.*, 1994). 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 microsphere-encapsulated tetanus toxoid 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).

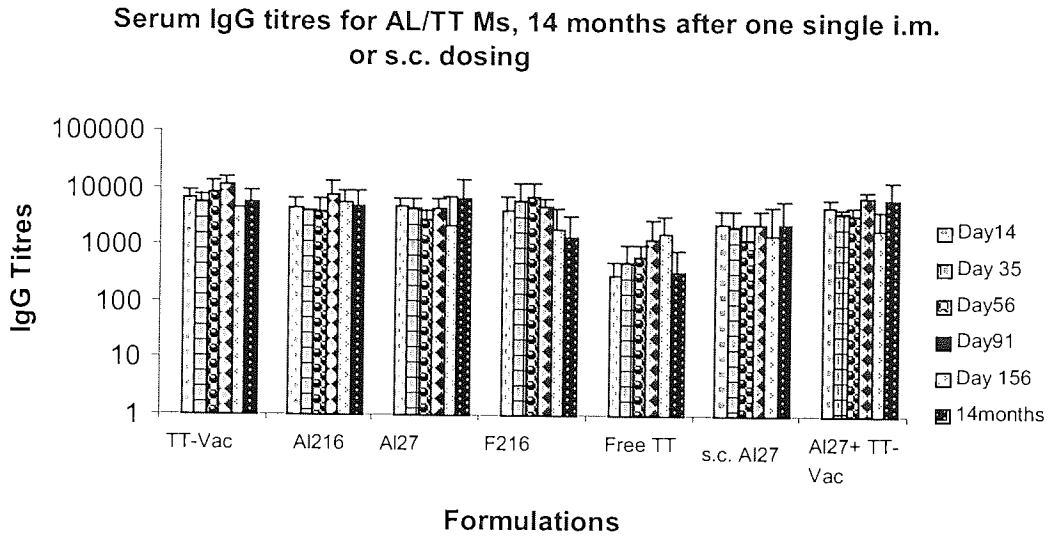


Figure 6.1. Serum IgG responses to a single dose (1 Lf) of PLLA microspheres encapsulating commercial alum-adsorbed tetanus toxoid vaccine, TT-Vac= commercial vaccine, Al27 and Al216= PLLA microspheres containing alum-adsorbed 216 and 27 Lf in the original preparation, F216= PLLA microspheres containing 216 Lf soluble TT, Al27+TT-Vac= combination dosing of Al27 and commercial alum-adsorbed TT vaccine, s.c.=subcutaneous dosing.

In the present studies it has been shown that encapsulation of alum-adsorbed tetanus toxoid within PLLA microspheres has not affected the immunogenicity of the commercial vaccine and comparable immune responses have been achieved with those of the commercial vaccine. These results suggest that tetanus toxoid upon release from the polymer has retained its biological activity, thus stimulating antibody production similar to those of the commercial vaccine on its own.

6.4.2 Intramuscular immunisation with microspheres encapsulating alum-adsorbed antigens prepared in-house

As evident from figure 6.2, the immune response to formulations containing in-house, alum-adsorbed TT did not produce comparable results to the first batches of microspheres

containing the commercially available alum-adsorbed TT vaccine. On day 35 most of the original formulations had antibody titres up to 9000, only one formulation (HAL 100kDa), from the new batches showed a similar response. The immune response to these formulations on day 35 produced comparable results to those on day 14 (Fig.6.2). The immune response to the above formulations was followed up to day 215 post-immunisation. The formulation HAL (PLLA 100kDa) induced immune response comparable to the commercial and in-house vaccines. The reason for the positive performance of this particular formulation compared to the others could be the nature of the adsorption in different solutions, which were used in the antigen adsorption process. It is well established that the presence of excess anions, particularly phosphate ions, and impurities such as amino acids, peptides and polysaccharides, reduce protein adsorption (Lindblad and Sparck, 1987). It has also been demonstrated that lower immunogenicity in mice and guinea pigs have been achieved following dilution of aluminium-adsorbed vaccines in saline (Hennesen, 1965)(Relyveld, 1985). It could be that alum-based formulations, which contained saline and phosphate buffer as media in the initial antigen adsorption process, perform less favourably due to the above reasons. The presence of residual anions within the core of microspheres might have detrimental effects on the alum-tetanus toxoid complex, thus lowering the levels of antibody production by these formulations. Most formulation however, maintained IgG responses higher than the soluble antigen.

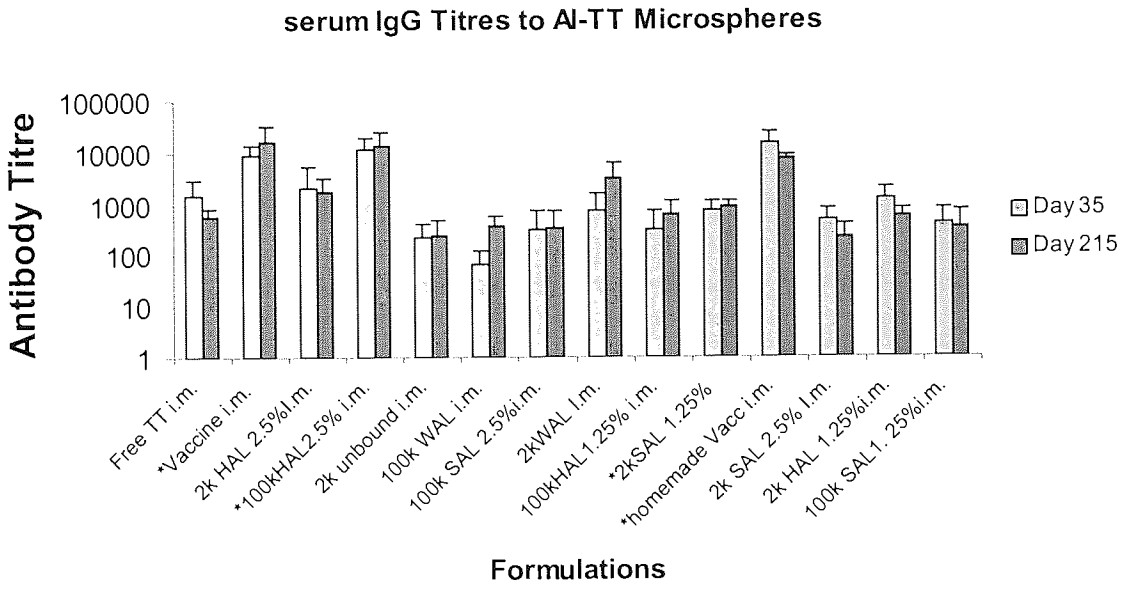


Figure 6.2. Serum IgG responses to a single dose (1Lf) of PLLA microspheres encapsulating in-house alum-adsorbed tetanus toxoid vaccine. *Vaccine= commercial vaccine. FreeTT= TT in solution. 2k HAL= 2kDa PLLA microspheres encapsulating alum in dH_20. 100k HAL= 100kDa PLLA microspheres encapsulating alum in dH_20. 2k WAL= 2kDa PLLA microspheres encapsulating whole undiluted alum. 100kWAL= 100kDa PLLA microspheres encapsulating whole undiluted alum.100k. 100k SAL2.5%= 100kDa PLLA microspheres encapsulating alum in saline (2.5% v/v). 2k SAL1.25%= 2kDa PLLA microspheres encapsulating alum in saline (1.25% v/v). 100k unbound= 100kDa PLLA microspheres encapsulating TT in solution without any alum. 2k unbound= 2kDa PLLA microspheres encapsulating TT in solution without any alum. i.m.= intramuscular injection. 100k unbound= 100kDa PLLA microspheres encapsulating TT in solution without any alum.

6.4.3. Intramuscular immunisation of microspheres encapsulating a mixture of alum and antigen without prior adsorption of antigen onto alum

The immune response to the above particles was measured in terms of released IgG titres on days 7, 14 and 56 post-intramuscular immunisation (Fig 6.3). Most preparations induced immune responses, which were significantly higher than soluble TT. On day 14 the commercial vaccine and three of alum-based microsphere preparations (AL-TT1i, PLLA 100kDa, PLGA 50:50 65kDa, and PLGA 75:25,113.2kDa) produced the highest immune response (There was no significant difference between IgG titres for these four preparations, $p<0.08$). Although the initial release profile for the above formulations differs, the general trend seems to be similar. The particle size of these formulations also appears to be similar and in the range of 4-8 μm. These formulations on average had between 5-10 times lower amounts of aluminium adjuvants than the conventional vaccine, yet producing comparable immune response (Fig 6.3). The continued production of total IgG may be due to the retention of the tetanus toxoid integrity within the microspheres and its subsequent release in a an integral form thereafter. One study which investigated the microencapsulation of alum-adsorbed tetanus toxoid proved to be unsuccessful in achieving increased immunogenicity of the antigen, compared to formulations encapsulating the plain toxoid (Esparza and Kissel, 1992). The dose of TT used in this experiment was 5 Lf and as such a direct comparison of our results is not possible. The presence of unbound aluminium hydroxide within the microspheres could be asserting a stabilizing effect on the antigen. Changes in the physico-chemical and antigenic/ immunogenic properties of encapsulated antigens may be induced during microsphere preparation. These changes might also occur when the antigens are released from microsphere particles. These changes are typically brought about by mechanical forces,

elevated temperature, organic solvent and stringent drying processes (Johansen *et al.*, 1998).

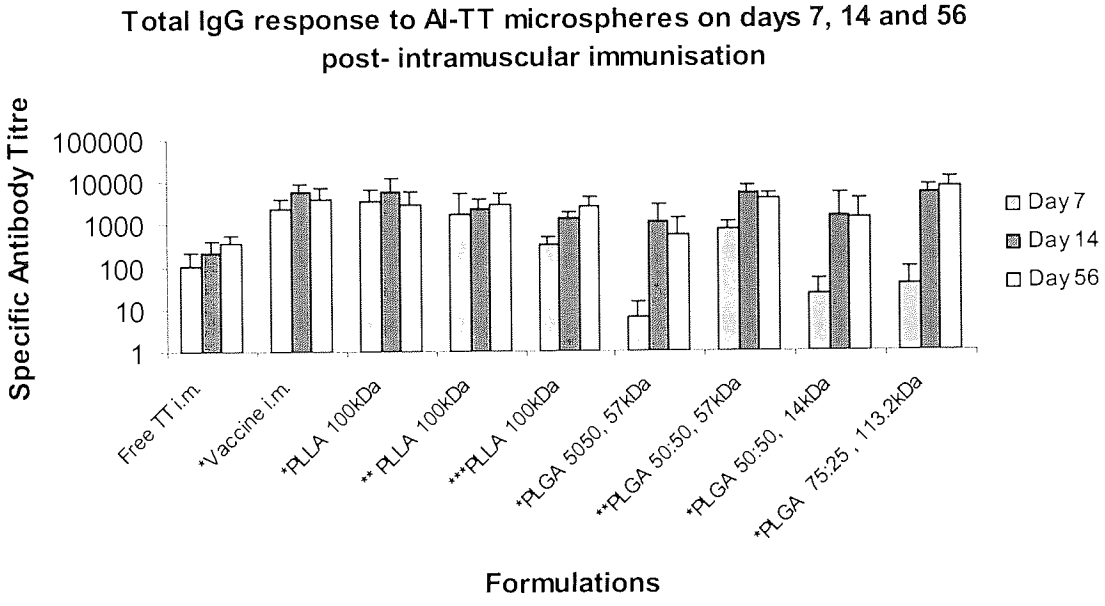


Figure 6.3. Serum IgG responses to a single dose (1 Lf) of PLLA microspheres encapsulating a mixture of alum and tetanus toxoid without prior incubation for adsorption, Vaccine = commercial vaccine. *=theoretical loading of 4.4 mg alum. **= theoretical loading of 2.2 mg alum, ***= theoretical loading of 1.1 mg alum in original microsphere preparation.

Improvement of tetanus antigenicity has been achieved by co-encapsulating the antigen with albumin, (Johansen *et al.*, 1998), gelatin and dextran (Schwedeman, *et al.* 1998). The latter investigation showed the ability of gelatin and dextran to inhibit inactivation of TT during exposure to 86% relative humidity and 37 °C. In their study, Alhydrogel® (stabilized by glycerol) was also incorporated into microparticles, but because of the poor

formation of microcores, further investigation into stabilising effects of this gel was not pursued. Schwendeman and co-workers have confirmed the findings of other researches in suggesting that exposure to moisture, which is a known characteristic of the microclimate of polymers such as PLGA (Hutchinson and Furr, 1990), is possibly responsible for the dramatic loss of activity within PLGA microsphere (Gupta *et al.*, 1993). The formulations presented in the present study containing this gel have formed microspheres with smooth outer surfaces and have performed as well as the commercial tetanus vaccine *in vivo*. Audran and co-workers investigated the effect of co-encapsulating different additives in microspheres containing TT on the specific antibody response (Audran *et al.*, 1998). The additives used in their study were known for their protein or pH stabilising properties. Their findings shows that microspheres containing those additives gave rise to antibody responses higher than those obtained in the absence of stabiliser and that the presence of additives elicited at least equivalent antibody responses with a 5-fold lower dose of microspheres without additives (Audran *et al.*, 1998). Calcium carbonate and calcium phosphate used as additives increased the responses shortly after immunisation and sustained antibody responses for the following three months. Other additives such as BSA, trehalose and cyclodextrin also enhanced the early immune responses, and stimulated antibody responses which were equal or even superior to those obtained with alum-adsorbed TT. These additives were found to be particularly efficient since even low antigen dose (0.2Lf) induced high antibody responses. BSA has been shown to reduce the water content in microspheres due to preferential hydration, which might stabilise the antigen during microsphere storage (Chang and Gupta, 1996).

6.4.4. Splenic cell proliferation and cytokine assays

One of the limitations of aluminium compounds is the induction of mainly humoral immunity by eliciting primary Th2-type immune response in mice (Bomford, 1980, Audibert and Lise, 1993). The inability of aluminium adjuvants to elicit cell-mediated immune responses, particularly cytotoxic T-cell responses, may prove to be a major limitation, particularly in vaccines against intracellular parasites and viral infections such as human immunodeficiency virus. There is evidence however, that microparticles composed of biodegradable polymers can exert an adjuvant effect for the induction of cell-mediated immunity (Moore *et al.*, 1995, Nixon *et al.*, 1996, O'Hagan *et al.*, 1993, Maloy *et al.*, 1994). It has been shown that microparticles stimulated cytotoxic lymphocyte (CTL) responses in mice following systemic (Moore *et al.*, 1995) and mucosal immunisation (Maloy *et al.*, 1994), with protein (Moore *et al.*, 1995) (Maloy *et al.*, 1994) and peptide (Nixon *et al.*, 1996) antigens. Recent studies in which cationic microparticles were used to adsorb DNA plasmids have shown these particles to be capable of enhancing both antibody and cytotoxic T lymphocyte responses in a range of animal models (Briones *et al.*, 2001, O'Hagan *et al.*, 2001). Microparticles also induced a delayed-type hypersensitivity (DTH) response (Maloy *et al.*, 1994), which is thought to be mediated by Th1 cells and strong T cell proliferative activities. Phagocytosis and presentation of microparticles are thought to be carried out by macrophages, through the cytosolic MHC class I-restricted pathway (Kovacsovics-Bankowski and Rock, 1995). The involvement of dendritic cells has also been indicated in the presentation of particulate antigens and the release of cytokines to promote a Th1-type response (Scheicher *et al.*, 1995). Nixon and co-workers have reported that the particle size may be an important factor in the induction of CTL responses by microparticles (Nixon *et al.*, 1996). To complete the *in vivo* studies with microspheres

encapsulating alum-adsorbed TT antigen, T cell proliferative responses and cytokine assays were carried out.

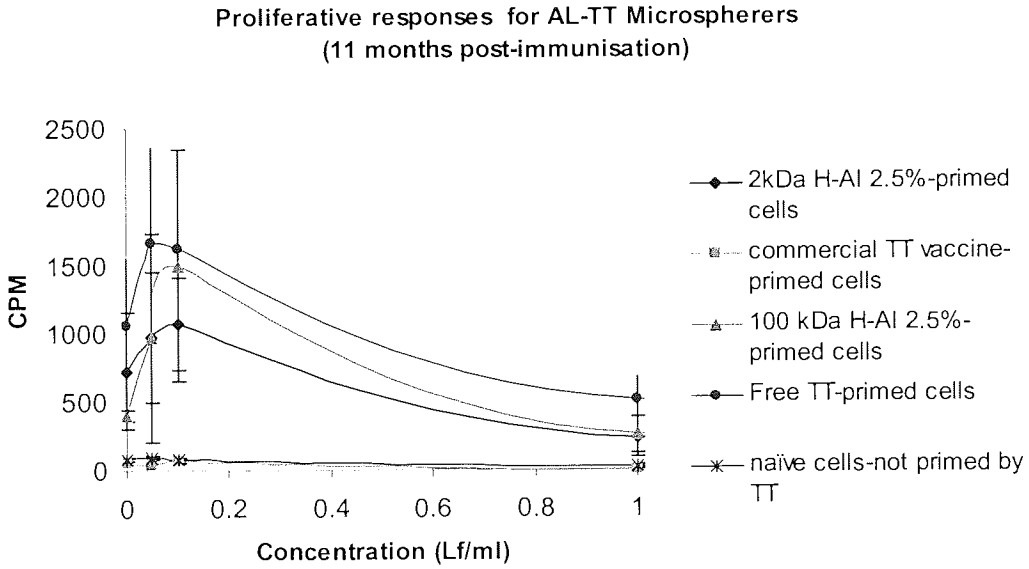


Figure 6.4. Proliferative responses in BALB/c mice following injection of TT formulations. Mice received a total dose of 1Lf of TT in a single injection with four different TT preparations 11 months prior to this assay, Vaccine= commercial alum-adsorbed TT vaccine, Free TT= TT in solution, 100kDa H-Al= microspheres composed of 100kDa PLLA, incorporating in-house alum adsorbed TT (H-Al= original solution used in the adsorption of TT onto alum), 2kDa H-Al= microspheres composed of 2kDa PLLA, incorporating in-house alum adsorbed TT, naïve = non-immunised mice mice used as negative control. Results expressed as CPM (counts per minute), showing [^{3}H] thymidine uptake by newly synthesised DNA molecules following stimulation of splenocytes.

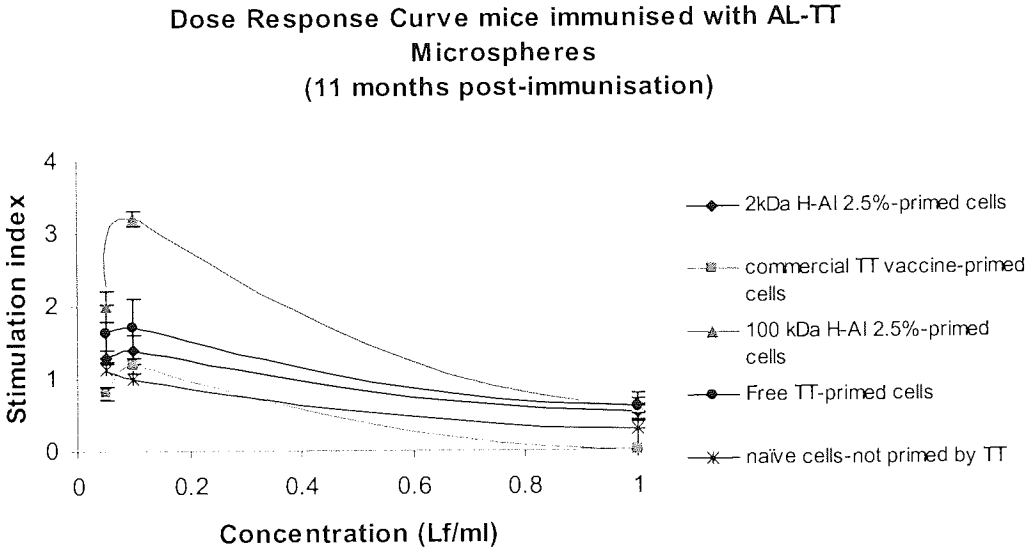


Figure 6.5. Proliferative responses in BALB/c mice following i.m. injection of TT formulations. Mice received a total dose of 1Lf of TT in a single injection with four different TT preparations 11 months prior to this assay, vaccine= commercial alum-adsorbed TT vaccine, Free TT= TT in solution, 100kDa H-Al= microspheres composed of 100kDa PLLA, incorporating in-house alum adsorbed TT (H-Al= original solution used in the adsorption of TT onto alum), 2kDa HAL= microspheres composed of 2kDa PLLA, incorporating in-house alum adsorbed TT, naïve = non-immunised mice mice used as negative control. Results expressed as stimulation index= cpm of stimulated cells with TT/ cpm of unstimulated cells incubated with the medium alone.

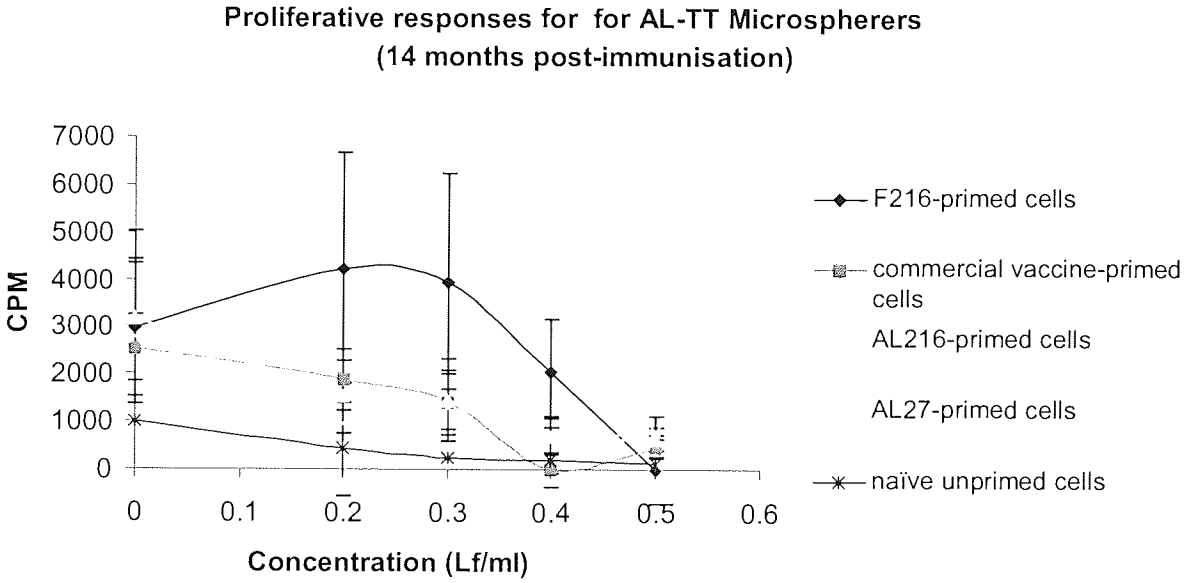


Figure 6.6. Proliferative responses in BALB/c mice following injection of TT formulations. Mice received a total dose of 1Lf of TT in a single injection with four different TT preparations 14 months prior to this assay. Vaccine= commercial alum-adsorbed TT vaccine. Free TT= TT in solution. Al216= microspheres composed of 100kDa PLLA, incorporating commercial alum adsorbed TT (216Lf TT in the original preparation). Al27= microspheres composed of 100kDa PLLA, incorporating commercial alum adsorbed TT (27Lf TT in the original preparation). F216= microspheres composed of 100K PLLA, incorporating TT in a non-adsorbed form (216Lf TT in the original preparation). naïve = non-immunised mice mice used as negative control. n= 5 ± SD.

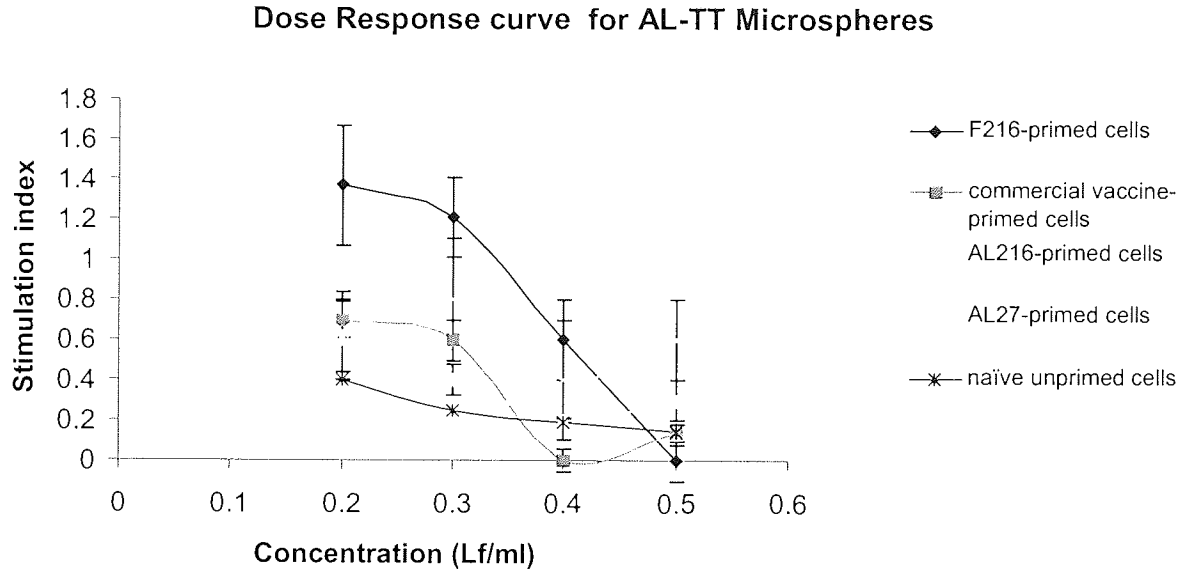


Figure 6.7. Proliferative responses in BALB/c mice following injection of TT formulations. Mice received a total dose of 1Lf of TT in a single i.m. injection with four different TT preparations 11 months prior to this assay Vaccine= commercial alum-adsorbed TT vaccine. Free TT= TT in solution. Al 216= microspheres composed of 100kDa PLLA, incorporating commercial alum adsorbed TT (216Lf TT in the original preparation). Al27= microspheres composed of 100kDa PLLA, incorporating commercial alum adsorbed TT (27Lf TT in the original preparation). F216= microspheres composed of 100K PLLA incorporating TT in a non-adsorbed form (216Lf TT in the original preparation). naïve = non-immunised mice mice used as negative control.

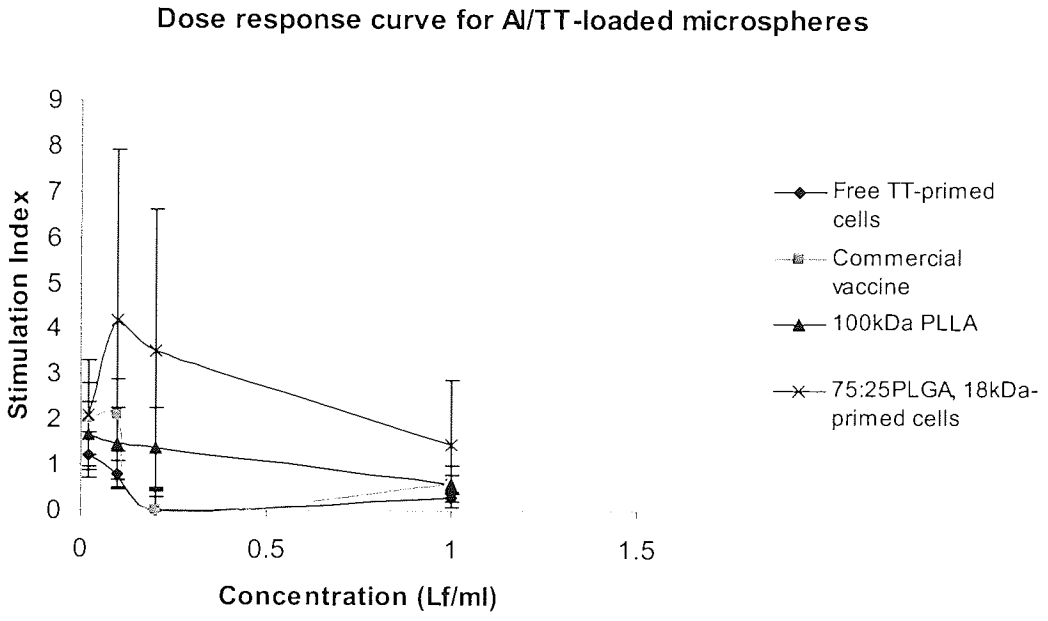


Figure 6.8. Proliferative responses in BALB/c mice following injection of TT formulations. Mice received a total dose of 1Lf of TT in a single injection with three different TT preparations 4 months prior to this assay, vaccine= commercial alum-adsorbed TT vaccine, Free TT= TT in solution, 100kDaPLLA = microspheres composed of 100kDa PLLA encapsulating alum and TT mixture without prior incubation for adsorption. PLGA 75:25, 113.2kDa= microspheres composed of PLGA 75:25, 113.2kDa polymer, incorporating alum and TT mixture without prior incubation for adsorption, naïve = non-immunised mice mice used as negative control. n=5; ± SD.

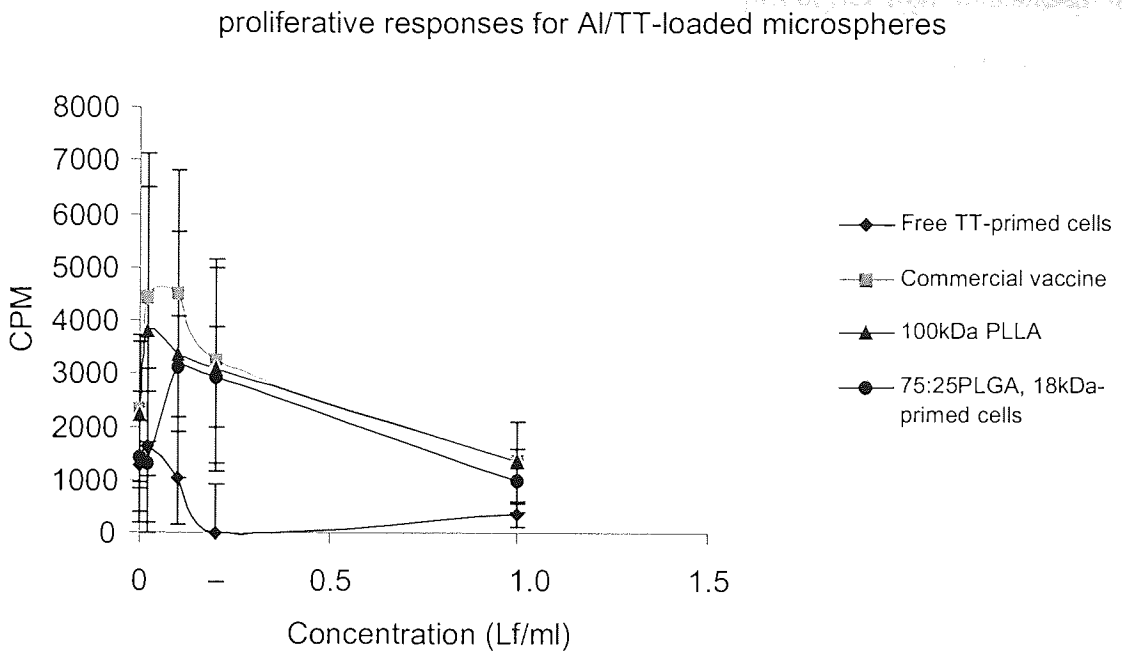


Figure 6.9. Proliferative responses in BALB/c mice following injection of TT formulations. Mice received a total dose of 1Lf of TT in a single injection with three different TT preparations 4 months prior to this assay. Vaccine= commercial alum-adsorbed TT vaccine, Free TT= TT in solution, 100kDaPLLA = microspheres composed of 100kDa PLLA encapsulating alum and TT mixture without prior incubation for adsorption. PLGA 75:25, 113.2kDa= microspheres composed of PLGA 75:25, 113.2kDa polymer, incorporating alum and TT mixture without prior incubation for adsorption, naïve = non-immunised mice used as negative control. n=5; ± SD.

These results show that proliferation was observed in all formulations at the three different time points (4, 11, and 14 months post-immunisation). The splenocytes from immunised animals had a higher proliferation activity compared to basal stimulation levels in the control mice. The highest stimulation index for mice immunised eleven months prior to the cytotoxic assay, was seen in the microspheres composed of 100kDa PLLA polymers encapsulating the in-house alum-adsorbed TT (100kDa 2.5% H-Al), followed by the free TT, formulation 2kDa 2.5% HAl, and finally the commercial alum-adsorbed TT vaccine. For mice immunised fourteen months prior to the T cell proliferation assays, formulation F216 (composed of 100kDa PLLA polymer containing unbound TT) had the highest stimulation index, followed by Al27, the commercial vaccine and Al216 formulation (100KPLLA, microspheres containing commercially available alum-adsorbed TT). In the more recent formulations (4 months post-immunisation), the microspheres composed of 75:25 PLGA 113.2 kDa polymer, followed by the commercial vaccine and then the 100kDa PLLA microspheres had the highest stimulation index. The cytokine release from all our formulations however were comparable to those of the commercial vaccine and no significant difference was observed between different formulations. In a recent study Men and co-workers investigated the effect of a single administration of TT within biodegradable microspheres on the stimulation f T cell responses and compared those with aluminium hydroxide (Men *et al.*, 1995). The authors found that significant T cell proliferation responses were obtained in all their microsphere-based formulations at an early stage (day 10) of immunisation. However, it was found that on day10, small microspheres (1-15μm) resulted in the induction of strong proliferation, whereas larger microparticles (32-70μm) induced a weak response. They related this observation to the findings in the literature on the uptake of microspheres with diameter size of less than 30μm by macrophages.

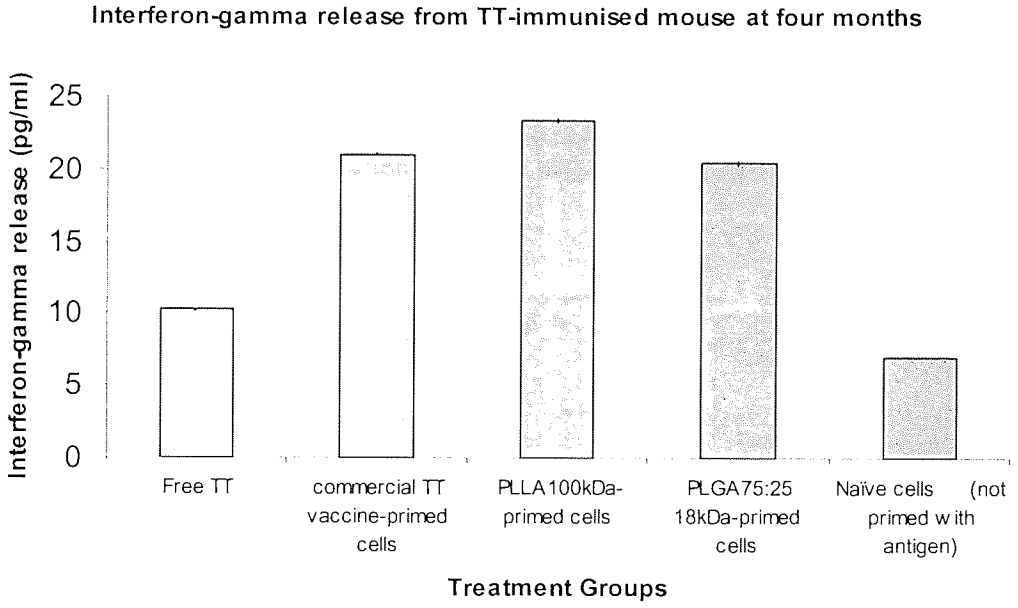


Figure 6.10. IFN-γ release from mice splenocytes stimulated with different Al/ TT formulations 4 months after immunisation with one single dose of intramuscular TT (1 Lf). Vaccine= commercial alum-adsorbed TT vaccine, Free TT= TT in solution. 100kDaPLLA = microspheres composed of 100kDa PLLA encapsulating alum and TT mixture without prior incubation for adsorption. PLGA 75:25, 113.2kDa= microspheres composed of PLGA 75:25, 113.2kDa polymer, incorporating alum and TT mixture without prior incubation for adsorption, naïve = non-immunised mice used as negative control. n=5; ± SD.

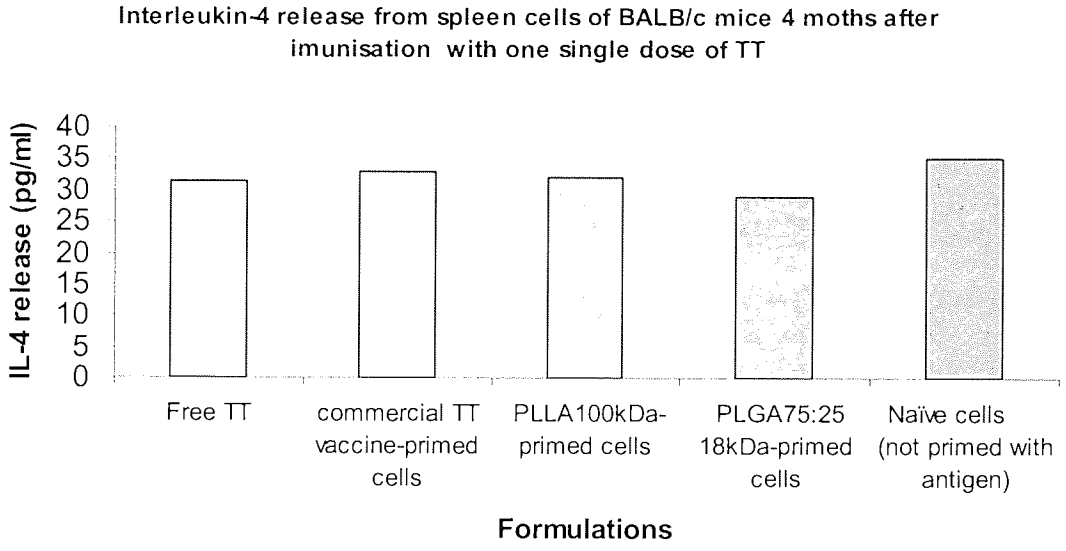


Figure 6.11. IL-4 release from mice splenocytes stimulated with different Al/ TT formulations 4 months after immunisation with one single dose of intramuscular TT (1 Lf). Vaccine= commercial alum-adsorbed TT vaccine, Free TT= TT in solution. 100kDaPLLA = microspheres composed of 100kDa PLLA encapsulating alum and TT mixture without prior incubation for adsorption. PLGA 75:25, 113.2kDa= microspheres composed of PLGA 75:25, 113.2kDa polymer, incorporating alum and TT mixture without prior incubation for adsorption, naïve = non-immunised mice used as negative control. n=5; ± SD.

Their overall findings shows that a combined effect of strong burst release, prolonged release, small particle size and possibly, specific polymer characteristics might be necessary for a long-term proliferative response (Men *et al.*, 1995).

7.0. Storage and stability of alum-loaded PLLA/PLGA microspheres

7.1. Introduction

Microspheres of biodegradable PLLA and PLGA polymers have been shown to harbour great potentials for vaccine delivery (Aguado and Lambert, 1992; Men *et al.*, 1997; Hanes *et al.*, 1997). However, it has also been shown that the functional stability of microencapsulated protein antigens is a critical factor. It is likely that protein stability is one of the most important obstacles for the formulation of single-dose delivery microsphere vehicles. It is therefore important to explore certain aspects of protein structure that may play a role ensuring the successful attainment of a 'super-vaccine' recipe. Proteins as biopolymers have very large globular structures (typically 2-8 nm a side or even larger) (Squire and Himmel, 1979). They possess complex internal architecture, which defines their unique biological functions, and also contain numerous chemically reactive moieties on their side chains, as well as possessing chemically labile bonds. In contrast, small molecules and even most peptides lack a higher-order structure that may be lost during microsphere preparation. There are a number of chemical alterations as well as denaturation and aggregation that may lead to the loss of protein activity (Volkin and Kilbanov, 1985; Manning *et al.*, 1989). Proteins are also susceptible to complex adsorption processes that could result in their denaturation, particularly when the surface is hydrophobic (Andrade and Hlady, 1985). In addition the size of the protein will also present special mass transport issues. This can be explained with the observation that small molecular weight stabilisers in the formulation that can regulate the environment of the protein (i.e. pH, ionic strength, surface tension, and viscosity) will be transported more freely through the degrading polymer than will the protein (Schwendemen *et al.*, 1995). The term stability of proteins can be defined in a variety of ways. From the

biochemical point of view, it is thought that a protein is usually most stable at its isoelectric point (Creighton, 1993). Yet to avoid aggregation or irreversible conformational changes at surfaces, it is suggested that one move the pH away from the isoelectric point (Andrade and Hlady, 1985; Volkin and Kilbonov, 1985). The pharmaceutical definition according to the US FDA considers a *stable pharmaceutical product* as one that deteriorates no more than 10% in two years (Cleland and Langer, 1994). On the other hand the *conformational or physical stability* of a protein has been described as its ability to retain its tertiary structure (Schwendemen *et al.*, 1995). The tertiary structure determines the functional properties of proteins; for example one that allows enzymes to recognise and bind their natural substrates as well as having their reactive functional groups properly aligned for catalysis. The issue of conformational stability has been debated amongst the immunologists, as being necessary for a long-lasting and neutralising immune response following administration of proteinaceous vaccines. It has become clearer that for achieving protection upon the use of such vaccines, it is essential that the native antigenic determinants on the antigen are retained (Levine and Chain, 1991). There is also the *chemical stability* of the protein to consider which describes the reactivity of the side chains and lability of the peptide bonds. It is known that the alteration of even one essential amino acid can seriously impair or abolish the protein's function. This is evidenced in sickle cell anaemia when single amino acid substitution in the haemoglobins can cause this serious disorder (Eaton and Hofrichter, 1990). The biochemical stability of the protein is elated to the magnitude of the change in Gibbs free energy between the folded and unfolded state of the protein (Pace, 1990) and depends on thermodynamic factors. This distinguishes this form of stability from that of the physical stability as the latter is a function of both thermodynamic and kinetic factors. The stability of proteins within biodegradable microspheres can be affected in different

stages, which include the microsphere preparation from polymers, hydration and erosion of the polymer during release incubation. The first process, involves the use of organic solvents (Alonso *et al.*, 1994), formation of the microspheres (Tabata *et al.*, 1993), and the process of lyophilisation, which all could lead to the inactivation of the protein (Pikal, 1994). During release incubation, slow hydration of the protein (slower than direct reconstitution), leads to the so-called moisture-induced aggregation, thus rendering the protein inactive (Liu *et al.*, 1991; Constantino *et al.*, 1994). Degradation of the polymeric matrix which results in lowering the pH surrounding the protein, the presence of hydrophobic polymer surface, and the formation of water-soluble polymer fragments and monomers are amongst other processes taking place during release incubation. All of these processes could lead to inactivation of proteins, as well as introducing the possibility of chemical reactions between the polymer and protein (Domb *et al.*, 1994; Lin *et al.*, 1994). The intrinsic stability of the protein is another important factor to be considered in its microsphere encapsulation. This determines the susceptibility of the protein to such processes as deamidation, oxidation of methionine and cystein residues and, or many other deleterious reactions involved in microsphere formulation, release and storage. In order to better understand how any of the above processes could affect the protein function, an overview of the protein structure and the mechanisms of protein inactivation will be presented shortly.

7.1.1. Protein structure

Globular proteins have internal architecture, comprised of a combination of irregular structures, such as random coils, and of regular structures such as α-helices, β-sheets, and β-turns. There are four separate but interrelated/ interdependent levels within the structure of protein hierarchy. These are primary (Anfinsen, 1956), secondary (Gruenewald *et al.*,

1979), tertiary (Rossman and Liljas, 1974) and quaternary structures (Branden *et al.*, 1984). Primary structure refers to the amino acids sequence or, more precisely, all the covalently bonded amino acids that constitute the polypeptide backbone of the protein (Anifesen, 1956, 1973). Secondary structure refers to the spatial arrangement of amino acid residues that are near one another in the linear sequence. Some of these steric relationships are of a regular kind, resulting in a periodic structure. These include the α–helices, β-sheets and β–turns, which are characterised by specific torsional angles (Ptitsyn and Finkelstein, 1980; Rossman and Argos, 1981). The latter serve as nucleation sites for folding because they can flicker in and out of the conformation that they occupy in the final protein (Anfinsen, 1973). They are thought to be entirely due to the interactions of short-range hydrogen bonds between the amide hydrogen and nitrogen atoms and the carbonyl oxygen atoms of the peptide linkage of one or more poly peptide chains (Rossman and Argos, 1981). These structures are thought to occur spontaneously. There is also a further level of hierarchy, which was introduced by Rao and Rossman, termed super-secondary structure. The term was used to describe a recurring fold consisting of several discrete secondary structural elements that do not complete the tertiary or domain structure of the protein (Rao and Rossman, 1973). The tertiary structure refers to the spatial arrangements of amino acid residues that are far apart in the linear sequence. Proteins containing more than one polypeptide chain exhibit an additional level of structural organisation. Each of these polypeptides is called a subunit. Some polypeptide chains fold into two or more compact regions that may be joined by a flexible segment of polypeptide chain. These compact globular units are called domains, and range in size from about 10 to 400 amino acid residues. Such domains may have a particular function such as being a binding site for another molecule. The three dimensional structure of the "active site" of a protein often consists of the interfaces between domains which permits

the individual function of each to be brought together to form a more sophisticated structure (Rosmn and Lijas, 1974). Quaternary structure refers to the spatial arrangements of protein subunits and the nature of their contacts. The constituent chains of a multi-subunit protein can be identical or different. The same covalent and non-covalent forces which maintain the tertiary structure of each subunit are responsible for maintaining the subunits geometry in amore stable and ordered structure (Branden *et al*, 1983). In a complete assessment of protein stability, knowledge of the amino acid sequence, secondary, tertiary and quaternary structures of the protein is essential. This information can be attained if a complete X-ray crystallographic analysis is carried out.

7.1.2. Mechanisms of protein inactivation

The known mechanisms of protein inactivation include reversible unfolding, aggregation, and chemical inactivation (Manning *et al.*, 1989). The most general of kinetic pathways leading to inactivation is summarised in the following scheme:

$$N \rightarrow \quad pU \rightarrow D$$
$$\downarrow$$
$$(pU)_n$$

where N is a native protein in equilibrium with a partially unfolded state (pU) (Volkin and Kilbanov, 1985; Manning *et al.*, 1989). The pU possesses less tertiary structure and is much more reactive to most side reactions, leading to an irreversibly denatured state D or to irreversible aggregates $(pU)_n$.

7.1.2.1. Physical (conformational) inactivation

Non-covalent interactions such as hydrogen bonding, van der Waals interactions, salt bridges and hydrophobic interaction, are responsible for cementing together the native

structure of proteins. As indicated in the above equation, the physical stability of the protein is critical, since on losing much of the native tertiary structure to form *pU*, the protein becomes more reactive. In this state the hydrophobic regions of its structure become exposed and the previously buried reactive side chaines interact with the solvent environment. There are several factors which contribute to the loss of physical stability, which include elevated temperature, extremes of pH, denaturants, and adsorption to hydrophobic surfaces (Schwendeman *et al.*, 1995). At physiological temperature, the folded state of protein is favoured by some 50 kcal/mol. When the pH is adjusted away from the protein's isoelectric point, the changes in the amino acid residues may lead to unfolding due to the presence of electrostatic repulsion that results (Volkin and Kilbanov, 1985). Salts, chelating agents and organic solvents cause denaturation of proteins. Salting-out salts (decreasing the solubility of hydrophobic groups in the protein) favour the native state, as opposed to the salting-in salts. The latter, raise the solubility of hydrophobic groups in water, and can destabilise the protein (Volkin and Kilbanov, 1985). Chelating agent such as ethylenediamine-tetraacetic acid (EDTA), can cause the unfolding of proteins which are stabilised by a heavy-metal ion. Water-immiscible organic solvents such as ethyl acetate can bind directly to hydrophobic groups of proteins, displacing the bound water molecules (Volkin and Kilbanov, 1985). Saturating the solvent with water adds to protein flexibility through diffusion of water molecules through the protein molecules (Zaks and Kilbonov, 1988). Water-miscible solvents can either stabilise (e.g. glycerol), or destabilise proteins depending on whether the solvent increases the amount of water bound to the protein molecules (preferential hydration), or decreases it (Timasheff and Arakwa, 1990). Detergents such as SDS cause the unfolding of proteins by direct binding. Non ionic surfactants on the other hand cause less unfolding since they inhibit adsorption to surfaces. Proteins can desorb from hydrophilic surfaces, without

conformational changes, whereas their conformation is changed to promote stronger binding with hydrophobic surfaces, as they expose their hydrophobic regions for extra contacts.

7.1.2.2. Aggregation of proteins

Several mechanisms are known for the non-specific aggregation of proteins by non-covalent interactions between unfolded protein molecules or by covalent cross-links. These include hydrophobic interactions following partial unfolding of two or more native protein molecules to form soluble aggregates, thiol-disulfide interchange, β-elimination related aggregation and isopeptide aggregation when lysine is added to an asparagine or glutamine residue (Constantino *et al.*, 1994).

7.1.2.3. Chemical inactivation

Chemical changes resulting in the inactivation of proteins include deamidation, oxidation, hydrolysis, β-elimination, and racemisation. Deamidation of asparagine and glutamine residues in proteins is a common covalent pathway, which can lead to protein inactivation. This reaction can take place under both acidic and basic conditions and also elevated temperatures. Deamidation is thought to be catalysed non-enzymatically by water and possibly by functional groups within the protein (Wright, 1991). Maximal stability against deamidation is ensured at pH of 6. Deamidation is increased when an asaparagine or glutamine residue is adjacent to certain amino acids in the primary structure (Wright, 1991). Oxidation reactions can occur simply in the presence of atmospheric oxygen (e.g. oxidation of methionine) as well as other oxidising agents (Manning *et al.*, 1989). These include hydrogen peroxide, hydroxyl and superoxide radicals, hypochlorite ion, and visible light (e.g. tryptophan oxidation)(Manning *et al.*, 1989; Volkin and Kibanov, 1989). Non-

enzymatic hydrolysis of proteins is favoured under acidic conditions and occurs primarily at peptide bonds adjacent to aspartic acid residues (Zale and Kilbanov, 1986). Natural, L-amino acids are susceptible to racemisation resulting in the formation of the corresponding D-enantiomers (Manning *et al.*, 1989). This is thought to occur *via* a carbanion intermediate, predominantly for aspartic acid, which forms a cyclic imide.

7.1.3. Protein inactivation during preparation, storage, and release incubation of microspheres

One of the most often used methods of microsphere preparation is the so-called double emulsion solvent evaporation method (Cohen *et al.*, 1991; Ogawa *et al.*, 1988; Alonso *et al.*, 1993). In this method as has been mentioned earlier, an aqueous protein solution is added to an organic polymer solution resulting in the formation of the first emulsion following sonication or homogenisation. The second emulsion is then formed by the addition of the primary emulsion into an aqueous phase containing an emulsifier such as PVA, and the subsequent mixing of the two phases (eg. vortexing or homogenisation). Subsequent addition of a second aqueous phase (larger volume) onto the second emulsion, and the removal of the organic solvent with continuous stirring and simultaneous hardening of the particles completes the process. The microspheres are then collected, washed and lyophilised. As the two aqueous and organic phases are mixed in the first emulsion, the two phases begin to partition into one another to the extent allowed by their solubility (Thies, 1992), for example 2% methylene chloride in water at 20 °C (Perry *et al.*, 1984). The presence of the organic solvent in the protein-containing inner water phase causes the denaturation of the protein. The organic solvent forms a large hydrophobic surface around the inner water phase, and any surface-active components, including proteins, of the two phases will diffuse towards and occupy this water-oil surface. Proteins

and other excipients within the inner water phase start diffusing into the organic polymer solution if any solubility exists. The homogenisation process creates a certain degree of thermal energy, as large pressure gradients are created. The thermal energy is particularly evident for viscous polymer solutions, since the higher flow resistance must favour the thermal route of energy dissipation. In the process of secondary emulsification, some of the inner water phase droplets containing protein are lost to the second aqueous phase (Ogawa *et al.*, 1988). One variation of the double emulsion method is the addition of solid protein directly to the polymer solution without water (no first emulsion). In this method during the second emulsification process, the protein may become hydrated, because very small amounts of water can diffuse through the organic phase, as the volatile organic solvent diffuses into the aqueous phase (if not pre-saturated with organic solvent), and begins to evaporate into the surroundings. At this stage a phase separation occurs at the surface of the polymer and second aqueous solution, leading to a polymer-rich phase at the microsphere surface, and a more dilute polymer solution in the interior. Thus discrete microsphere particles are formed encapsulating the protein within. The hardening process must proceed either by particle shrinkage or co-transport of organic phase and second aqueous phase components (mass must be conserved across the inter-phase if particle volume is fixed), thus there is the potential of introducing such compounds as isopropanol into the protein-containing inner water phase. During the collection and lyophilisation, residual solvent remains which is then removed under vacuum, leaving pores behind (visible by SEM)(Mathiowitz and Langer, 1992). The above analytical view of the physico-chemical processes during double emulsion method, indicates that importance of such factors as the organic solvent used (Alonso *et al.*, 1994), the presence of water (protein mobility) (Volkin and Kilbanov, 1985; Zaks and Kibanov, 1988), and the method of emulsification (Tabata *et al.*, 1993) in relation to protein stability.

Source of protein inactivation	Mechanism of protein inactivation
Initial rehydration Reduced water content	Moisture-induced aggregation aggregation
Adsorption to polymer surface	Unfolding and aggregation
Acidic microclimate pH	Chemical inactivation
Interaction with erosion products	Binding and unfolding
Dialysis effect	Loss of stabiliser, addition of extra intracellular elements

Table7.1. Potential sources of protein inactivation during microsphere release incubation, adapted from Schwendeman *et al.*, 1995.

The actual events following the addition of lyophilised proteins onto organic solvents are not as straight forward as they may appear The actual formation of the lyophilised proteins is uncertain (whether in the native or reversibly unfolded state), and can be evaluated using infrared spectroscopy (Prestrelski, 1993). It is understood, within the enzyme literature that the conformational mobility of the protein is particularly restricted in the hydrophobic solvents, typically those used for polymer dissolution such as methylene chloride. Addition of small quantities of water to such enzymes which have been suspended in the organic solvent, increases the protein's flexibility, thus enhancing the activity by orders of magnitude (Zaks and Kilbanov, 1988). The residual water content during the lyophilisation process is another important factor. The less hydrophobic organic solvents can "strip away'' the water molecules more vigorously, and thus disrupt the catalytic centres (Dabulis and Kilbanov, 1993). Addition of large quantities of water to the organic solvents can provide the proteins with sufficient mobility to denature and then aggregate (Zaks and Kilbanov, 1988). The direct addition of protein solution to organic solvents

maximises the protein mobility. As the organic phase diffuses through to the inner water phase, it can alter the aqueous ionic strength, binds or bind directly to the protein, thus exposing the protein's hydrophobic regions (Volkin and Kibanov, 1985). It has been observed that upon addition of tetanus toxoid to methylene chloride, first soluble and then insoluble aggregates are formed, when a single interface is formed between the solvent and the aqueous protein solution (Schwendeman *et al.*, 1995). Emulsification processes include homogenisation and ultrasonication, all of which increase the rate of mass transfer between the bulk liquid and air-liquid interface. It is thought that this exposure is responsible for protein inactivation due to the hydrophobicity of air, which favours unfolding of the protein (Volkin and Kilbanov, 1985). Ultrasonication has also been found to result in 40% loss in lysozyme activity during the double emulsion technique (Tabata *et al.*, 1993). Homogenisation involves pumping the liquid past a narrow orifice, introduces the protein to both pressure and shear forces, which increase its unfolding (Volkin and Kilbanov, 1985). Protein inactivation can also occur during freezing, drying and storage of the microspheres. During freezing, high solute concentrations can be reached which may lead to protein denaturation (Volkin and Kilbanov, 1985). The preferential crystallisation of one of the multiple buffering species can alter the pH dramatically (Pikal, 1994). The presence of contaminants such as metal catalysts, in polymers and excipients can inactivate proteins during storage (Volkin and Kilbanov, 1985). The mechanisms of protein inactivation during release incubation have been outlined in table 7.1.

7.1.4. Protective measures to inhibit protein inactivation

In general two approaches are used towards stabilisation of proteins. The first is to minimise its reversible unfolding (loss of physical stability), and the greater exposure of the reactive hydrophobic regions which follows, thus inhibiting aggregation. The second approach involves the prevention of irreversible aggregation or chemical reactions, which

follows the unfolding of the protein, thus facilitating protein refolding to its native conformation (Volkin and Kilbanov, 1985). These two approaches could prove to be mutually exclusive at times and prioritising them seems necessary under certain circumstances. For example maintaining the pH close to neutral rather than acidic, prevents aggregation due to adsorption of a protein with isoelectric point of 4.8. However, disulfide interchange is inhibited at acidic pH. In maintaining the stability of proteins in the process of microencapsulation, it is advisable to use methods in which water is absent or controlled at an optimal level in order to avoid mobility-related aggregation of the proteins (Volkin and Kilbanov. 1985). The choice of organic solvent is also important as ethyl acetate for example decreases the soluble aggregates formed and increases the recovery of antigenically-active tetanus toxoids compared to methylene chloride (Alonso *et al.,* 1994). It is not known whether homogenisation or sonication has the least detrimental effects on proteins. The use of organic solvents can be omitted in microencapsulation techniques. For example aqueous protein solutions have been air-atomised in liquid nitrogen (Gombotz, 1990). Once these microparticles have been lyophilised, their subsequent microencapsulation within biodegradable polymers, should do the least damage, since it can be assumed that the protein will be most stable as its exposure to the organic solvent occurs when it is immobile in the solid state (Gombotz, 1990). The preservation of protein stability during freezing and drying is mechanistically distinct for each process. It is thought that cryoprotectants stabilise proteins by preferential exclusion of the solute, which stabilises the native protein in the freeze-concentrate during freezing (Carpenter *et al.*, 1994). However, some cryoprotectants provide little protection during subsequent drying (Carpenter *et al.*, 1990). Lyoprotectants are formulation additives, which maintain the stability of the product during freezing, dehydration, and possibly storage. According to Pikal's description two hypotheses exist for stabilisation during

drying and storage. The first one is the kinetic *vitrification hypothesis,* which states that the protein is stabilised during storage when lyophilised in the presence of good glass formers. Thus the protein mobility is prevented and deleterious reactions are inhibited. The second hypothesis is the thermodynamic *water substitute hypothesis*, stating that hydrogen bonding between the protein and the sugar promotes the native conformation once water has been removed (Pikal, 1994). Both of the above hypotheses have been shown to be quite important (Prestrelski, 1993; Slade and Levine, 1991). Other approaches to stabilise proteins in the process of microencapsulation include the use of ''protein-friendly'' polymers. These synthetic biodegradable polymers are likely to either provide a suitable aqueous environment, or are capable of maintaining the rigidity of the protein by preventing its contact with water (Schwendeman *et al.*, 1995). The former is represented by certain polyphosphazenes (e.g. poly [bis(carboxylatophenoxy)phosphazene]), which require no organic solvents or rehydration steps. The latter type of polymers may be represented by the polyanhydrides, because extremely hydrophobic polyanhdrides limit water penetration due to their hydrophobic nature (Ron *et al.*, 1993).

7.1.5. Stability of tetanus toxoid during microencapsulation

Tetanus toxoid has been studied for use in single injection vaccine delivery systems (Singh *et al.*, 1991; Alonso *et al.*, 1994; Men *et al.*, 1995; Thomasin *et al.*, 1996). Basic studies on the molecular structure and biological activity of this protein has revealed that, the mature tetanus neurotoxin is a 1351-amino-acid protein consisting of two chains. The N-terminal light chain is 52 kDa and C-terminal heavy chain is 98 kDa. These two chains are linked by a single disulfide bridge (Eisel *et al.*, 1986; Halpern and Lofthus, 1993). Conformational studies have shown the importance of conformational domains for such biological activities as toxicity and antigenicity (Halpern and Lofthus, 1993; Robinson *et*

al., 1982; Kreiglstein *et al.*, 1990). However, there is still little data on the mechanisms of activity loss and related chemical and structural changes. Loss of antigen stability during microsphere preparation has been illustrated which is typically aggregation-related inactivation of the protein (Schewendeman *et al.*, 1996). Other studies have shown that the secondary structure of the toxoid had undergone reversible changes during lyophilisation (Constantino *et al.*, 1996). Tetanus toxoid has been microencapsulated in the presence of stabilising excipients such as sugars, polyols, surfactants and proteins in order to diminish loss of antiginicity (Johansen, *et al.*, 1998; Chang *et al.*, 1996). For example albumin has been shown to greatly improve the stability of tetanus toxoid both during microencapsulation and release (Johansen, *et al.*, 1998; Audran *et al.*, 1998). Other studies have shown that dextran and heparin provide a particularly stabilising environment for tetanus toxoid inside the microspheres during polymer degradation process (Sanchez *et al.*, 1999). Therefore an understanding of the possible mechanisms involved in the loss of activity of this antigen and the implementation of appropriate protective measures seems to be necessary for its successful encapsulation and release in a fully functional toxoid. The studies presented in this manuscript have evaluated the stability of formulations by measuring *in vivo* immune responses and later on using high performance liquid chromatography (HPLC). The effect of co-encapsulation of human serum albumin (HSA) and trehalose on the stability of the microencapsulated toxoid was investigated. The following represents the experimental methods and the subsequent results.

7.2. Materials and Methods

7.2.1. Polymers

See section 5.2.3.

7.2.2. Antigens

See section 5.2.2.

7.2.3. Excipients

Trehalose D (+) was obtained from Fluka Biochemicka (Messerschmittstr, Switzerland). Albumin (human source), HSA was provided in powder form of fraction V from Sigma Chemicals (Poole, UK).

7.2.4. Chemicals

See section 5.2.4.

7.3. Methods

7.3.1. Microsphere preparation

See section 5.3.1. for original formulations encapsulating the commercial alum-adsorbed TT vaccine. The trehalose and HSA stabilised formulations were prepared by modifying the preparation procedure described in section 5.3.3. The internal phase consisted of 1.5 ml of aqueous solution containing 200Lf (TT), alum (2.2 mg) and HSA (5%) or trehalose (15%) and PVA (2.5% w/v) PVA. The secondary phase consisted of 5ml of dichloromethane containing 250 mg of PLGA (50:50, 0.4 dL/g 57kDa) polymer. The external phase consisted of 75 ml PVA (5% w/v). The particles were prepared as described before.

7.3.2. Storage of microspheres under different temperature conditions

The original alum-loaded formulations, encapsulating the commercial vaccine (AL27 and AL 216) were investigated for antigenicity and immunogenicity of the encapsulated TT after dry storage at three different temperatures (RT, 4°C and 37 °C) for 6 months. More recent formulations were co-encapsulated in the presence of HSA and trehalose, and were stored in a dry form at two different temperatures (4°C and RT). These formulations were analysed using HPLC and SDS-PAGE gel electrophoresis.

7.3.3. Analysis of antigen stability using ELISA

See section 2.7.

7.3.4. Analysis of antigen stability using high performance liquid chromatography

See section 2.9.

7.4. Results and Discussions

7.4.1. Effect of different storage temperatures on the release characteristics of antigens

The original alum-loaded formulations were stored dry at three different temperatures and were analysed for immunogenicity of the tetanus toxoid after six months of incubation. As evident in figure 7.2, the immunogeniciy of these particles was adversely affected by long-term storage.

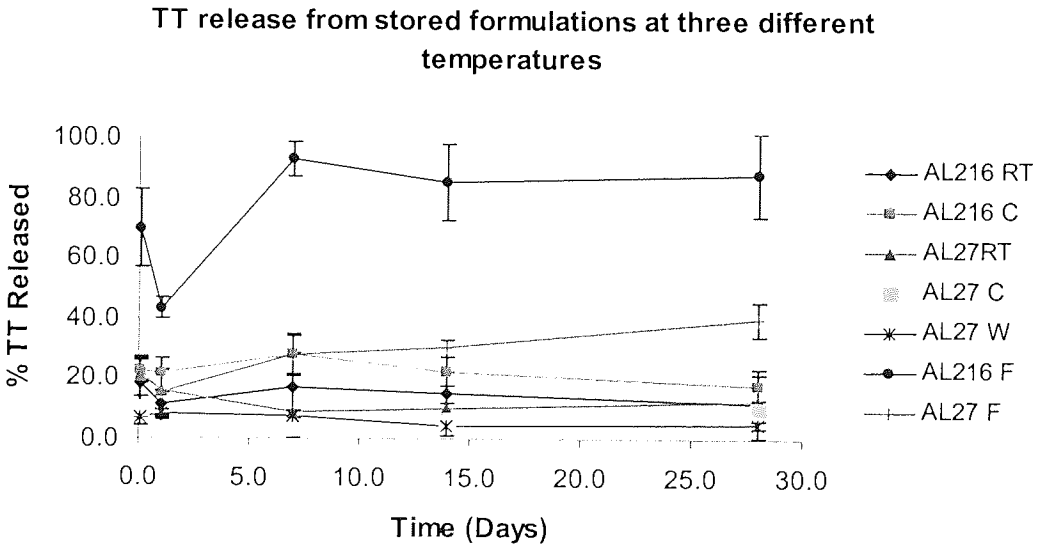


Figure 7.1. *In vitro* release of tetanus toxoid from microspheres encapsulating (commercial TT vaccine, Al216 and Al27) after six months of dry storage at different temperatures (Room temperature RT, 37 °C W, and at 4 °C, C). Al216 and Al27 = preparations containing 216 and 27 Lf alum-adsorbed TT. AL216 F and AL27 F= freshly prepared formulations.

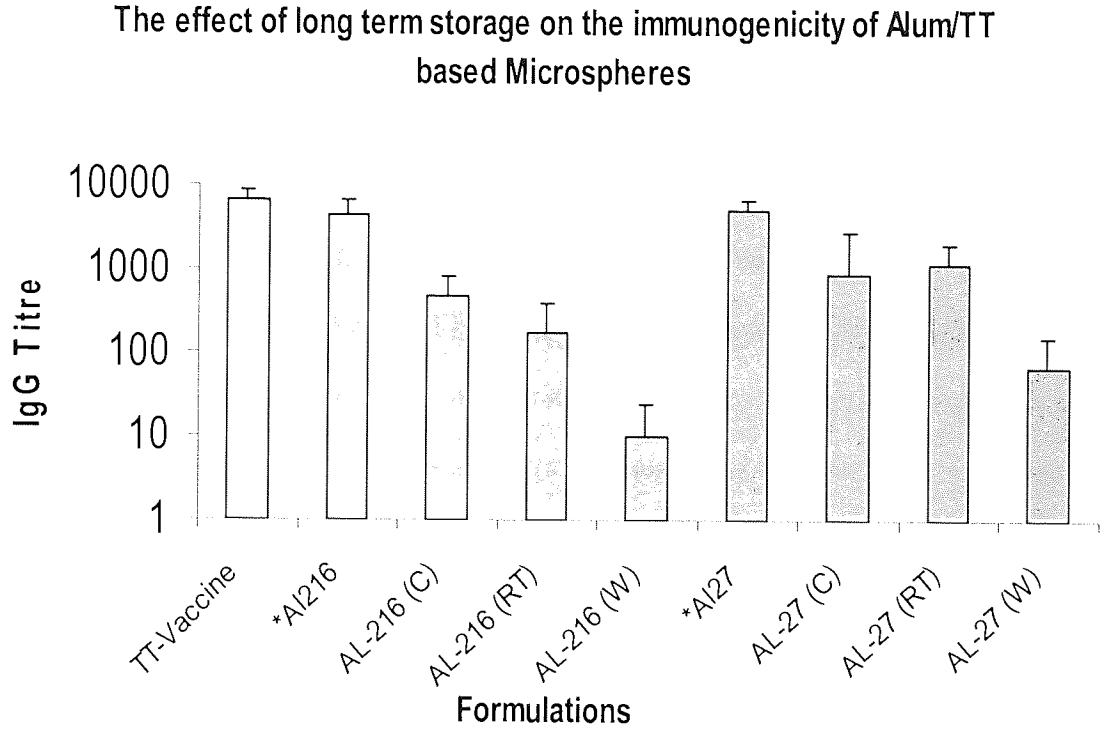


Figure 7.2. Total serum IgG antibody response in BALB/c mice 14 days following injection with tetanus toxoid delivered in different formulations from microspheres encapsulating (commercial TT vaccine, Al216 and Al27) after six months of dry storage in different temperatures. RT= room temperature. W= 37 °C. C= 4 °C. Al216 and Al27 = preparations containing 216 and 27 Lf alum-adsorbed TT, F216 and F27= preparations containing 216 and 27 Lf soluble TT. *= freshly-prepared formulations.

The release profile of stored formulations (Fig 7.1) exhibits the detection of reduced amounts of TT compared to the freshly prepared samples. The release profile seems to

present an initial burst followed by a lag period in which only a small amount of the protein is being released. The release profile also shows a reduction in the amount of released antigen after the initial burst. This could be due to the re-absorption of the antigen onto the test vials and or the microspheres. Initial burst release of the stored samples varied between 10-20% as opposed to 25-70% for the freshly prepared particles. This indicates that storage conditions had affected the release of the protein adversely and that may be due to aggregation of the protein within the polymeric particles which would cause its incomplete release. The hydrophobic interactions between polymer and protein can unfold proteins, thus resulting in the loss of conformational epitopes in vaccine antigens (Chang and Gupta, 1996). The initial lyophilisation of microspheres, would lead to water loss, which in turn increases the local concentrations of proteins. Thus the rate of chemical reactions is increased leading to inter- and intramolecular bond formation. Tetanus toxoid may also undergo aggregation due to the low pH environment inside the microspheres, which would result in loss of antigenicity and non-release of TT due to large size (Chang and Gupta, 1996). Xing and co-workers have shown that TT undergoes conformational changes both at low pH and high temperature. It is suggested that in both cases, a loosening of the structure occurs, leading to an increase in the exposure of tryptophan side-chains to organic solvent. This appears to cause an irreversible modification of this side-chain (Xing *et al.*, 1996).

7.4.2. Effect of long-term storage at different temperatures on the immune response to microspheres encapsulating alum-adsorbed particles

The immune response to the alum-based formulations was also found to be affected due to long-term storage of the microspheres (Fig 7.2). Formulation Al216 (containing the higher load of TT) was affected more than Al27, and showed a reduced immune response of 10%

and 4% for formulations that were kept at 4 °C and room temperature respectively. Formulation Al27 when kept at 4 °C produced 18% of the total immune response first observed for the freshly prepared particles. This value was 24% for the same particles kept at room temperature. The formulations, which were kept at 37 °C, exhibited 1% of the original immune response observed with these formulations. These findings indicate the adverse effects of long-term storage on the stability of tetanus toxoid, and are also more complicated in view of the inclusion of commercial TT vaccine within them. The commercial vaccine is supplied in an aqueous buffer and contains a measurable amount of ions, which might also influence the nature and the rate of the polymer degradation. These ions can also degrade the protein if their concentration rises due to loss of moisture particularly in the case of high temperature storage. In view of these findings and based on the findings of other investigators, it was decided to formulate TT within biodegradable polymers in the presence of known stabilising agents such as trehalose and human serum albumin.

7.4.3. Effect of different excipients on the long-term storage of antigens

There is a wealth of literature on protein formulations investigating protein stability under typical pharmaceutical conditions such as lyophilisation, storage and rehydration (Constantino *et al.*, 1994). In recent years this research has focused more on stabilisation of the proteins within biodegradable polymers such as PLGA and PLLA (Meniel *et al.*, 2001, Van de Weert, 2000a). As it is becoming clearer that the success of these polymeric vehicles for drug delivery relies on the stability of the protein during microencapsulation and also in the course of the polymer degradation (Crotts and Park, 1997; Van de Weert, 2000b). Stabilisation of TT within PLGA and PLLA microspheres has been extensively investigated as the potential of the above systems for the single-dose delivery of TT has

been realised (Alonso *et al.*, 1993, 1994; Sasiak *et al.*, 2001). Many stabilising agents have been evaluated as potential candidates for co-encapsulation with TT. These, include BSA, trehalose, calcium salts, γ-hydroxypropylcyclodextrin, alginate, poloxamer 188, gelatin and HSA (Audran *et al.*, 1998; Sanchez *et al.*, 1999; Chang and Gupta, 1996; Schwendeman *et al.*, 1998). In the present study, TT was co-encapsulated with aluminium hydroxide and either 5% HSA or 15% trehalose. The average size of micospheres was 7 μm and 11 μm for the above particles respectively. Both preparations resulted in a high yield of particles (94% and 88%) for HSA and trehalose-containing preparations respectively. The total protein concentration was determined by BCA assay. This presented loading efficiencies of 92% for HSA and 97% for trehalose-stabilised particles. The main problem with the use of total protein concentration assay in these studies is the reaction of excipients (stabilisers) in the spectrophotometeric method used in the BCA assay and the non-specificity of this method for different proteins. To overcome these problems standard solutions of TT and of stabilisers (HSA and trehalose) were included in the BCA assay, and the results were adjusted for the background absorbance. Gupta and co-workers evaluated various methods to determine loading of vaccine in biodegradable polymer microspheres encapsulating tetanus toxoid (Gupta *et al.*, 1997). In order to overcome the cross-reactions of excipients within protein assays, they propose the complete digestion of microspheres with acids or base followed by amino acid analysis by colorimeteric assays such as ninhydrin method or using amino acid analysers. Chang and Gupta validated the results of the total protein concentration measured by the BCA assay, using the radioactivity of microspheres containing ^{14}C-labelled TT when co-encapsulating TT with HSA (0.4%) (Chang and Gupta, 1996). The formulations prepared in the present study were stored at dry conditions at room temperature or 4 °C for over nine months before analysis. The effect of long-term storage on the above particles was investigated using HPLC. The HPLC results presented

a peculiar picture as the TT in solution had the characteristic of higher molecular weight fractions which have been observed in the impure WHO preparation (Fig 7.3), whereas the chromatogram of TT extracted from alum-based formulations exhibited more similarity to the purified form of TT preparations. This peculiarity nevertheless indicates that the TT has been stabilised within these formulations despite an incubation time of over nine months and that detectable and well-preserved protein can be visualised extracted out of the stored microspheres (Fig 7.4). Amongst the different preparations, only the HSA-stabilised samples, which were kept at room temperature, produced the above results. No detectable TT was observed from any of the samples kept at 4 °C. The HPLC peaks are small on the whole and represent the limitations in the amount of the TT that could be extracted from the stored samples. Human serum albumin has been used as a stabiliser for stabilising enzymes and proteins. Chang and Gupta have shown that HSA-stabilised (0.4%) TT-containing microspheres continued to release antigenic TT for 4 weeks at 37 °C and that the total antigenic protein release from the above microspheres was higher than the gelatin-stabilised (0.2%) microspheres. It is thought that inclusion of HSA could have an effect on stabilising the pH within the microenvironment of the polymer during degradation and also the release buffer (Chang and Gupta, 1996). Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide of glucose found in several organisms that are able to survive drying (Elbein, 1974). The prevalence of this sugar in such organisms termed ''anhydrobiosis'' (able to survive long periods of drought) is suggestive of the effectiveness of trehalose as a desiccation protectant. Trehalose has been shown to protect cells from freezing injury (Honadel and Killian, 1988), preserve and maintain activity of biomolecules (Crowe *et al.*, 1984) and protect various proteins, viruses and antibodies during drying (Crowe *et al.*, 1983). Audran and co-workers used trehalose to stabilise TT within their PLLA, and PLGA microsphere preparations and found that

addition of trehalose (15% w/w) was associated with higher antibody responses specially, in the early phase. The antibody response was found to be at least equal or even superior to those obtained with alum-adsorbed TT (Audran *et al.*, 1998). Interestingly, the same authors found that trehalose did not preserve the antigenicity of the encapsulated TT as determined by ELISA after TT extraction from microsphere. Trehalose was found to increase TT loading efficiency and mediated the highest burst release which could explain its efficacy in induction of high antibody responses (Audran *et al.*, 1998). Trehalose was also used in a recent study carried out by Sanchez and co-workers. These authors found that inclusion of trehaose (10%) in their PLGA microspheres resulted in the retention of 89% of antigenic TT after lyophilisation. However, these authors found that only 10-15% of the released protein was antigenic from day 1 to day 40 of the release period (Sanchez *et al.*, 1999). The ability of non-reducing sugars to increase protein stability within microspheres has been evaluated and trehalose and mannitol have been shown to improve the recovery of the native protein after encapsulation and to increase the amount of native protein released from the microspheres (Cleland and Jones, 1996). In a recent study Fu and co-workers were able to directly quantify the effect of trehalose on the secondary structure of two model proteins (BSA and lysozyme) encapsulated within microspheres. Trehalose (10% w/w) was added directly to the aqueous protein phase during microsphere preparation, and its effect on the secondary structure of the model proteins were studied using Fourier transform infrared (FTIR) spectroscopy. Trehalose was shown to be able to fully restore the α–helix content of the encapsulated BSA to that of freeze-dried BSA in the absence of sugar (Fu *et al.*, 1999). Microspheres containing trehalose were also shown to release more monomeric BSA molecules compared to those lacking the sugar. A possible mechanism by which trehalose may slow down the dimerisation of BSA could be

due to dilution of the protein, at high sugar to protein ratio (10:1) trehalose may shield the BSA molecules from each other and reduce dimerisation (Fu *et al.*, 1999).

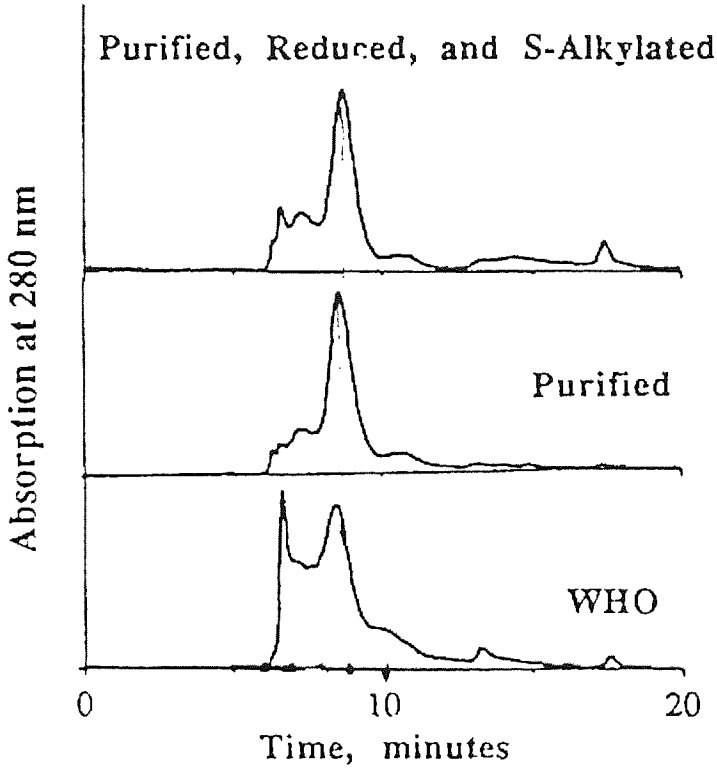
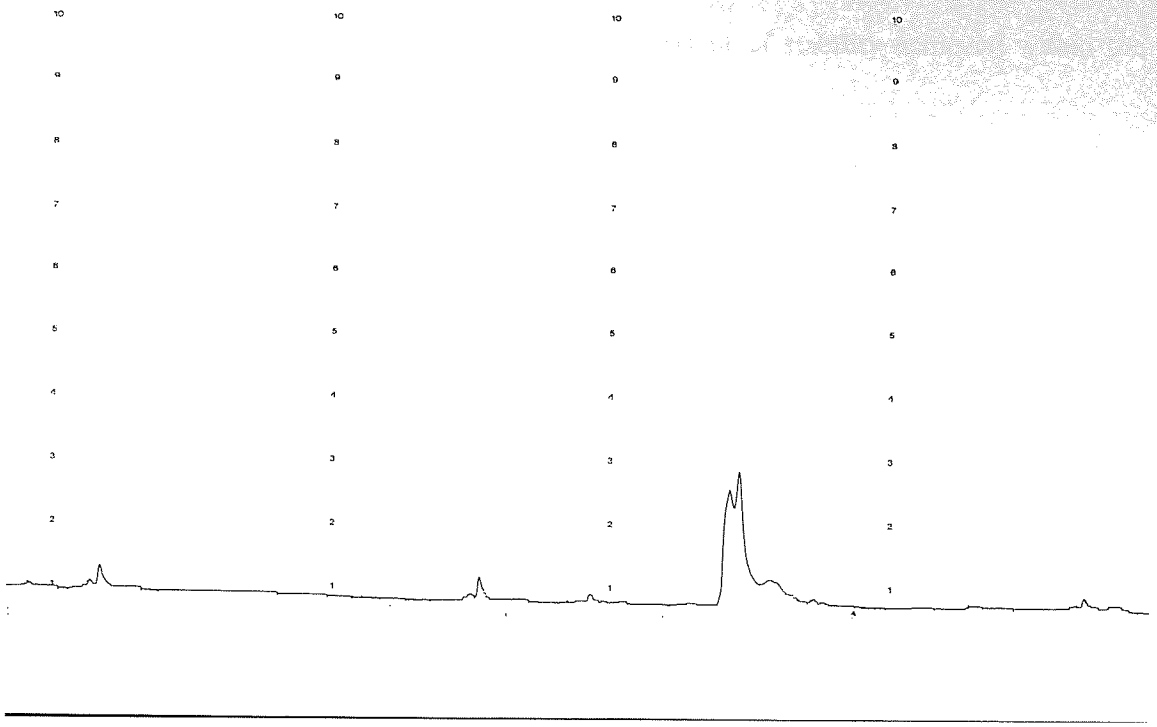


Figure 7.3. Size-exclusion chromatograms of three tetanus toxoids following protein analysis (adapted from Schwendeman *et al.*, 1994).



1 2 3 4

Figure 7.4. Size-exclusion chromatograms of TT antigen extracted from alum-TT microspheres stored for nine months at room temperature. 1,2, 4=alum-TT microspheres containing HSA (5%) within formulation. 3= TT standard in solution. The trehalose-stabilised particles were not included in this analysis.

In conclusion, the results presented in this chapter indicate that the original alum/TT formulations encapsulating the commercial alum-adsorbed TT vaccine were adversely affected during a storage period of six month at dry conditions at RT and 4 °C. The long storage resulted in decreased immune response and rate of protein release. These effects could be related to the presence of unsuitable ions within the commercial vaccine buffer increasing the rate of polymer degradation and or degrading the released protein. The effect of co-encapsulating alum/ TT particles with 5% HSA and 15% trehalose was assessed using HPLC after a nine month period of dry storage at dry conditions. The results indicate that TT may be preserved co-encapsulated with alum particles and 5% HSA at room temperature during the above incubation period. The nature of this study has

been a qualitative analysis of the effects of long-term storage on the stability of TT and therefore no conclusions can be drawn as to the extent of stability achieved within the above formulations

8.0. Microspheres for the delivery of Hepatitis B plasmid DNA

8.1. Introduction

DNA vaccines represent a type of a subunit vaccine where only the antigen required for protective immunisation is presented to the host immune system. Recombinant DNA technology is used to clone DNA sequences encoding the protein (i.e. immunogen) into an eukaryotic expression vector (Ristove *et al.*, 1998). DNA vaccine is injected intramuscularly where it is taken up by cells, transcribed into mRNA and, is then expressed as protein. This delivery system has the advantage of inducing both humoral and cellular immune responses as the immunogen is synthesised within the host (Ulmer *et al.*, 1993; Wolff *et al.*, 1990). No adverse effects of DNA inoculation have been reported as yet, and compared with live vaccines, no infectious agent is set free in the body. The antigen is produced within the cells of the host and the resulting *in situ* production of the protein can involve biosynthetic processing and post-translational modifications. Therefore, using this method of vaccination, may offer the advantage of live virus vaccines while providing the safety of subunit vaccines. Cellular immune responses induced by DNA vaccines against conserved internal proteins of a virus together with antibodies to viral surface proteins have the potential to provide protection against antigenic variants that differ in their surface proteins (Donnelly *et al.*, 1995). This was first demonstrated for influenza DNA vaccines (Ulmer *et al.*, 1993), in which the current vaccines are only effective in a strain-specific manner. The use of pure plasmid DNA offers additional advantages for the purpose of immunisation. These include ease and speed of production, easier quality control and non-integration of the DNA (Wolff *et al.*, 1990; Danko and Wolff, 1994). At present vaccination with naked DNA is limited to intramuscular, cutaneous and intradermal routes of immunisation (Torres *et al.*, 1997; Condon *et al.*, 1996). The mucosal routes of immunisation (gastrointestinal, nasal, pulmonary and vaginal routes) are

more desirable (Ruedl and Wolf, 1995; McGhee *et al.*, 1994), since most pathogens enter host cells through these routes, and induction of mucosal immunity offers the most effective line of defence at the port of entry (Elson, 1997). Since naked DNA is ineffective in crossing mucosal barriers, and it is rapidly degraded by nucleases, delivery systems that protect plasmid DNA and target it to APCs seem essential for the successful design of plasmid DNA-based mucosal vaccines. Particulate carrier systems such as microspheres and liposomes offer promising prospects for the effective delivery of plasmid DNA by mucosal routes (Klavinskis *et al.*, 1997; Mathiowitz *et al.*, 1997; Alpar *et al.*, 1997). The molecular form of the plasmid DNA has been reported to effect the efficiency with which it will transform or transfect cells. Most reports indicate that linear plasmid DNA is much less efficient in transfection or transformation protocols than open circular or super-coiled plasmid DNA, and that there is little difference between the super-coiled and open circle forms (Kimoto and Taketo, 1996; Xie *et al.*, 1992). The integrity of the encapsulated plasmid DNA will consequently have a significant effect on the *in vivo* activity of the microparticles. Since our NIBC/PDLA formulations were prepared using extremely mild manufacturing conditions, and offered strong and sustained immune response following mucosal (intranasal) delivery we set out to investigate their suitability for the encapsulation of model plasmid DNA as novel delivery systems. The model plasmid DNA used in our studies was one encoding for the Hepatitis B virus, pRc/CMV-HBs(S). The following section gives a brief description of the structure and nature of the Hepatitis B virus infection.

8.1.1. Hepatitis B infection

Hepatitis B virus (HBV) remains an important world wide health problem and the availability of a safe and effective vaccine is essential for the control of infection and disease. This is particularly true for areas of the world where HBV is endemic, such as

sub-Saharan Africa and much of Asia. The majority of individuals infected as adults will recover completely and are thereafter protected against subsequent infection. However, about 5-10% of the infected individuals become chronic carriers. It is estimated, that there are over 250 million chronic carriers of HBV in the world today and although, some of them are healthy carriers without liver pathology, approximately 70% remain in a state of chronic hepatitis and are particularly susceptible to developing cirrhosis and hepatocellular carcinoma later in life. (Tiollais and Buendia, 1991; Hadler and Margolis, 1993).

8.1.2. The structure of Hepatitis B virus

The HBV genome contains only four genes: *S, C, P and X* which encode surface (HBs), core (HBc, nucleocapsid), pol and X proteins respectively. The surface and core proteins are structural whereas *pol* serves various functions, which are associated with transcription. The function of the X protein is not clear and although it appears to be non-essential, it may have transcription-activating properties and as such increase the viability of the virus in its host (Ganem and Varmus, 1987; Gerlich and Bruss, 1993). The structural gene coding for the envelope protein (HBs) is a single large open reading frame containing three in-frame ATG start codons. Thus the gene is divided into three domains designated pre-S1, pre-S2 and S (from 5' to 3'), and a single stop codon. The different sized polypeptides produced are known as small or major (S), middle (M= pre-S2 +S) and large (L=pre-S1 + pre-S2 +S) and since each can exist in glycosylated or unglycosylated forms, there are a total of six different forms of envelope protein.

8.2. Materials and Methods

8.2.1. Polymers

Poly (dl-lactide124kDa, Resomer 206) was purchased from Boehringer Ingelheim KG (Ingelheim, Germany). PLGA (50:50, 57kDa) was purchased from Medisorb Products,

(CA, USA). Polyvinyl alcohol (PVA, Ma 13-23000) was obtained from Aldrich chemicals (Gillingham, UK).

8.2.2. Plasmid DNA (pDNA)

The plasmid pRc/CMV-HBs(S) (2.7 mg/ml in PBS), encoding the S (small) region of hepatitis B surface antigen was obtained from Aldevron (Fargo, ND, USA).

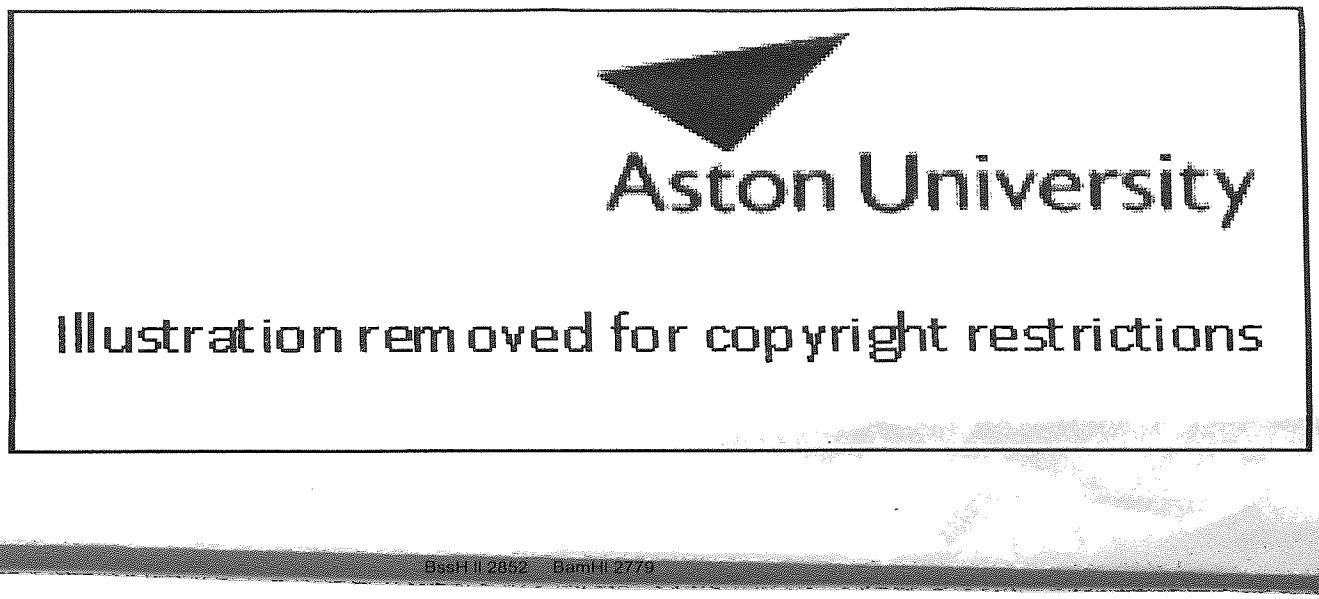


Figure 8.1. Plasmid map of HBsAg-expressing plasmid, derived from pRc/CMV (Aldevron, Fargo, ND, USA)

8.2.3. Chemicals

All chemicals and reagents were supplied by BDH Chemicals Ltd (Poole, Dorset, UK), Sigma Chemical Co (Poole, Dorset, UK) and Fisons (Loughborough, Leis, UK) unless otherwise stated and were of Analar grade or equivalent. Double distilled water was used in all experimental procedures unless otherwise stated.

8.3. Methods

8.3.1. Microsphere preparation of PDLA/ block copolymer microspheres encapsulating plasmid DNA

Microspheres were prepared using L101 as a stabiliser and a surfactant. The polymer poly (dl-lactide, MW 124 kDa, Boehringer Ingelheim KG, Germany) was used in these preparations. The polymer (10.86 mg ml^{-1}), L101 (2.0 mg ml^{-1}) and plasmid pRc/CMV-HBs(S) (331 μg) were dissolved in and dispersed in the acetone (20 ml). The acetone was then added to the aqueous solution (0.75% w/v PVA, 40 ml), at a low speed stirring (100 rpm) using a magnetic stirrer at room temperature. The acetone was allowed to evaporate overnight by gentle stirring. The particles were then, collected by ultracentrifugation at 30,000 rpm for 25 minutes and lyophilised. All formulations were prepared in triplicate.

8.3.2. Preparation of PLGA 50:50 microspheres encapsulating plasmid DNA

Microspheres were prepared using a (w/o/w) double-emulsion solvent evaporation method. The internal phase consisted of 1.5 ml of aqueous solution containing 331 μg of plasmid pRc/CMV-HBs(S) and PVA (Mw, 13-23000, 88% hydrolysed, 2.5% w/v). The secondary phase included 5 ml of dichloromethane containing 250 mg of PLGA (50:50; 57kDa, Medisorb Products, USA). Both phases were emulsified using the small probe Silverson homogeniser for 2 minutes at full speed. The primary emulsion formed was added drop-wise to the external phase (75 ml aqueous solution of 5% w/v PVA). The secondary emulsion was homogenised for 6 minutes with a large Silverson probe at maximum speed (~ 16,000 rpm). The final emulsion was washed three times with the aid of ultracentrifugation at 30,000 rpm for 25 minutes. The microparticles were then freeze-dried (VIRTIS ADVANTAGE, Biopharma process system, Winchester, UK) at a shelf temperature of –20 °C for 48 hours. All formulations were prepared in triplicates.

8.3.3. Determination of plasmid DNA in microspheres

Aliquots of microspheres (10-15 mg) were vortexed with 200 mM NaOH (1ml) and incubated at 120 °C for 10 minutes. The aqueous phase was recovered by centrifuging at 10,000 rpm for 10 minutes in a microcentrifuge and the plasmid DNA concentration determined by UV absorption at 260 nm blanked against 200 mM NaOH. Breifly, DNA solution (500 µl) was poured into a quartz cuvette (700 µl capacity) and placed in the spectrophotometer. The absorption at 240, 260 and 280 nm were measured. Samples were diluted where necessary to meet the recommended absorption of between 0.3 and 0.7 (Manchester KL, 1995). The concentration of DNA solutions were then calculated by multiplying the absorption at 260 nm by 50, and then by any dilution factors (when appropriate). The ratio of the absorption at 260 nm and 280 nm is indicative of the purity of the DNA sample, a value of between 1.8 to 2.0 being favourable (Manchester KL, 1995).

8.3.4. Particle size analysis by laser diffraction

See section 2.2.2.

8.3.5. Agarose gel electrophoresis

The pDNA samples were recovered from the microparticles by adding dichloromethane (1ml) and extracting into PBS solution (2x 300 µl and 1x400 µl) were analysed using a submarine gel electrophoresis system (Bio-RAD, UK). Samples of control and recovered pDNA (10 µl) were applied to a 0.8% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2mM EDTA, pH 8.0). Adequate separation of the bands for supercoiled, open circular and linear pDNA was obtained by running the gel for 1 hour at 100 V, 400 mA, and visualised under UV illumination after ethidium bromide staining.

8.4. Results and Discussion

The physical characteristics of microspheres encapsulating the pDNA is summarised in the following table (8.1).

Table 8.1. Physical characteristics of microspheres encapsulating pDNA. *= NIBC/Block copolymers, **= Double emulsion solvent evaporation method, n=3.

Formulation	Number mean (μm±SD)	Volume mean (μm±SD)	pDNA incorporation (μg/mg)	Entrapment efficiency	Yield (%)
*MS/HepB	0.59 (±0.01)	3.83 (±0.14)	0.36	27.7	62.3
**MS/HepB	1.22 (±0.11)	8.54 (±0.04)	0.24	18.2	57.8

The incorporation of pDNA was greater in the microspheres prepared with emulsification-diffusion method compared with the double-emulsion solvent evaporation method. The former preparations also seemed to produce slightly greater yield. These differences could be due to the use of acetone in the NIBC/block copolymer preparations, which is a milder organic solvent in comparison with dichloromethane. The particle diameters differed, with smaller particles being produced by the emulsification-diffusion method. This method is often used to formulate small particles (nanometers to micron range) and was expected to result in smaller microspheres in this study. The particle size distribution obtained by both methods (3-8 μm) is suitable for the delivery of the encapsulated DNA by all delivery routes including oral route, which requires microparticles of less than 10 μm in size to be taken up by Peyer's patches of the gut (Eldridge *et al.*, 1990). The integrity of pDNA was investigated using agarose gel electrophoresis. As evident in figure 8.2. the emulsification-diffusion method used to prepare the NIBC/Block copolymer (L101) resulted in the preservation of integrity and also in the recovery of larger amount of pDNA (Gel2). The w/o/w double emulsion solvent evaporation method resulted in lesser amount of the pDNA

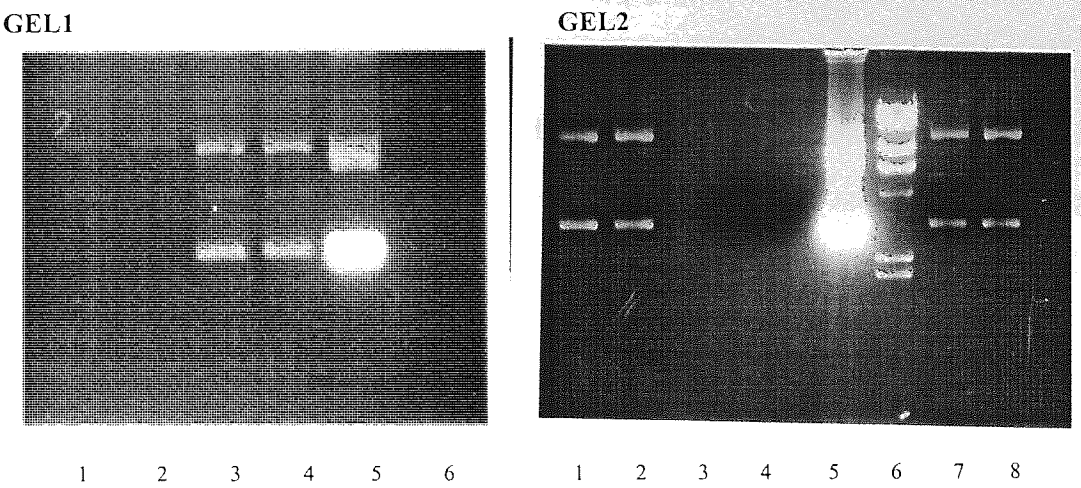


Figure 8.2. Agarose gel of pDNA, pRc/CMV-HBs(S) recovered from different preparations of microspheres prepared using emulsification-diffusion and double emulsion solvent evaporation methods. Gel1: Lanes 1,2 contain pDNA extracted from double-emulsion solvent evaporation method. Lanes 3 and 4 contain pDNA extracted from NIBC/Block copolymers prepared by emulsification-diffusion method. Lane 5, contains the control pDNA plasmid. Gel2: Lanes 1,2,7, and 8 contain pDNA ectracted from NIBC/Block copolymers prepared by emulsification-diffusion method. Lane 5, contains the contol pDNA plasmid. Lane 6, contains λ/HINDIII ladder. Lanes 3, and 4 contain pDNA extracted from double-emulsion solvent evaporation method.

recovery, but the faint bands present in gel 1, indicate that DNA integrity might have been preserved in microspheres prepared by this method. The molecular form of the pDNA has been reported to effect the efficiency with which the pDNA will transform or transfect cells. The reported literature indicates that linear pDNA is much less efficient in transfection or transformation protocols than the open circle or supercoiled pDNA (the lower band in Fig. 8.2), and that there is little difference between the supercoiled and open circle forms (the upper band in Fig. 8.2.) (Kimoto and Taketo, 1996, Xie *et al.*, 1992). Therefore, the integrity of the encapsulated pDNA has a significant effect on the *in vivo* activity of the microparticles.

9.0. Summary and Conclusions

The concept of controlled release for the delivery of antigens using biodegradable microspheres as vaccine carriers has attracted intensive research activities over the past decade (Aquado and Lambert, 1992). The focus of these research efforts has been on the development of single-dose delivery vehicles, which are easily manufactured and easy to use with emphasis on reducing the number of antigen doses needed to induce long-lasting protection. This study was set out to develop microsphere type drug vaccine delivery systems composed of biodegradable polymers with the above mentioned objectives in mind. Namely there was focus on the manufacture of microspheres which would act as a controlled release system for the entrapped antigens which were easy to manufacture. This was established by using non-ionic block copolymers (L101 and L121) to serve as both adjuvants and stabilisers in a preparation method which was simple, quick and had the least detrimental effects on the entrapped antigens as compared with the most conventional methods of microencapsulation (chapter 2). Microspheres were prepared using the emulsification-diffusion technique, which involves the formation of a conventional oil-in-water emulsion between a partially water-miscible solvent containing the polymer and the drug, and an aqueous phase containing a stabiliser. The subsequent addition of water to the system causes the solvent to diffuse into the external phase, resulting in the formation of microparticles (Fessi *et al.*, 1989). The microsphere preparations were first evaluated using egg white lysozyme, OVA and finally DT. In order to create the optimal conditions for microencapsulation of the above antigens, different formulations were prepared by varying the ratios of the polymer to the NIBC component, the organic phase to the aqueous phase, varying the aqueous phase components and by the inclusion of additional excipients such as Tween 80. The results of these initial investigations provided the required data

needed to optimise the NIBC microspheres and make them suitable for *in vivo* studies. The optimised formulations included those, which contained low concentrations of PVA (0.75 % w/v) in the external aqueous phase, aqueous phase to organic phase ratio of 1: 0.5 and the inclusion of L101 as the stabilising/ adjuvant component. The above formulations were then evaluated using different proteins. OVA and DT were used to compare the relevance of the entrapped protein to both the physical characteristics and the nature and magnitude of the immune response produced *in vivo*. The comparison between the physical characteristics of the above formulations showed no significant difference in terms of entrapment efficiency, size, and yield. One of the major problems encountered in optimising the NIBC/PDLA microspheres was that due to the aggregation of the particles which is an inherent problem of the emulsification-diffusion technique. The most successful approach which was adapted to overcome this problem was the suspension of particles in PVA (0.75% w/v) prior to freeze-drying. This approach produced fine, free flowing particles, which were easily dispersed in PBS. These formulations were then administered *via* intramuscular and intranasal routes to BALB/c mice in order to evaluate their immune response. The immune response for the above formulations were examined and compared for two model antigens (OVA and DT) up to day 75 after administration of one-single dose. Both routes of delivery (i.m. and i.n.) induced high serum IgG titres, which were significantly higher than the free antigens in solution. The immune response to different formulations encapsulating DT was then followed for 286 days after administration of one single dose. These formulations compared the effects of polymer molecular weight, the surface coating of particles with PVA (0.75%), and the role of the NIBCs on the quality of the immune response (i.e. Th1 Vs Th2). Formulations containing the NIBCs, all resulted in significantly higher immune response at early time points (i.e days 7 and 14) and sustained this high response up to day 286. The role of the NIBC

seemed more prominent at the early stages of the immunisation as with later time points all formulations including those without the NIBCs had resulted in significantly higher production of specific serum IgG than that of those produced by the free antigen. The surface coating of the polymers with PVA also affected the immune response at the early stages (days 7 and 14 post-immunisation), presenting the highest IgG responses amongst all the formulations. The IgG subfractions were also investigated as an indirect measure for the involvement of Th1 or Th2 immune response. The results are in agreement with published data suggested that low molecular weight NIBCs, such as L101 used in formulations, results mainly in the induction of IgG1 compared with IgG2a and IgG2b. In the same chapter (chapter 3), the effect of combination dosing of NIBC/PDLA formulations with Quil A (efficient stimulant of CMI i.e. a prominent Th1 stimulation), was evaluated in order to manipulate the type of immune response induced by microsphere preparations. The combination dosing resulted in a significant shift towards production of IgG2a compared with other sub-types. The splenic cell proliferation was also investigated caused by one single four months after the initial immunisation. It was found that microsphere preparations resulted in splenic cell proliferative responses administered both *via* i.m. and i.n. routes, but with the latter route inducing a greater effect. Since the stability of the entrapped protein is of uttermost importance in designing new vaccine delivery systems, the effect of long-term dry storage on our preparations was also investigated. The stability aspects of DT were examined by i) comparing the immune response of the stored samples to that of the freshly prepared PDLA particles and ii) by structure analysis using SDS-PAGE. The intranasal administration of the stored particles (6-month storage) resulted in comparable and in some cases higher than the intramuscular administration of newly prepared microspheres. The SDS-PAGE analysis of the entrapped DT also indicated that the structural integrity of the protein was maintained. The results

therefore, indicate that NIBC/PDLA formulations are effective delivery systems for development of novel vaccines for clinically relevant proteins such as DT. These formulations used easy and mild manufacturing conditions, which could be easily scaled up and applied in an industrial setting. The preservation of the DT within these formulations will ensure the reproducibility of immunisation responses, which is necessary for their suitability for clinical trials. The initial successful *in vivo* studies could pave the way for larger *in vivo* studies leading to clinical trials of such preparations. In chapter five the encapsulation of alum within biodegradable polymers of different molecular weights (PLLA 2kDa and 100 kDa, PLGA 50:50 57 kDa and 14 kDa, 75:25 113.2 kDa) was investigated. In designing experiments, efforts were made to overcome the shortcomings of alum in its current form of application, and thus prepared microspheres which encapsulated both the alum and the desired antigen, TT. Three types of formulations were prepared using, the commercially available TT vaccines, in-house alum-adsorbed TT vaccines, and new mixtures of alum and TT without prior incubations. All these preparations formed microspheres with smooth surfaces encapsulating the TT associated with alum. In direct mixing of alum and TT, it was envisaged that the alum might serve as both an adjuvant and a stabilising excipient for the entrapped TT. The release characteristics for these formulations were typical of a tri-phasic *in vitro* release for PLLA and PLGA microspheres. The protein release was as high as 84% for PLGA 75:25 on day 30 indicating the continuous and sustained release of the entrapped antigen. In chapter six, the immune response to the above formulations was evaluated and was found that following encapsulation of alum-adsorbed TT within biodegradable microspheres, these particles induced immune responses comparable with the commercial alum-adsorbed vaccine. One of the main objectives in designing the microspheres encapsulating a mixture of alum and TT, was to ensure that much lower amounts of alum is used in these

preparations compared with the amount present within the commercial vaccine (5-10 times less alum). These formulations were also effective inducers of serum IgG after one single administration and resulted in high titres comparable with the commercial TT vaccine. The splenic cell proliferation in response to the above preparations at 4 months, 11 months and 14 months following a single injection of TT formulations was also examined. All formulations were seen to cause proliferation at these time points with TT encapsulated within higher molecular weight polymer (PLLA 100kDa), inducing the highest indices at 11 months and 14 months post-immunisation. In chapter seven the stability of the entrapped antigen within microspheres stored at dry storage conditions at two temperatures (4 °C and room temperature) was examined using HPLC analysis. The effectiveness of HSA (5%) and trehalose (15%) on the antigen stability within the microspheres was also investigated. The storage of these formulations over a nine month time (dry storage at RT) period did not result in the loss of structural integrity as evidenced in the chromatograms of the extracted TT from alum-TT loaded microspheres. The stability of the antigen was only observed in formulations containing 5% HSA kept at room temperature. Overall, the present work on the encapsulation of alum-TT shows the potential of such delivery system as an alternative improvement on the existing vaccines, when lower amounts of alum are used to produce high and sustained immune response over a long period of time (up to 14 months). One of the main objectives which was met within this work, has been the development of such delivery systems containing alum which can be kept as a dry powder and which can be used a long time after its initial preparation. The potential of these formulations in combination dosing with other established and novel adjuvants is an exciting area of research which can be focused on in continuing these studies. Finally in the last chapter, the suitability of NIBC/PDLA microspheres as means of plasmid DNA delivery was evaluated using Hepatitis B plasmid as a model. These formulations resulted

in a good entrapment efficiency of the plasmid and a high yield of particles. On investigation of the stability of the plasmid DNA, it was observed that these preparations maintained the structural integrity of the plasmid compared with particles prepared using a w/o/w double emulsion solvent evaporation method. In conclusion, this report presents the effectiveness of NIBCs as effective adjuvants and point towards new avenues of incorporating well-established adjuvants such as alum in vaccine delivery systems.

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