THE DELIVERY OF BIOACTIVE PROTEINS USING BIODEGRADABLE MICROSPHERES

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SUMMARY

The development of controlled release vaccines is an important area of research, with the emergence of a large number of subunit antigens and synthetic peptides which are generally weakly immunogenic. An ideal vaccine should deliver the antigen in such a way that a long-lasting boosting effect is achieved with a single administration. Recent publications have investigated the adjuvant properties of biodegradable microspheres when administered by parenteral routes. Another exciting area is the development of these antigen carriers for administration by non-parenteral routes for the generation of a mucosal immune response. In this project, antigen-containing microspheres were produced using a range of biodegradable polymers by single and double emulsion solvent evaporation and spray drying techniques. The proteins used in this study were mainly BSA, tetanus toxoid, F1 and V *Y. pestis* subunit vaccines and the cytokine, interferon-gamma. The polymer chosen for use in the vaccine preparation will directly determine the characteristics of the formulation. Full *in vitro* analysis of the preparations was carried out, including surface hydrophobicity and drug release profiles. The influence of the surfactants employed on microsphere surface hydrophobicity was demonstrated. Preparations produced with polyhydroxybutyrate and poly(DTH carbonate) polymers were also shown to be more hydrophobic than PLA microspheres, which may enhance particle uptake by antigen presenting cells and Peyer's patches. Systemic immunisation with microspheres with a range of properties showed differences in the time course and extent of the immune response generated, which would allow optimisation of the dosing schedule to provide maximal response in a single dose preparation. Both systemic and mucosal responses were induced following oral delivery of microencapsulated tetanus toxoid indicating that the encapsulation of the antigen into a microsphere preparation provides protection in the gut and allows targeting of the mucosal-associated lymphoid tissue. Co-encapsulation of adjuvants for further enhancement of immune response was also carried out and the effect on loading and release pattern assessed. Co-encapsulated F1 and interferon-gamma was administered i.p. and the immune responses compared with singly encapsulated and free subunit antigen.

Key words Biodegradable microspheres; vaccines; protein; controlled release; mucosal delivery

TO MY FAMILY

In memory of my father

Patrick J. Conway

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ABBREVIATIONS

ABTS	(2,2' azino-bis)3-ethylbenzthia-zoline-6-sulphonic acid
AMPS	ammonium persulphate
APC	antigen presenting cell
BALT	bronchial-associated lymphoid tissue
BCA	bicinchoninic acid
BCG	bacille Calmette-Guérin
BSA	bovine serum albumin
CMIS	common mucosal immune system
CT	cholera toxin
CTB	cholera toxin B subunit
CTL	cytotoxic T-lymphocyte
CTTH	*N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine hexyl ester
DCM	dichloromethane
d/e	double emulsion solvent evaporation technique
DMEM	Dulbecco's Modification of Eagle's Medium
DSC	differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
EthAc	ethylacetate
F1	fraction 1 antigen
FCA	Freund's complete adjuvant
FDA	Federal Drug Administration
FIA	Freund's incomplete adjuvant
GA	glycolic acid
GALT	gut-associated lymphoid tissue
GI	gastro-intestinal
GPC	gel permeation chromatography
HBSA	Hepatitis B surface antigen
HIC	hydrophobic interaction chromatography
HIV	human immunovirus
HSA	human serum albumin
HV	hydroxyvalerate
IEF	isoelectric focusing
IEL	intra-epithelial lymphocytes
IFN	interferon
I-IFN-γ	3-[^{125}I] iodotyrosyl)γ-interferon

IFN-γ	interferon-gamma
i.g.	intra-gastric
i.m.	intramuscular
i.p.	intra-peritoneal
i.n.	intranasal
ISCOMS	immune stimulating complexes
LA	lactic acid
LHRH	luteinisinig hormone-releasing hormone
LP	lamina propria
LPS	lipopolysaccharide
M cells	microfold cells
MALT	mucosal-associated lymphoid tissue
MC	methylcellulose
MDP	N-acetyl-muramyl-L-alanyl-D-isoglutamine
MHC	major histocompatibility complex
Mn	number average molecular weight
Mw	molecular weight
NALT	nasal-associated lymphoid tissue
o/w	oil-in-water
OVA	ovalbumin
PBS	phosphate-buffered saline
PBST	0.05% v/v Tween 20 in PBS
PDLA	poly(DL)lactide
PDTHC	poly (deamino-tyr-tyr carbonate ester)
PGA	polyglycolic acid
PHB	polyhydroxybutyrate
PHBV	polyhydroxybutyrate-co-valerate
PLA	polylactide
PLGA	polylactide-co-glycolide
PLLA	poly(L)lactide
PMSF	phenylmethylsulfonylfluoride
PPs	Peyer's patches
PVA	polyvinyl alcohol
PVP	polyvinylpyrrolidone
RES	reticuloendothelial system
S-IgA	secretory IgA
s.c.	subcutaneous
s.d.	standard deviation of the mean

s.e.	standard error of the mean
SC	secretory component
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
s/e	single emulsion solvent evaporation technique
SEB	staphylococcal enterotoxoid B
SEM	scanning electron microscopy
TB	tuberculosis
T_c	crystallisation exotherm
TEMED	N,N,N,'N'- Tetramethylethylene diamine
T_g	glass transition temperature
T_m	crystalline melting temperature
Tris.	tris(hydroxymethyl)-aminomethane
TT	tetanus toxoid
W.H.O.	World Health Organisation
w/o/w	water-in-oil-in-water

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1.0 INTRODUCTION

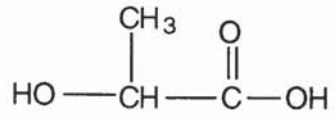
1.1 MICROENCAPSULATION USING POLYLACTIDE POLYMERS

1.1.1 Biodegradable lactide/glycolide polymers

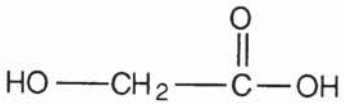
When related to biomedical devices, the term biodegradable polymers is assigned to those which break down in the human body. They may be synthetic or natural polymers and may degrade either enzymatically or non-enzymatically *in vivo* to produce biocompatible or non-toxic by-products which can be further metabolised or excreted *via* normal physiological pathways (Jalil & Nixon, 1990a). The application of natural polymers such as albumin and gelatin is more restricted due to uncertain purity and immunogenicity. Polymers can degrade either by homogeneous degradation, with random cleavage of the polymer chains in the polymer matrix, or by heterogeneous degradation, where the degradation is restricted to the surface of the matrix (Vert *et al.*, 1994). Polyesters (of lactic and glycolic acid) are the most widely studied synthetic polymers for drug delivery and degrade by random hydrolysis of ester bonds producing organic acids. In 1979, Beck *et al.* proposed the first long-acting injectable polylactide (PLA) microcapsule system for the controlled release of progesterone. Polylactides (PLAs) have been used for over 20 years for resorbable sutures and drug delivery systems and have therefore been extensively studied. Commercialisation can be seen in the development of depot-forms of luteinising hormone-releasing hormone (LHRH) agonists using biodegradable polylactide-co-glycolide (PLGA) polymers for the treatment of advanced prostatic cancer and endometriosis. These PLGA formulations have been approved for use by the FDA, e.g. leuprolide acetate- Lupron Depot®; goserelin acetate- Zoladex®; triptorelin- Dé-Capeptyl® (Cleland, 1995).

1.1.2 Synthesis of lactide/glycolide polymers

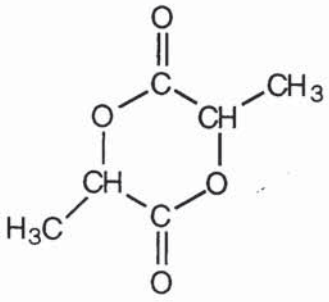
Low molecular weight PLAs and PLGAs (Mw $\leq$ 3000) can be formed by the direct condensation of lactic or glycolic acid with antimony trioxide (Gilding & Reed, 1979), (figure 1.1). The preferred method for production of high molecular polymers is the



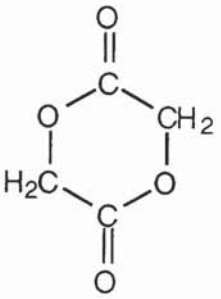
D(-)- or L(+)-LACTIC ACID



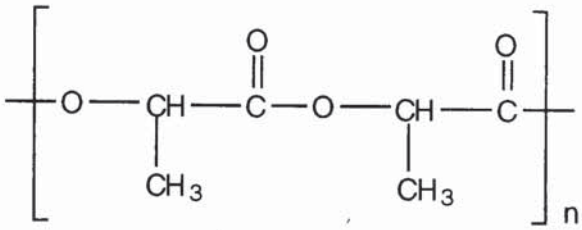
GLYCOLIC ACID



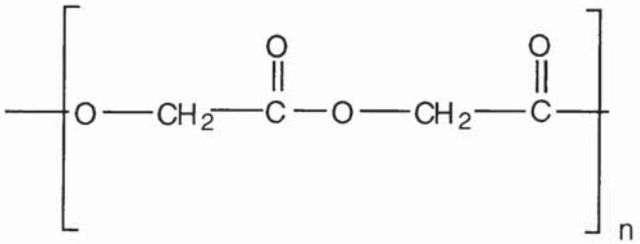
D(+)- or L(-)-LACTIDE



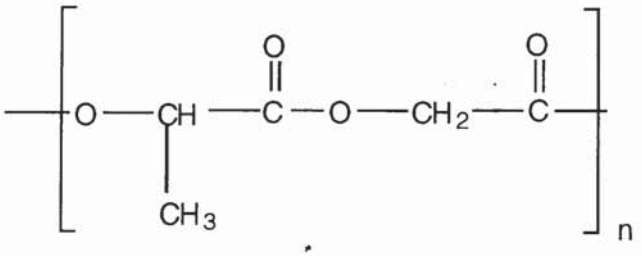
GLYCOLIDE



L- or DL-POLYLACTIDE



POLYGLYCOLIDE



POLY(LACTIDE-CO-GLYCOLIDE)

Figure 1.1. Structures of polylactides and related compounds

ring opening polymerisation of the cyclic diesters (lactides and glycolides) using antimony, lead or tin catalysts (figure 1.1). The molecular weight of the polymers can be controlled by the choice of adequate polymerisation conditions. PLA can be synthesised from either optically active D- and L-lactic acid or the optically inactive racemic mixture DL-lactic acid. Poly(L)lactide (PLLA) is a semi-crystalline polymer (with up to 80% crystallinity) but the introduction of a non-stereospecific methyl group as in poly(DL)lactide (PDLA) eliminates this crystallinity probably due to the random arrangement of the asymmetric centre. The co-polymer with glycolic acid can involve various molar ratios of monomer components and this controls the degree of crystallinity. PLGA co-polymers containing less than 70% of the glycolide are amorphous (Gilding & Reed, 1979).

1.1.3 Degradation of polylactides

Degradation of this group of polymers is dependant on molecular weight, polydispersity, geometry, polymer composition and polymer structure. Degradation ultimately leads to enhanced water uptake and the generation of porosity (Holland *et al.*, 1989). High molecular weight polymers degrade to lower molecular weights, as measured by viscosity changes, but retain their water-insolubility (Hutchinson & Furr, 1990). Lower molecular weight polymers degrade with immediate weight loss (figure 1.2). These results are consistent with the degradation of these polymers *in vivo*, suggesting that even in subcutaneous tissue, enzyme-mediated degradation is less significant then simple hydrolysis. It is generally accepted that chain scission occurs through simple hydrolysis with no contribution of enzymes (Vert *et al.*, 1994). Random hydrolytic scission of ester linkages occurs, eventually yielding lactic and glycolic acid which, in humans, are generally processed through normal metabolic pathways and ultimately eliminated from the body *via* the respiratory system as carbon dioxide (Makino *et al.*, 1985). Lactic acid is a natural metabolic product of all higher animals and glycolic acid is also a metabolic product of some amino acids.

For low molecular weight polymers, the polymer chains should break into oligomers too short to be detected by conventional techniques or short enough to be immediately solubilised leading to immediate weight loss (figure 1.2). Soluble oligomers which are close to the surface of the particles, can leach out before total degradation occurs. Those which are entrapped within the matrix remain and contribute to the autocatalytic degradation. Release of these oligomers depends on the rate of ester bond cleavage and the diffusion rate of the species involved which is controlled by molecular mass, degree of matrix swelling, macromolecular confirmation and rigidity (Vert *et al.*, 1994).

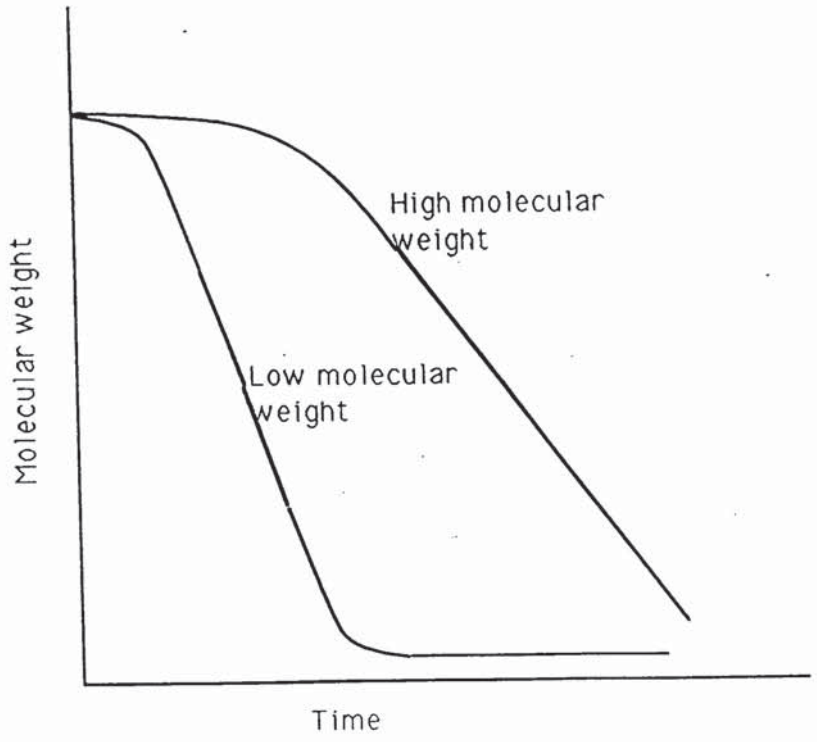


Figure 1.2. Schematic representation of the decrease in polymer molecular weight for high and low molecular weight polylactides as a function of time

Polymers with a high lactide content are more stable to hydrolytic attack than those with intermediate ratios (Holland *et al.*, 1986). The introduction of chemically different (glycolide), or configurationally different (DL-lactide) residues into the polymer chains of the semi-crystalline PLLA, rapidly decreases the ability to crystallise leading to intrinsical amorphousness (Kissel *et al.*, 1991). Thus, PLLA is degraded more slowly than the amorphous PDLA, the crystalline regions being less accessible to water. The degradation of such semi-crystalline polymers proceeds in two stages, firstly in the amorphous regions and then in crystalline ones (Vert *et al.*, 1991). The mechanism of PLLA degradation involves the hydrolysis of the polyester bond with a rate dependent on the polymer crystallinity, the amount of lactic acid released during PLLA degradation is very small but increases as low molecular weight oligomers are formed. Polyglycolic acid (PGA), is the most hydrophilic of the group owing to the lack of pendant methyl groups. Although a biodegradable polymér, it is of limited use due to its poor solubility in common organic solvents. It has been used by Lee *et al.* (1991) for production of porous microspheres containing a polypeptide, salmon calcitonin.

The extra methyl group in polylactide makes it more hydrophobic than polyglycolide and the reduction in polymer hydrophobicity when the lactide content is reduced from

100% to 80% results in an increased hydrolysis rate (Floy *et al.*, 1993) (figure 1.3). Most hydrophobic and crystalline polymers exhibit a relatively slow degradation rate due to the low hydration degree in the microspheres, related to the water accessibility to the hydrolytically unstable ester linkages in the polymer backbone (Park, 1994). The 50:50 co-polymer of DL-lactide and glycolide degrades most rapidly as it is the least likely to possess crystalline blocks of either of the monomers. The glycolide units, being more hydrophilic than the lactide units, are thought to promote water uptake into the polymer, thereby encouraging hydrolytic degradation (O'Hagan *et al.*, 1994). For an amorphous polymer, water uptake is governed by the hydrophilicity of the repeat units and by end group effects. As the molecular weight falls, the end group numbers increase and the essentially hydrophobic polymer becomes more hydrophilic. High molecular weight polymers therefore exhibit two phases of water uptake separated by a lag period where little water uptake is observed. For low molecular weight polymers, the processes are practically continuous as the molecular weight of the polymer decreases.

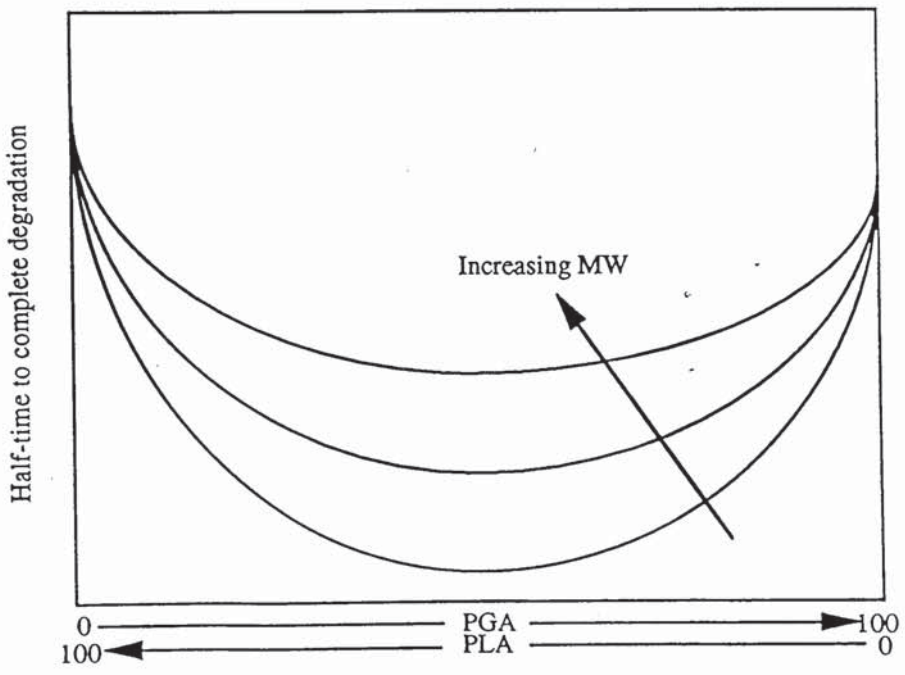


Figure 1.3. Schematic representation of degradation profile for polylactides (adapted from Miller *et al.*, 1977)

Degradation not only depends on the molecular arrangement of the chiral and achiral units, and the release of the carboxyl-terminated oligomers depends on their solubility in the surrounding medium and thus on its pH, ionic strength, temperature and

buffering capacity. Degradation of PLA is enhanced in alkaline pH and in solutions of high ionic strength (Makino *et al.*, 1986). Sah & Chien (1993) observed that the internal structure of the microsphere was degraded faster than the surface, confirming the autocatalytic effect of microsphere degradation suggested by Spenlehauer *et al.* (1989) and Pitt *et al.* (1981). Due to an inability to diffuse out of the microsphere, the acidic fragments of the polymers of the polyester backbone may become trapped, causing an acceleration in the autocatalytic process inside the particles while the surface is diluted by the surrounding fluid.

The degradation is also dependant on monomer sequencing and cross-linking with the polymer backbone and the time of solubilisation of the matrix is influenced by the surface area available, water penetration and the matrix porosity. Degradation of the polymer may also be affected by ultrasound, light, magnetism, gamma-radiation and other external modalities (Morris *et al.*, 1994). γ-irradiation (normal sterilisation dose of 25kGy) causes scission of the polymer chains which is more pronounced in amorphous than crystalline regions (Horacek & Kudlacek, 1993). Degradation can also be accelerated in plasma, due to the adsorption of proteins, increasing solubility of PLA (Makino *et al.*, 1987). Thus, bulk degradation is too general a term to describe polyester hydrolysis and the degradability of polymers is dependant on both the chemical and configurational structure, residual compounds and adsorbed substances. It is also affected by crystallinity, water absorption, ion diffusion and ionic strength or pH of the receiving buffer.

1.1.4 Release of macromolecules from polylactide microspheres

The release of peptides from PLGA was described by Sanders *et al.* (1984 and 1986) as a three stage process (figure 1.4). The lag period (B) after release of drug from superficial regions occurred as the polymer was hydrolysed *in situ* to lower molecular weight polymers which were still water-insoluble. However, when a low molecular polymer is used, the lag phase is reduced leading eventually to a virtual overlap of the two phases of release and a steady release (C) can be achieved. Thus, water soluble macromolecules can be released by diffusion through the aqueous pores generated in the polymer matrix leading to a continuous release profile.

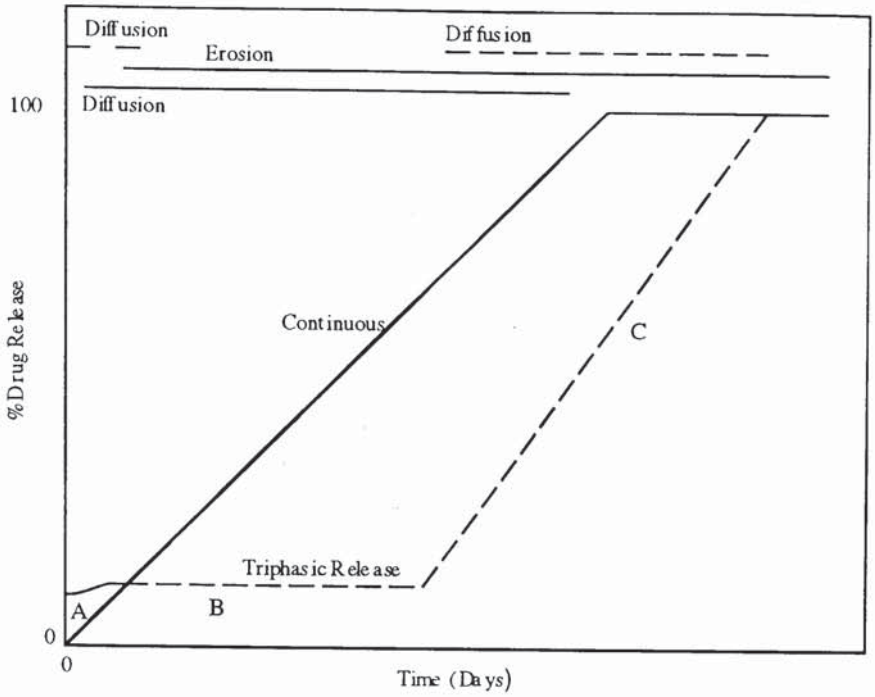


Figure 1.4. Schematic representation of two types of release pattern of drug from PLA polymers (adapted from Cleland, 1995)

For both profiles, protein is initially released by diffusion. Protein which is contained at or near the surface of the particles diffuses into the surrounding media in a matter of minutes to hours after contact with an aqueous environment. This is known as the "burst effect". High molecular weight polymers usually display reduced burst effects on protein release (Morris *et al.*, 1994). Continued diffusion of the drug is dependant on microsphere porosity, the number of channels available for diffusion of the antigen determining the rate of release. In some cases, the initial diffusive state lasts only for a few hours followed by little or no protein release for several days. This lag period is controlled by the erosion of the polymer which may need to be extensive in some cases before the macromolecule can diffuse out of the particles. This triphasic release could be manipulated for formulation as a single immunisation vaccine (see section 1.2.1). If the microspheres are sufficiently porous to allow continuous diffusion, the diffusive stage can occur simultaneously with the erosion of the polymer, resulting in a zero order release.

The dissolution of the substrate in the release medium and/or the diffusional resistance afforded by the tortuous and porous structure of the polymer matrix are likely to be involved in the controlling of substance release. A hydrophilic protein cannot diffuse through the intact polymer matrix. However, as the drug is dissolved, it can diffuse out

of the matrix and new drug/matrix is thus exposed, the polymer is hydrolysed and the process of diffusion and erosion continues. Even large molecules such as tetanus toxoid (Mw 160kDa) may diffuse through the pores or channels formed by the degradation of low molecular weight PLLA to soluble degradation products (Alonso *et al.*, 1993).

Employing a double emulsion method of microsphere formation, may result in microparticles with different internal structures (Florence & Whitehill, 1982). Depending on the formulation and conditions used, particles with microcapsular (A), multi-vesicular (B) or microsphere matrix-type structures (C) may be produced. If the protein is distributed evenly throughout the polymeric matrix (figure 1.5C), the initial formation of pores at or near the surface of the spheres will facilitate the early release of protein from these domains. As degradation proceeds, the channels go deeper into the structure and more protein is released. The higher the loading of a hydrophilic material, the more readily channel formation proceeds and the faster the protein is released (Hora *et al.*, 1990a).

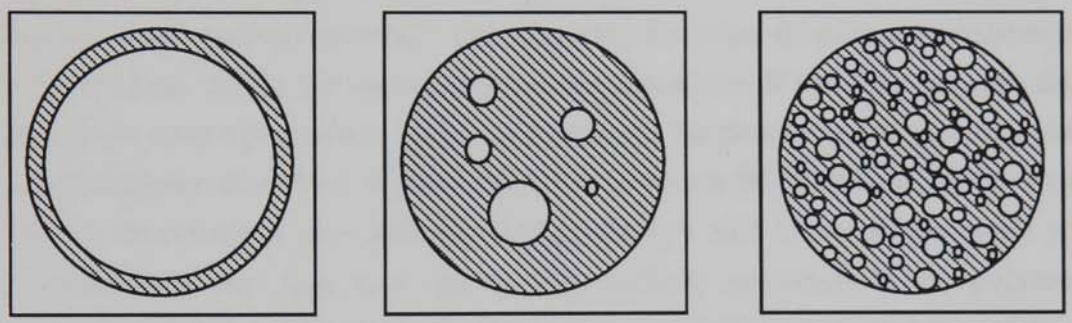


Figure 1.5. Diagram of the main types of internal morphology for microspheres formed by w/o/w double emulsions. A: microcapsule; B: multi-vesicular structure; C: microsphere matrix-like structure, adapted from Nihant *et al.* (1994)

1.1.5 Crystallinity and thermal behaviour of polylactide microspheres

Differential scanning calorimetry (DSC) is a well established technique used to study polymers and generally measures two finite properties - the glass transition temperature (T_g) and, depending on the degree of crystallinity, the crystalline melting temperature (T_m). The T_g value for a polymer indicates a transition from a rigid to a flexible structure causing a change in heat capacity and hence a shift in the baseline. Below this temperature, the polymer loses its flexible working behaviour. In addition to the

crystallinity already present in the polymer, further crystallisation may be induced in the sample on heating accompanied by release of heat visible as a crystallisation exotherm (T_c).

Polylactide appears with various degrees of crystallinity ranging from completely amorphous for the racemic form to a maximum of 37% crystallinity for PLLA (Cohn *et al.*, 1987). Co-polymerisation of lactic acid (LA) with glycolic acid (GA) at different compositions results in completely amorphous polymers between 22-66% w/w GA. Biodegradability and other properties of polymers, e.g. hydrophobicity, depend on their physical structure and it is important to control the amorphous/crystalline balance. Semi-crystalline polymers, such as PLLA, are characterised by both their glass transition temperatures and their melting points which increase with increasing polymer molecular weights (Jalil & Nixon, 1990a).

Under certain circumstances, thermal analyses may provide insight into the distribution of drug within the polymer matrix and the effects of the drug and other parameters on the gross morphology of the microparticles (Thies, 1992). Depending on the encapsulation method, generally the drug may be either dissolved or dispersed in the organic phase during the initial stage of the procedure. If the drug remains dispersed during the entire procedure, then it is physically suspended in the polymer matrix (a solid solution). However, if it is dissolved, there are more options; some drugs may end up dissolved in the polymer matrix though this is not likely with proteins. Alternatively, the drug may remain molecularly dispersed in the polymer with interactions between the drug molecules and polymer chains too weak to sustain a stable state (Dubernet, 1995). Stronger interactions between the individual drug molecules lead to diffusion through the polymer matrix. This process may be extremely slow, taking several years. In the third situation, the drug may crystallise during the microencapsulation procedure and remains physically dispersed in the polymer matrix in the form of a crystalline dispersion.

Due to plasticisation of the polymer in a solid solution, the T_g is decreased. In the molecular dispersion situation, the drug remains dispersed only due to the high viscosity of the medium inhibiting diffusion. In an ideal situation, the polymer characteristics are unaffected as there is no interaction between the drug molecules and polymer chains. The viscosity of the polymer is reduced as the T_g of the polymer is approached and the mobility of compounds in the polymeric matrix is dramatically increased. Thermal analysis is therefore useful in studying the state of the drug molecules within the polymer matrix. The presence of additives, e.g. surfactants, may

act as a plasticiser and reduce the T_g of PLA and PLGA polymers leading to a subsequent increased degradation and diffusion rate through these polymers (Pitt *et al.*, 1979).

1.1.6 Biocompatibility of polylactide polymers

Polymer selection is of prime importance when designing a controlled release product and the availability of homo- and co-polymers of lactic and glycolic acid in a wide variety of polymer ratios and molecular weights make them very suitable for this purpose. They have a long and well accepted safety profile, reviewed by Vert *et al.* (1992). Most early literature reports are concerning PLLA, as the L-form of lactic acid is metabolised in the body and this was considered the most useful polymer (Holland *et al.*, 1986). The suitability of the amorphous and semi-crystalline forms for drug delivery has prompted more recent work into biocompatibility studies for both forms and their co-polymers with glycolic acid.

Injected PLA microspheres induce only a minimal inflammatory response. This response includes the infiltration of immune cells and a minimal foreign body response including some small foreign body giant cells (Visscher *et al.*, 1985 and 1986). Even when traces, up to a few 10 parts *per* million, of polymerisation initiators or solvents, such as dichloromethane (DCM), are present, no significant adverse reaction has been detected after many years of experimentation on animals and humans (Vert *et al.*, 1992). Excellent tissue compatibility was also demonstrated by Csernus *et al.* (1990), who detected no significant foreign body tissue reaction to PLGA microcapsules and microspheres. Little or no fibrous tissue forms around the injection site (Visscher *et al.*, 1985). Typical tissue responses include mononuclear macrophages, proliferating fibroblasts and mature vascularised fibrous capsules. The time course for polymer degradation and the tissue returning to normal is dependant on the polymer selected and the inflammatory response is less for amorphous PDLA. Results are consistent with the pattern of increasing degradation rate with decreasing molecular weight of the initial preparation. Throughout implantation time, there were no degenerative changes, necrosis nor abscess formation observed in the tissue (Yamaguchi & Anderson, 1993).

Tissue responses to different polymers may be influenced by the administration route. Following i.p. injection, only small differences in inflammatory reaction occur related to molecular weight (van Sliedregt, 1992). The particles rapidly form aggregates (4 hours after injection) and differences observed are more likely due to differences in hydrophilicity of the preparations.

1.1.7 Formulation of microspheres using polylactide polymers

The selection of a particular manufacturing technique for the production of microspheres depends on the nature of the polymer and the drug and the intended use. Microspheres have been produced by a variety of methods including coacervation phase separation, solvent extraction and evaporation, spray coating and drying and by a melting method. The selection of a particular technique will depend on the nature of the core material, the polymer and its intended use. Only a few are acceptable for peptide and protein formulation, their physicochemical properties making incorporation difficult.

1.1.7.1 Single emulsion-solvent evaporation techniques

One of the more suitable methods for incorporation of macromolecules, including proteins involves emulsion solvent evaporation. The procedure, first described by Beck *et al.* (1979) involves the emulsification of a polymer/drug solution into an immiscible phase containing an emulsifier, to form droplets (figure 1.6). The solvent is then removed forming a suspension of hardened drug containing microspheres which can be harvested, washed and dried. In the single emulsion (o/w) technique, the polymer is dissolved in an organic solvent, usually dichloromethane or chloroform. The drug to be incorporated is either dissolved or suspended in this solution which is then emulsified into an aqueous phase in which the polymer does not dissolve. The aqueous phase may contain a variety of emulsifiers and the removal of the solvent may be by application of heat or vacuum or may take place under atmospheric conditions. This technique has been adapted to encapsulate a variety of drugs and manufacturing parameters have been widely investigated.

As the solvent evaporates, the droplets become more viscous leading to an increased tendency for coalescence. This can be reduced by the addition of a small amount of a droplet stabiliser in the external medium, providing a protective layer surrounding the droplets, reducing coalescence at the liquid/liquid interface (Arshady, 1991). Once the polymer has precipitated, the role of the agent is to stabilise a solid/liquid or solid/air interface preventing aggregation of the microparticles in aqueous suspension. The type and concentration of emulsifying agent has been reported to affect particle size, morphology and encapsulation efficiency (Watts *et al.*, 1990). Most o/w techniques employ polyvinyl alcohol (PVA), gelatin or methylcellulose (MC), agents which increase the viscosity of the solution.

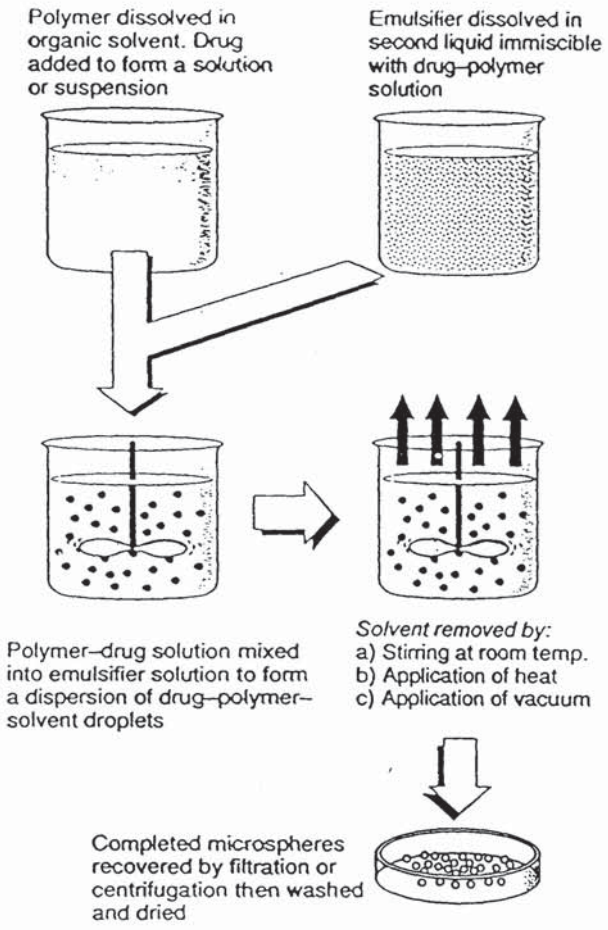


Figure 1.6. Microsphere formation using a single emulsion (o/w) solvent evaporation method (adapted from Watts *et al.*, 1990)

Factors influencing solvent evaporation procedures include the specific organic solvent, temperature of solvent evaporation, volume of organic phase *per* unit volume of aqueous phase, nature and amount of emulsifier, polymer structure and molecular weight and protein solubility. Very small microspheres may be produced using this technique but smaller sizes may result in a diminished carrying capacity of the microsphere and adsorption of the antigen onto the surface rather than the inside of the spheres. Microspheres with less than 30% of their weight consisting of protein are produced using this method.

Disadvantages with the solvent evaporation method include aggregation of the microparticles due to the organic solvent or the temperatures employed, loss of the drug into the external phase and potential formation of drug crystals. Dichloromethane, with a relatively high water solubility and low heat of evaporation, is generally the most successful solvent employed in this technique. A potential problem is the difficulty in removal of solvent contaminants as the particle size decreases (see section 3.3.9). With high molecular weight PLLA, being less soluble in the solvent, precipitation occurs more rapidly and larger microspheres are produced which have a tendency to aggregate due to the inherent tackiness of the polymer. The higher viscosity of these solutions of high-molecular-weight polymers also leads to their more difficult dispersion into the external aqueous phase and consequently a larger particle size. Using low molecular weight PLLA should lead to the production of smooth, non-porous microspheres while highly porous particles are produced with the polymers of higher molecular weight (Jalil & Nixon, 1990b).

1.1.7.2 Double emulsion-solvent evaporation techniques

Of particular interest for drugs that are sensitive to organic solvents and those with high aqueous solubility, particularly proteins, is the double emulsion-solvent evaporation technique. The formation of these complex emulsion systems was described by Ogawa *et al.* (1988) for the preparation of PLA or PLGA microspheres incorporating a LHRH analogue, leuprolide acetate. An aqueous solution of the drug is emulsified into a polymer/organic solvent mixture. The resultant w/o product is further emulsified into an external aqueous phase containing PVA (figure 1.7). The burst effect was reduced and incorporation efficiency was improved. With selection of formulation and control of processing parameters, the result is a free flowing powder, consisting of homogeneously dispersed drug throughout the polymer matrix (figure 1.5). The w/o/w emulsion solvent-evaporation technique has been proposed for the encapsulation of hydrophilic drugs minimising the loss in drug activity by contact with the organic solvent. Experimental parameters involved in the double emulsion technique and their effects on microsphere properties, e.g. particle size, morphology and porosity have not been fully explored. The subsequent effects on drug content and kinetics of drug release also require further investigation.

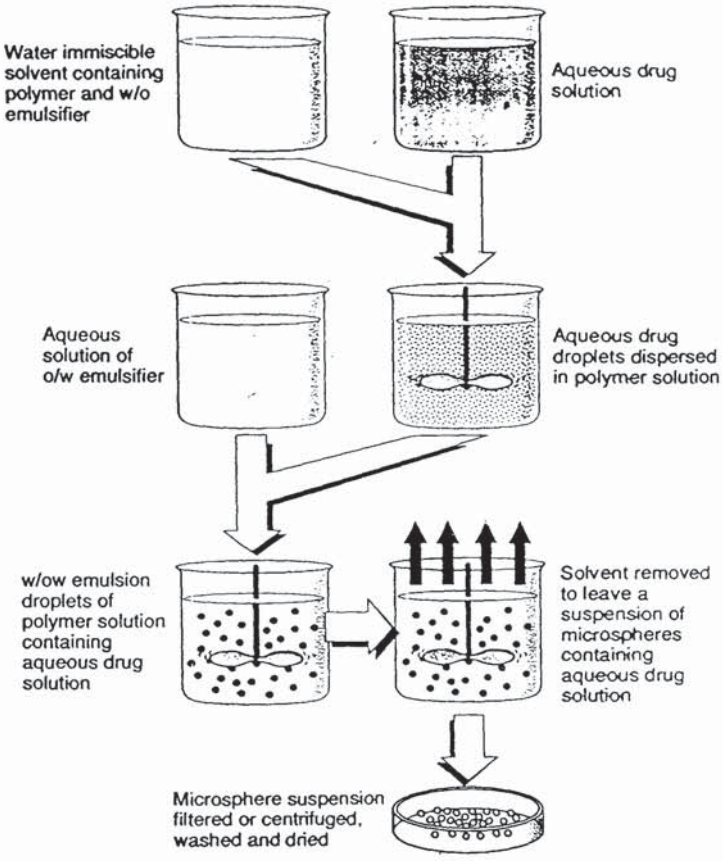


Figure 1.7. Microsphere formation using a double emulsion (w/o/w) solvent evaporation method (adapted from Watts *et al.*, 1990)

1.1.7.3 Protein adsorption to the surface of polylactide microspheres

The hydrophobic properties of the polymers of the lactide/glycolide series offer the opportunity to bind hydrophobic peptides to the surface of microspheres for use in controlled drug delivery (Mehta *et al.*, 1994).The relatively high surface concentration of antigen adsorbed onto a hydrophobic microsphere may afford protection by reducing the ability of the proteolytic enzymes in the gut to gain access to and degrade the antigen following oral delivery. Adsorption of peptides or proteins to polymer surfaces involves a series of complex interactions, both polymer and protein dependant and therefore, not easily explained or predicted. Adsorption onto polymeric surfaces such as the polyesters can be employed in a drug delivery system as the release rate can be altered by changing the polymer-drug interaction.

Eldridge *et al.* (1991a) did not detect any adjuvant effect from the mixing of staphylococcal enterotoxoid B (SEB) with empty microspheres prior to immunisation. However, this adjuvant effect has previously been reported (Almeida *et al.*, 1993; O'Hagan *et al.*, 1993b) leading to significant promotion of antibody responses following adsorption of the antigen involved to the surface of the microsphere. However, responses are reduced relative to entrapped antigen, due to either desorption *in vivo* or reduced capacity for protection of the antigen against degradation. Thus, the conditions of adsorption must be controlled in order to promote maximal protein adsorption. It must also be established that the sorption procedure and the conditions encountered during delivery, e.g. the harsh acidic environment of the stomach, have no adverse effect on the protein stability.

1.2 SYSTEMIC VACCINE DELIVERY

Recent scientific advances have produced many potential vaccine antigens in the form of isolated sub-units and recombinant proteins expressed in a variety of vectors. Recombinant, sub-unit, synthetic and purified antigens are, in many cases only poor immunogens and it is important to develop pharmaceutically acceptable delivery systems and adjuvants for enhancing their antibody response. Antigen-loaded liposomes have a limited shelf life and if a modified bacterial vector is employed, the immune response to the carrier organism is generally limited to a single immunisation. Typically, a minimum of three immunisations is required with new vaccine formulations and due to poor patient compliance, repeated immunisations are generally inefficient. The children's vaccine initiative aims to reduce the number of doses, with an earlier dosing schedule. It would like to combine multiple antigens, improve heat stability and achieve oral administration. Topics of high priority for the W.H.O. are the use of controlled release formulations for vaccine delivery, thus overcoming the need for multiple administrations and the development of vaccines for oral administration, e.g. as the incidence of tuberculosis (TB) is increasing in industrialised countries, the desire to improve BCG-based vaccines is becoming more important.

Delivery systems capable of releasing the antigen in a sustained and controlled manner, e.g. microspheres and liposomes, may be capable of potentiating the immune response, achieving single-step immunisation with an associated reduction in vaccination costs. The continuous release of antigen over time, pulsatile release of the antigen and induction of an initial high response with adjuvants are also important considerations.

1.2.1 Single-step immunisation

The development of single-dose vaccines, mainly those administered during childhood, providing effective protection against a number of diseases, would be a very important advance towards better immunisation coverage and protection against the respective pathogens. To date, small multiple doses have been successful in achieving protective immunity, but this schedule, requiring multiple injections of antigens, limits the practical value of such immunisation protocols. The time intervals between injections can be prolonged by using oil-based media such as Freund's complete adjuvant (FCA) to deliver antigens but more than one injection is still required (see section 1.4). Moreover, FCA and other oil emulsions are too toxic to be used in humans, eliciting severe granulomatous reactions with some containing toxic biological substances (Vogel & Powell, 1995).

The use of polymers to control the release of antigens was first reported in 1979 by Pries & Langer demonstrating sustained release from a non-biodegradable polymer and an enhanced immune response for over six months. They showed the induction of IgG antibodies following a pulsatile plus trickle delivery of the antigen and a secondary response was obtained with this delivery profile without the need for a secondary burst delivery effect. It has been established that the sustained release of large proteins is an effective way of raising antibodies to them (Langer, 1981). Therefore, excipients, including the polymer matrix, used to attain sustained release of macromolecular drugs may provoke an adjuvant-induced immunological response, which may be related to the nature of the excipient, the delivery rate or the release profile. An advantage of microsphere delivery of vaccines is their versatility. By modifying the co-polymer ratio or the molecular weight, the degradation pattern and subsequently the release of their contents can occur at different times, eliminating the need for booster injections.

The adjuvant activity of biodegradable microspheres during primary responses is explained by their rapid uptake by the mononuclear cells of the reticuloendothelial system (RES), resulting in high local concentrations of the antigens in cells and also in their ability to be rapidly degraded in the lysosomal milieu of the macrophages. During booster injections, the antigens exposed on the surface of the microspheres are thought to be important for adjuvant activity due to their ready availability for binding to non-specific antibodies in the circulation (Khan *et al.*, 1994).

According to Eldridge *et al.* (1993) antigen released from microspheres into the extra-cellular milieu is no more effective than antigen injected in solution. The predominant

mechanism of immune enhancement is a directed intracellular delivery of antigen to accessory cells. Microspheres larger than 10μm remain at the site of injection until bulk hydrolysis of the matrix results in microspheres fragments being engulfed by phagocytic cells. By this time the bulk of the antigen has been released but the antigen phagocytosed with the polymer fragments accounts for the lesser adjuvanticity of larger particles. Eldridge *et al.* (1991b) found a strongly enhanced serum IgG and an enhanced early antibody response following subcutaneous administration of microencapsulated SEB compared with the free form. There was no evidence of tolerance at higher doses and systemic immunisation proved successful priming for a subsequent orally administered booster dose. The injection of a mixture of PLGA microspheres of various sizes (1-10μm and 20-50μm) containing SEB resulted in a synergistic effect enhancing immune response. The adjuvant effect of PLGA microspheres containing SEB injected subcutaneously into mice was comparable to that of FCA but with no induction of the characteristic inflammation and granulomata of the emulsion and stimulated up to 500-fold higher antibody responses than the free antigen (Eldridge *et al.*, 1991a).

O'Hagan *et al.* (1993a) confirmed the ability of small microspheres (1.5μm) to induce more potent immune responses after subcutaneous (s.c.) administration than larger particles (72.6μm) which were too large to be phagocytosed The latter still induced a significantly enhanced immune response compared to soluble antigen. This may be due to controlled release of the antigen and also phagocytosis of fragments of the polymer as degradation and breakdown occurs.

1.3 MUCOSAL DELIVERY OF ANTIGENS

1.3.1 The mucosal immune response

Although immunisation strategies have been successful in the reduction of disease incidence and mortality when applied to disease associated with systemic organs, there has been a remarkable lack of success in vaccination to control mucosal disease. Traditionally, vaccines have been administered parenterally to evoke antibody production and cell-mediated immunity in systemic lymphoid tissues and the bloodstream. However, using antigen delivery systems applied at mucosal surfaces, immune responses may be stimulated in both systemic and mucosal compartments due to the dissemination of antigen-sensitised cells to other tissues (Moldoveanu *et al.*, 1993). It has been shown that protection against influenza correlates better with the presence of antibodies at the mucosal surfaces of the respiratory tract than with serum

antibodies. It is likely that most bacterial and viral diseases gain entry into the host *via* mucosal tissues including HIV, cholera and plague. The induction of a mucosal immune response exerting its effect at the local mucosal site of entry while capable of translation to distant mucosae and their secretions would lead to a more effective vaccination against many pathogens (Bienenstock, 1988).

Effective immunity at mucous membranes can prevent both infection and disease. As most vaccines in widespread use are administered systemically, consequently they mainly induce serum and/or systemic cell mediated immunity but little, if any secretory immunity. Direct immunisation of the mucosa, as illustrated by the Sabin oral polio vaccine, can lead to secretory immune responses which are effective in preventing colonisation of the mucous membranes and disease. For mucosal immunisation to be successful, there are obstacles which must be overcome, such as the peristaltic motion and the mucus membrane, and other mucosal environmental factors including the presence of proteases, bile salts and unfavourable pH. These factors have probably contributed to poor success rates with attempted mucosal immunisations (Nedrud & Lamm, 1991). Any process which facilitates the binding and transport of antigens into Peyer's patches (and other similar organised lymphoid tissues) might be expected to promote mucosal immune responses.

1.3.1.1 The common mucosal immune system (CMIS)

Mucosal membranes are the most frequent portals of entry of almost all infectious agents. They possess a large area of contact with the environment and are often exposed to antigenic substances that induce specific humoral, as well as cell-mediated immune responses, not only at the site of stimulation, mucosa-associated lymphoid tissues, but also in the draining lymph nodes, spleen and bone marrow. The intestines and other mucosa-associated tissues contain the largest accumulation of lymphoid cells, including B- and T-lymphocytes and plasma cells, and accessory and antigen presenting and processing cells (Mestecky *et al.*, 1994). The organisms entering by this route may either remain at the site of infection, e.g. *Shigella* or may spread to extra-mucosal sites, e.g. *Hemophilus influenzae*. The mucosal immune system is distinct from the systemic compartment and consists of distinct lymphoid sites where antigens are encountered and processed and initial B- and T-cell triggering occurs (inductive sites). The separate areas where immune cells actually function are called effector sites (figure 1.8). The Peyer's patches (PP), for example, are well characterised inductive sites for IgA memory B-cells while the lamina propria regions of the gut are major effector regions rich in IgA plasma cells. Oral administration of proteins may be expected to induce IgA responses

in the gut and other distant mucosal sites, but in reality such responses are poor and short-lived. Also, oral delivery of large amounts of protein can induce oral tolerance (Jackson *et al.*, 1993).

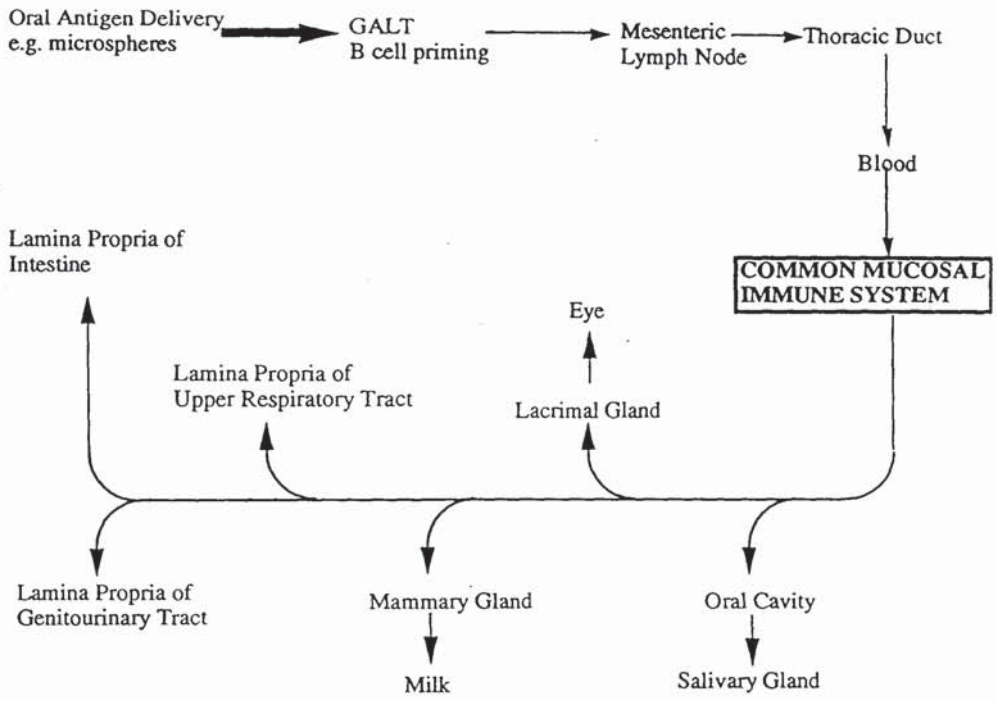


Figure 1.8. Induction of antigen-specific mucosal immune responses by the common mucosal system (adapted from McGhee *et al.*, 1992)

Secondary lymphoid tissues are highly organised with structurally recognisable compartments consisting of a variety of cell types. A common feature of most secondary lymphoid tissues is lymphoid nodules or follicles which represent the building blocks of the immune system and appear to have a major role in the development and maintenance of humoral immune responses. They are found as solitary nodules or as aggregates associated with the mucosa of the respiratory and digestive tracts, e.g. Peyer's patches. The mucus bathing the membranes serves to non-specifically sweep infectious micro-organisms and other antigenic material from the body.

The existence, in the respiratory tract, of the bronchial-associated lymphoid tissue (BALT) has been established for some time and it shares many anatomical similarities with the gut-associated lymphoid tissue (GALT) consisting of aggregates of lymphoid follicles extending into the submucosal layer, differing in quantity and morphology between different species. Similarities between the BALT and GALT strongly suggest

that they function together as part of the common mucosal system (O'Hagan & Illum, 1990; Almeida & Alpar, 1995). The existence of the CMIS in both humans and animals is now well established with demonstration of the induction of mucosal immunity at distant mucosal sites such as tears, saliva and genital secretions following oral immunisation (Bergmann & Waldman, 1988). In humans, the immunisation of volunteers with oral cholera toxin elicited gut-derived antibody-producing cells in peripheral blood and in distant mucosal tissues, in this case, labial minor salivary glands (Czerkinsky *et al.*, 1991).

1.3.1.2 Secretory IgA (S-IgA)

For organisms that enter the body through the gastro-intestinal, upper pulmonary, nasopharyngeal and genitourinary tract the need for effective vaccination procedures is paramount. The IgA class of immunoglobulins characterises the antibody response at mucosal surfaces and a unique transport mechanism has evolved to ensure their selective transport into mucosal surfaces. These surfaces are covered in secretions containing secretory IgA, the most important protective humoral factors. Current opinion is that S-IgA is locally derived from large numbers of IgA producing plasma cells that infiltrate the lamina propria regions underlying the mucosal membranes and it is specifically transported to the lumenal surface through the action of secretory component (SC). These S-IgA antibodies are induced, regulated and transported by mechanisms distinct from those involved in systemic immunity. The intestine comprises 70-80% of all immunoglobulin-producing cells in the body and produces more S-IgA *per* day than the whole body production of IgG (Shalaby, 1995). Generally currently used systemic immunisation regimes are ineffective at the induction of secretory IgA. These antibodies can limit the absorption of antigens through the mucosal membranes, inhibit the attachment of bacteria to the epithelial cells and neutralise the viruses which can cause local or more generalised infection. A recent review of the mucosal immune system has been published by McGhee *et al.* (1992).

The organised lymphoepithelial structures of the GALT and the BALT are the main sources of the precursor cells of IgA synthesis, which mature in the mesenteric lymph nodes and enter the circulation through the thoracic duct, lodging in the lamina propria of the intestinal, respiratory and genital tracts and in the mammary, salivary and lachrymal glands. Terminal differentiation into IgA plasma cells occurs in these sites under the influence of locally produced cytokines derived from T-cells and mucosal epithelial cells. Mucosal-associated lymphoid tissue (MALT) of the upper respiratory tract and gastro-intestinal tracts are separated from lumenal spaces by a layer of

epithelium interspersed with specialised microfold cells which can phagocytose antigens and pass them to the underlying lymphoid tissue for recognition and processing. The poor absorption of proteins and their degradation in the lumenal space can to a large extent be overcome by microencapsulation-protecting the antigen, enhancing absorption and potentiating immune recognition. IgA and a mechanism for its trans-epithelial transport exist in the nasal and upper respiratory tracts and are both considered legitimate targets for local administration of vaccines.

In mammals, S-IgA is polymeric, consisting of two or more immunoglobulin monomers each with a pair of heavy and a pair of light chains. It also contains two additional polypeptide chains, J chain and secretory component, a glycoprotein produced by various epithelial cells lining mucosal surfaces. The serum IgA, a minor immunoglobulin in serum, contains no SC, can be either monomeric or polymeric and there is little transport of serum IgA into the mucous secretions (Nedrud & Lamm. 1991). Polymeric IgA, secreted by the plasma cells from the lamina propria, binds to the poly-immunoglobulin receptor located on the basolateral surface of the epithelial cell and the resulting complex is then endocytosed by and transcytosed through the epithelial cell to its apical surface. Here, the receptor is cleaved in such a way. that a portion of it remains associated with the IgA forming SC. IgM which is polymeric may also enter the lumenal secretions by this route, but not IgG or monomeric IgA. It is possible to prevent the initial infection of mucosal surfaces by stimulating production of mucosal S-IgA levels directed against relevant virulence determinants of an infecting organism. S-IgA may prevent the initial interaction of the pathogen with the mucosal surface by blocking attachment and/or colonisation, neutralising surface acting toxins or preventing invasion of host cells.

1.3.1.3 Secretory IgA cell cycle

The stimulation of IgA precursor B-cells in GALT with orally administered antigens leads to the dissemination of B- and T-cells to mucosal effector tissues such as lamina propria regions of the intestinal, respiratory and genitourinary tracts and various secretory glands for subsequent antigen-specific S-IgA antibody responses (Mestecky, 1987). Although Peyer's patch germinal centres contain many cells for expression of surface IgA, antigen driven maturation of these to IgA secreting plasma cells does not often occur within the Peyer's patches. Rather, following antigen stimulation and presentation to the B-and T-cells, antigen sensitised B-cells migrate from the Peyer's patch, possibly along with antigen presenting cells, to the mesenteric lymph nodes where they continue to mature before draining into the bloodstream *via* the thoracic

duct. From the blood, these IgA committed antigen sensitised B-lymphoblasts home back to mucous membranes where they become the mature IgA secreting cells of the lamina propria (LP). Although they tend to return to their tissue of origin, it is believed that IgA precursor cells from one mucosal site can seed plasma cells into both the originating and other mucosal sites. This seeding of cells has led to the concept of the common mucosal system. There are several points along this cycle where the B-cells can be influenced by other cells and/or factors. The common mucosal system is linked by emigrating antigen stimulated IgA precursor cells. The precise mechanism for B-cells switching to the IgA isotope remains unknown but a number of elements which may influence this phenomenon have been identified, including lipopolysaccharide, IL-4, and other chronic mitogens and antigens (O'Hagan, 1992).

1.3.1.4 Mucosal tolerance

Large or repeated doses of a variety of soluble and particulate antigens can lead to a state of systemic unresponsiveness, known as mucosal tolerance where appropriate (Tomasi, 1980). It can arise whether the antigen is administered by the oral or the respiratory route, oral or inhalation tolerance respectively, and both forms probably operate under the same mechanisms. It seems to serve as a means of protecting the host from activation of potentially deleterious systemic immune responses to commonly encountered environmental antigens, e.g. pollen or food antigens. If the vaccine is designed against environmental antigens or pathogenic micro-organisms limited to the mucous membranes, inducement of mucosal tolerance would be beneficial avoiding undesirable events resulting from these agents gaining access to the systemic circulation. The sites and mechanisms modulating this specific systemic hyporesponsiveness are yet unknown, but it is believed to be mediated by a combination of suppressor T-cells, inhibitory cytokines and factors inhibiting the inflammatory process (Shalaby, 1995) with oral tolerance being associated with a down regulation of all inflammatory cytokines, including interferon-gamma (IFN–γ).

1.3.2 Oral delivery of antigens

Strategies developed for oral administration of antigens include the use of attenuated mutants of bacteria as carriers of heterogeneous antigens (e.g. *Salmonella typhimurium* Oyston *et al.*, 1995) and the addition of bacterial products with well known adjuvant properties (Elson, 1989). Other strategies under investigation include encapsulation of antigens into liposomal formulations or microspheres of polylactic acid species.

Administration of unprotected or free protein or glycoprotein may result in a markedly reduced immunogenicity resulting from loss of critical epitopes due to enzymatic degradation. Important epitopes may also be lost due to irreversible conformational changes arising in freely dispersed or surface bound macromolecules under acidic or alkaline conditions. For human mucosal vaccines, the development of improved oral delivery systems is of paramount importance. New recombinant proteins are generally not effective after peroral administration and frequent injections or infusion therapy is required. Due to practical benefits and an ability to preferentially stimulate protective immune responses on the mucosal portals of entry of most infective agents, oral delivery could become a routine mode of administration for vaccines. Attractive attributes of oral vaccines include the simplicity of administration, potential for unlimited boosting, fewer side effects, less stringent requirements for the preparation of orally delivered antigens than liquid injectables, thereby being less expensive to manufacture and fewer problems with storage of dry, lyophilised oral vaccines, a particular advantage in developing countries. Orally administered pharmaceuticals are not required to be sterile but the unique uptake of microspheres into the Peyer's patches, and beyond, may necessitate sterilisation of microspheres intended for oral vaccine. The gamma radiation used for sterilisation has been shown to decrease molecular weight of the polymer, increasing the biodegradation rate. Although some authors have successfully used gamma radiation, others report a loss of tetanus toxoid antigenicity from an unspecified dose of gamma radiation (Esparza & Kissel, 1992).

Targeting to IgA inductive sites and protection from proteases in the GI tract are two of the major advantages for antigen delivery in microspheres. The logistic advantages of oral vaccination in any major immunisation programme are obvious and the immunological advantages are almost as great. The area of mucosal surface in mammals far exceeds the skin area and they are supplied with a similarly greater number of lymphoid cells. A circulation of lymphoid cells, primed at a mucosal surface such as the gut or the nose, to other mucosal regions is well established in animal models and is also thought to operate in man. Our increased knowledge of mechanisms of infection and immunisation at mucosal sites is bound to hasten the day when many additional vaccines will be administered *via* a mucosal route.

As early as 1961, Sanders & Ashworth were examining the body mechanisms of absorption, transportation and disposition of polystyrene latex particles following oral administration. The general preconception was that for oral immunisation, large amounts of vaccine were required and results were not very successful. The oral route of immunisation remains clinically underexploited partly because of the relatively recent

appreciation of the important protection of secretory IgA at mucosal sites (Mestecky & McGhee, 1987). However, a recent study by Maloy *et al.* (1994) showed that oral or parenteral administration of antigen in PLGA microspheres primes a wide range of humoral and cell-mediated immune responses both locally and systemically, extending previous reports and re-emphasising their importance as oral vaccine vectors, with the production of antigen-specific IgA responses in the small intestine. They also found that the spheres primed antigen specific systemic cytotoxic T-lymphocyte (CTL) responses, after both oral and parenteral administration, probably of the class I major histocompatibility complex (MHC)-restricted CTL. The induction of systemic responses after oral administration is different to the normal response to soluble antigens, resulting in oral tolerance. The ability of the drug entrapped in microspheres to induce secretory IgA and systemic IgG responses is most likely due to uptake of particles by the Peyer's patches.

1.3.2.1 Gut-associated lymphoid tissue and Peyer's patches

The extent of microparticulate absorption across the GI tract remains the subject of contention, though it is of primary importance to allow a realistic assessment of the potential of microspheres as vaccine or drug delivery systems. Both polystyrene and biodegradable microspheres are preferentially absorbed by the microfold (M) cells of the Peyer's Patches (Pappo & Ermak, 1989; Eldridge *et al.*, 1989 and 1990). It appears that microparticles are preferentially adsorbed and trancytosed largely by the Peyer's patches in a manner that results in distinct absorptive patterns and events. The particles adsorb to the lumenal surface of the intestine epithelium an a very highly localised concentration around the domes of the Peyer's patches (Jenkins *et al.*, 1994; Jepson *et al.*, 1993). There are three possible mechanisms of uptake, intracellular, paracellular and *via* the M cells and Peyer's patches (Kreuter, 1991) and evidence exists for all three pathways with the simultaneous occurrence of more than one pathway. The decreased amount of mucous associated with M cells would facilitate absorption but the mechanisms may be different in regions of the GI tract and may also depend on the surface characteristics of the particles and their size.

There are four lymphoid cell populations associated with the intestine, the first being lamina propria lymphocytes (LPL) which contain a high proportion of IgA producing cells as well as macrophages, neutrophils, eosinophils and mast cells. Another group, the intra-epithelial lymphocytes (IEL) are primarily T-lymphocytes dispersed between the epithelial cells of the mucosal membrane, the precise function of which is largely unknown in humans. The Peyer's patches, appendix and related solitary lymphoid

nodules or follicles, the organised lymphoid tissues, comprise the third group and are often referred to as the GALT. The fourth intestinal lymphoid element is the mesenteric lymph nodes which drain the GALT and in turn drain, *via* the thoracic duct, into the bloodstream. The other mucous membranes are populated by analogous populations of lymphoid cells, though they are less prominent. In the respiratory tract, the structures corresponding to the Peyer's patches are known as the BALT. It is these organised tissues of the mucous membranes where mucosal lymphocytes are thought to first encounter antigen and initiate immune responses (Gilligan & Li Wan Po, 1991).

Peyer's patches can be anatomically divided in three main regions, the dome area at the lumenal surface, the underlying follicles (B-cell areas) consisting of one or two germinal centres and the parafollicular region (T-cell area). The epithelium covering the dome consists of cuboidal epithelial cells and specialised membranous phagocytic epithelial cells called microfold (M) or follicle-associated epithelial cells (figure 1.9). M cells are both structurally and morphologically different from cuboidal and columnar epithelial cells being flatter and having a thin cytoplasm, short irregular microvilli, numerous small cytoplasmic vesicles and a close association with intraepithelial cells by tight junctions and desmosomes. M cells can take up macromolecular and particulate antigen and pass it largely intact to the lymphocytes and antigen presenting cells in the dome region by virtue of its lack of lysosomes and the small distance between the apical and basolateral membranes facilitates rapid transport. The dome region is enriched with lymphocytes, macrophages and plasma cells (Wolf & Bye, 1984).

Antigen presenting cells in Peyer's patches include follicular dendritic cells within the germinal centres, interdigitating cells in close contact with the lymphocytes of the parafollicular T-cell region and numerous macrophages in all regions including the dome. Adherence to the apical surface of M cells may result in increased transportation of antigen which correlates with increased immunogenicity. M cells may therefore be more accessible for micro and nanoparticles.

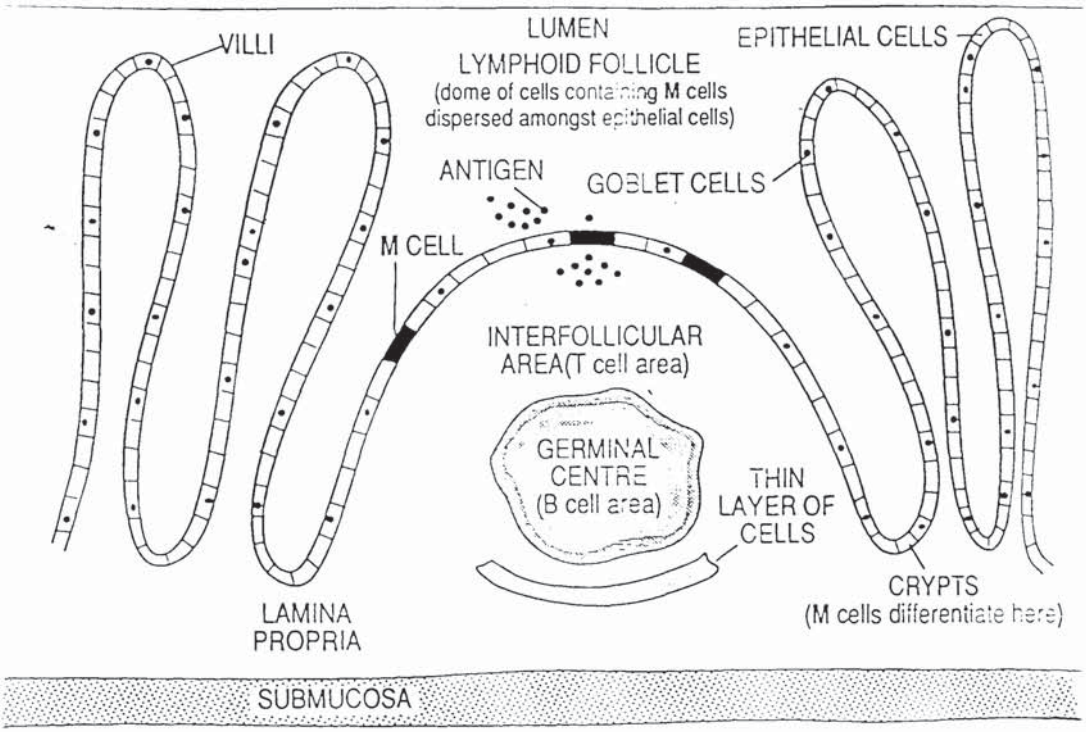


Figure 1.9. Simplified diagram of the structure of the Peyer's patches and M cells (adapted from O'Hagan, 1990)

Absorption through the Peyer's patch cells is thought to be largely by a transcellular process which may involve membrane bound vesicles or vacuoles originating in the apical cytoplasm of the absorptive cells (Sanders & Ashworth, 1961). These may be linked to the intracellular transport of microspheres within the MAC^{1+} macrophages. It is likely that particle absorption is a combination of many processes including the presence of sinus-like lymphatic cavities below the follicular domes allowing periodic retention and release of delivered particles. Thus, it can be envisaged that there could exist a biphasic absorption pattern, allowing mucosal retention and systemic dissemination of particles, providing specific targeting for vaccine antigens.

1.3.2.2 Oral delivery using microparticulate systems

Tissue penetration in the gut of fluorochrome-containing PLGA microspheres was found to be limited to the Peyer's Patches (Eldridge *et al.*, 1989). All of the microspheres in the PPs were contained in macrophages of which a proportion was carried into the mesenteric lymph nodes. The uptake and transport from the gut cells is strongly influenced by particle size with particles of 1-10μm being taken up by PPs and those with a size less than 5μm traversing the cells, entering mesenteric lymph nodes and entering the circulation and the spleen where the released antigen stimulates a serum antibody response (Eldridge *et al.*, 1990). Particles between 5-10μm remain in the PPs (for up to 35 days) and drug release and degradation may take place at that site. Fluorescence microscopy analysis of pathology sections revealed that orally administered microspheres larger than 10μm in diameter are not absorbed (Eldridge *et al.*, 1989b). The results of a recent study by Jenkins *et al.* (1994) confirm the theory that large particles are retained for longer periods within the Peyer's patches and that small particles are progressively extravasated to other major organs. Though they found that the extent of particle absorption is less than that previously thought (Alpar *et al.*, 1989; Jani *et al.*, 1989), it is thought that intestinally administered microparticles are capable of being absorbed in several thousands, retained in the lymph nodes and Peyer's patches and also systemically transported *via* the superior mesenteric lymph duct. Therefore, encapsulated antigens may be used to increase the local concentrations of antigens in the GALT by preferential tissue retention, protect antigens from lumenal and intracellular degradation, increase the antigen processing capability and direct the overall mucosal immunological network towards an enhanced response.

Hydrophobicity is a determinant of the Peyer's patch uptake, as demonstrated by the increased uptake of the more hydrophobic polyhydroxybutyrate (1000-1500 microspheres for every three PP examined) and PLA/PLGA (200-1000) microspheres compared with similarly sized cellulose type particles which were not taken up at all (Eldridge *et al.*, 1990). Although the precise mechanisms of absorption of particles across the intestinal epithelium have not yet been elucidated, but the particles are believed to be almost exclusively delivered to the mesenteric lymph and systemically disseminated within migrating macrophages *via* the local mesenteric lymph nodes. The subjects of levels and temporal characteristics of absorption and subsequent dissemination to other lymph nodes remain undecided (Jani *et al.*, 1989 and 1992; Alpar *et al.*, 1989; Eldridge *et al.*, 1990; Ebel, 1990).

Intraperitoneal (i.p.) priming with orogastric boosting was nearly as effective as tertiary oral immunisation for stimulating mucosal anti-toxin antibodies (Eldridge *et al.*, 1991b). Three oral doses of encapsulated toxoid stimulated circulating IgA, IgG and IgM antibodies and mucosal IgA was detected in the saliva. Following systemic administration, neither free nor encapsulated toxoid demonstrated a detectable mucosal IgA response. A T-cell proliferative response can be induced after (i.p.) administration of OVA in PLGA microparticles similar to that induced by OVA adsorbed to alum (O'Hagan *et al.*, 1993b). Also, i.p. administration of OVA in PLGA microspheres induced a more potent IgG antibody response after a single administration (O'Hagan *et al.*, 1991a) than FCA with OVA. Microsphere delivery systems for antigens are therefore capable of inducing CTL responses following i.p. injection. The mechanism for the induction of a CTL response with antigen entrapped in microspheres is unknown. Specific antibody-secreting cells were detected both in the mesenteric lymph nodes and in the spleens of animals orally immunised with the microencapsulated cholera toxin B (CTB), free CTB not exhibiting any such responses (see section 1.4.2).

Microencapsulated influenza virus induced higher titres when given by the oral route compared with free antigen (Moldoveanu *et al.*, 1989 and 1993). The elevated IgA and IgG levels were prolonged after oral microsphere immunisation but boosting by additional doses was required and neither antibody titre nor protection were comparable to systemic dosing with spheres or free antigen. Similar mean salivary antibody responses to free or PLGA microencapsulated OVA following intra-gastric immunisation PLGA have been reported (Challacombe *et al.*, 1992; O'Hagan *et al.*, 1993b; O'Hagan *et al.*, 1994). However, levels of salivary IgA antibodies after secondary immunisation were up to 50 times larger than those obtained with the soluble antigen. Systemic IgG antibodies were also induced by immunisation *via* this oral route. Faster releasing PLGA 50:50 co-polymer induced higher levels of secretory IgA than a PLGA 75:25 co-polymer. This could be due to the more rapid release of entrapped antigen following uptake into the Peyer's patches. Particles of the slower-releasing polymer were more effective at producing serum IgG responses possibly due to the delayed release allowing delivery of relatively large amounts of antigen to the lymph nodes and spleen. The S-IgA response was short-lived, decreasing after 8 weeks (Challacombe *et al.*, 1992) and it is possible that a slower degrading polymer could extend this time period.

Although the fate of particles after their oral absorption remains a controversial issue, it must be concluded that translocation of small-sized particles is possible. The extent of particle uptake will determine whether it is a route compatible with a strategy of drug

administration with therapeutic objectives. If the main limitation for the passage of microspheres from the intestine is that the process is sometimes erratic or restricted, it is still reasonable to believe that the systems have real prospects for the oral delivery of antigens. It is generally accepted that limited doses of antigen are sufficient for mucosal immunisation with oral delivery of antigens being considered the most convenient means of producing an IgA antibody response. With the protection of the antigen against enzymatic degradation afforded by microencapsulation, this may be a profitable and successful delivery strategy.

Microspheres have been used as a delivery system for an oral antigen, enterotoxigenic *E. coli* CFA/II, in human volunteers (Tacket *et al.*, 1994). Immune responses to two different protein antigens contained within the same microspheres were produced, providing evidence that the development of microspheres systems for a multivalent vaccine is possible. Failure of microencapsulated *Bordetella pertussis* antigen formulations to elicit an immune response, while maintaining immunogenicity *via* the nasal route, (Shahin *et al.*, 1995) may be due to the poor oral absorption of the PLGA microsphere preparations used in that study (Eldridge *et al.*, 1989a).

1.3.3 Nasal delivery of antigens

Due to its easier accessibility, the nasal cavity is a potential alternative to the parenteral route for delivery of peptides and proteins and also for the delivery of immunogenic substances. However, intranasal (i.n.) immunisation may not represent a purely local response as part of the antigenic load may be swallowed or inhaled leading to the stimulation of a more general immune response.

For many years, drugs have been administered nasally for local effect on the mucosa. Recently, there has been interest in the intranasal route for systemic administration of vaccines, hormones, peptides and other drugs which are susceptible to degradation in the GI tract or wall (Duchêne & Ponchet, 1993). Although small number of drugs appear to be well absorbed through the nasal route (e.g. propranolol and progesterone) showing bioavailabilities similar to the intravenous route, the majority of drugs show a lesser degree of absorption. Due to the relatively easy accessibility of the nasal route, it has been extensively investigated as a potential alternative system for the delivery of peptide drugs (for review see O'Hagan & Illum, 1990; Almeida & Alpar, 1996). The existence of nasal-associated, organised lymphoid tissue (NALT) has been demonstrated (Spit *et al.*, 1989). The potential of i.n. immunisation to produce a further line of defence, exploiting the common immune system providing a more

comprehensive defence against infection has also been demonstrated (Almeida *et al.*, 1993). Recent advances in the area include the intranasal administration of low doses of three different *Bordetella pertussis* antigens encapsulated within PLGA microspheres and all elicited strong IgG and IgA in respiratory secretions. Immunisation with a combination of the three antigen formulations was more effective than administration of a single antigen formulation, coinciding with protection against infection (Shahin *et al.*, 1995). Also, a low but detectable IgA antibody response was detected in the gut wash of a number of mice following nasal administration of 10μg microencapsulated doses. However, there have also been reports of the failure of intranasal delivery of microencapsulated parainfluenza virus to elicit an immune response. This may be due to the relatively large particle size (mean 5μm) used in the study (Ray *et al.*, 1993) and further investigations into the mechanism behind induction of this response are essential.

Microspheres administered to the respiratory tract have been reported to be both absorbed into the bronchus-associated lymphoid tissue and phagocytosed by alveolar macrophages. Large numbers of the latter were seen to enter the draining lymphatics and to migrate to the bronchial lymph nodes (Eldridge *et al.*, 1989). There are few reports on characterisation of the pathways by which particles reach the circulation following nasal delivery. Using confocal imaging techniques (Huang & Donovan, 1995) and model polystyrene microspheres, it has been shown that relatively hydrophilic microspheres with smaller diameters (0.01μm) can pass through the mucosa *via* the paracellular route. More hydrophobic particles are lodged intracellularly. However, these observations were carried out on excised rabbit nasal mucosa and accumulation of the particles in the submucosa would be reduced due to the vasculature *in vivo*. Absorption of polystyrene microspheres has also been studied by Alpar *et al.* (1994) in rats following intranasal administration. Subsequent translocation of 0.83μm through the nasal mucosa was reported. Quantification of the extent of absorption *via* this route is an essential step in understanding the mechanism behind this process.

1.4 IMMUNOLOGICAL ADJUVANTS

It may be necessary to administer an adjuvant with oral inactivated microbial vaccines and synthetic peptides as these do not elicit as strong an immune response as live attenuated vaccines. Many adjuvants are biological substances or their synthetic analogues however, some chemical substances are also known to possess adjuvant properties, e.g. dextran sulphate. The substances that exhibit adjuvanticity are diverse and their mode of action is not singular but generally, they may act on the antigen itself

and/or the host cells involved. Therefore, adjuvants increase the immunogenicity of antigens, reduce the number of injections and dose required for protective immunisation, increase responsiveness in immunologically immature, suppressed or senescent vaccines, promote cell-mediated immunity and permit the oral delivery of vaccines.

Freund's complete adjuvant is a mixture of paraffin oil and mannide monooleate which is given in a 1:1 w/o emulsion containing killed *Mycobacterium tuberculosis* in the oil phase and antigen in the aqueous phase. It has been recognised as the most potent adjuvant due to its ability to enhance strongly both humoral and cellular immunity, but reaction at the injection site, causing granulomas and abscesses, and sensitisation to the *Mycobacterium tuberculosis* limits its use (Vogel & Powell, 1995). Though the mycobacteria play a key role in potentiating the immune response, the oily phase alone acts as an adjuvant, although to a lesser extent, and it known as Freund's incomplete adjuvant (FIA). The general hypothesis for the mode of action of such emulsions is the retention of the antigen at the site of injection performing a depot function, protection of the antigen from rapid destruction, the facilitation of phagocytosis and stimulation of the cells of the immune system.

The adjuvant properties of liposomes has been successfully demonstrated (Gregoriadis, 1990) and these have been further enhanced by the inclusion of lipid adjuvants, e.g. Lipid A, and immuomodulators (reviewed by Alving, 1991). The application of this technology to microspheres has not been widespread and possible adjuvants for microencapsulation are discussed below.

1.4.1 Muramyl dipeptide

One adjuvant which has been shown to enhance the immune response when co-administered with an antigen is muramyl dipeptide, N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP), which is a purified peptidoglycan moiety of the cell wall of mycobacterium, the immunostimulating component of mycobacterial cells known to provide enhanced T-cell help. By itself, it is non-immunogenic, exhibits low toxicity and has none of the side-effects inherent in other adjuvants. Incorporation of MDP with an antigen into liposomes, has been shown to enhance the immune response compared to that generated by the antigen alone (Alving, 1991). Nellore *et al.* (1992) encapsulated MDP into polyglycolide microspheres along with Hepatitis B surface antigen (HBSA). A 10% loading of antigen was used but no details of the encapsulation efficiencies of either agent were discussed. PGA microspheres were injected i.p. and microspheres in

the size range 1-10μm showed an enhanced immune response during weeks 2 to 4. The larger microspheres (20-60μm) produced higher antibody levels after 6 weeks while a combination of the two groups produced a more durable response, maintained even in the sixteenth week. Antibodies produced by the co-encapsulated MDP formulation (1-10μm) were substantially higher than any other formulation and were maintained for a longer duration. The absence of a memory response following a second injection for the PGA microspheres was unusual but an anamnestic response was obtained for the co-encapsulated formulation indicating a need for an immunostimulant for microencapsulated HBSA.

1.4.2 Cholera Toxin

Currently, the most potent mucosal adjuvant is cholera toxin (CT), which in addition to its binding affinity for M cells and intestinal epithelium, appears to enhance antigen-presenting capacity of macrophages and modulate the growth of B- and T-cells (reviewed by Elson, 1989). It has been discovered that cholera toxin is a major exception to the poor immune response developed with orally administered proteins, being an exceptionally potent oral immunogen capable of producing strong mucosal S-IgA and plasma IgG antitoxin responses as well as immunological memory in the intestine. It does not appear to induce oral tolerance and can remarkably potentiate the immune responses to both immunologically related and unrelated protein antigens given orally. It should be administered concomitantly with the antigen and by the same route suggesting direct effects on mucosal tissues and lymphoid cells and generally both mucosal S-IgA and serum IgG responses are potentiated (Jackson *et al.*, 1994).

The mechanism of CT adjuvanticity is poorly defined but it is known to bind to GM_1 ganglioside receptors in the GALT, thus targeting the immunogen for increased uptake. The toxin of *Vibrio cholerae* is composed of two subunits, A and B. Subunit A activates adenylate cyclase triggering the biological activity, whereas subunit B (CTB) is responsible for binding to cell receptors. The mucosal adjuvant effect is thought to be due to a synergistic effect involving both the CTB and the adenylate cyclase activity of the A subunit and there is some debate about the ability of CTB alone to act as an adjuvant. The reports of adjuvanticity being conferred by CTB may be due to trace amounts of whole CT (O'Hagan, 1992). It is also likely that CT would need to be specifically conjugated to the vaccine antigen to avoid immune responses from unrelated bystander antigens in the gut. It may prove difficult to conjugate to the antigen and still maintain full antigenicity and adjuvant effects.

It is well established that oral immunisation of mice with protein antigens and vaccines together with CT induces serum antiprotein and anti-CT antibodies, notably IgG and IgA isotypes. As much work is still necessary to develop safe and effective oral immunoadjuvants to potentiate the immune response to orally administered antigens. O'Hagan *et al.* (1993b) encapsulated CTB into PLGA microspheres. SDS-PAGE analysis proved that the CTB was not degraded or aggregated by the process and Western blot analysis showed that an anti-serum raised against non-entrapped CTB recognised CTB from the microparticles. It induced immune responses following both parenteral and oral immunisation in mice.

Studies using soluble tetanus toxoid (TT) with CT as adjuvant have been carried out in order to formulate a protocol to optimise both systemic and mucosal immune responses to TT (Jackson *et al.*, 1994). It was found that 10μg of CT co-administered with 250μg of TT gave optimal antigen-specific anti-TT mucosal IgA and serum IgG responses on day 21 of the study. Using ELISPOT techniques, they determined that the antigen-specific anti-TT and anti-CT IgA antibodies found in faecal extracts were derived from activated B-cells located in the lamina propria of the gut, consistent with its role as a mucosal effector site. Mice orally immunised with soluble TT without CT showed only a weak serum IgG response after 21 days and no IgA or IgM. Data suggested that in mice orally immunised with TT and CT, a strong Th2-type response occurred both systemically (spleen) and in the intestinal mucosa (Peyer's patches). Intraperitoneal immunisation resulted in high frequencies of both Th1 and Th2-type cells in the spleen, suggesting that systemically administered CT does not discriminate between subsets. As the IgG1 serum response is predominantly larger than the IgG2a titres, it may be that other cytokines are involved.

The addition of 10μg of exogenous CT to a 100μg dose of TT in PLGA microspheres, improved immune response. Oral immunisation with TT in microspheres failed to produce a primary response, unlike the s.c control, but following two boosts, serum anti-TT-specific IgG levels reached end-point titres comparable with those of orally administered soluble antigen and CT. The administration and single boost of microspheres with CT produced anti-TT-specific IgG levels similar to those produced after three immunisations without the presence of CT. Significant mucosal IgA and anti-TT responses required a third immunisation with microspheres.

1.4.3 Cytokines as co-adjuvants

Preclinical and clinical studies involving cytokines and growth factors are focusing not only on their own therapeutic abilities but also the role of synergistic combinations with chemotherapeutic agents, antibiotics and other cytokines (Talmadge, 1993). A difficulty involved in the establishment of optimal administration of IFN-γ is the non-linear dose-effect relationship described by Talmadge *et al.* (1986).The administration and regulation of biological control of IFN-γ will initiate many undesirable physiological events, so delivery must be adapted to ensure targeting of the required physiological localisation.

Cytokines are proteins which demonstrate profound effects on specific immune responses and thus may be regarded as adjuvants. The cellular immunology and cytokine biology of mucosal immune responses suggest additional strategies for designing mucosal adjuvants. These approaches include altering the balance of cytokines at the site of immunisation and/or at the site of plasma cell/memory cell naturation. It may be possible to package and deliver mucosally relevant cytokines to the mucosa or the activity of the accessory cells, T cells, antigen presenting cells (APCs) and epithelial cells, responsible for particular cytokine secretion might be modulated with various agents. Xu-amano *et al.* (1994) found that oral immunisation preferentially induced Th2-type cells in PPs. It is well established that the isotype and level of B-cell and antibody responses are influenced by T-cell derived cytokines. Thus mucosal IgA responses appear to be highly dependent on Th2 cells.

Differentiation of T cells is regulated by a selective group of cytokines, IFN-γ, IL-2 and IL-12 favour Th1 cell development whereas IL-4 and IL-10 favour Th2 development. These activated T cells in turn produce cytokines, activated Th1 cells secrete IFN-γ, IL-2 and TGF-β whereas activated Th2 primarily express IL-4, IL-5 and IL-10. This complex network regulation of the development of immune responses is regulated by modulation of the differentiation and expansion of committed T-cells. The Th1 cell subset is preferentially involved in cell-mediated immunity, while Th2 cells are most effective in the regulation and support of B-cell responses. Different antigens, microbial infections and routes of immunisation may induce different subsets of Th cells.

Mature B-cells synthesise antibodies against immunogens in providing the host with humoral immunity. Like T-cell growth, the process is regulated by a cytokine network. IFN-γ along with IL-2, IL-4, IL-5 and IL-6 are all activators of B cells. Cytokines may enhance the potency of sub-unit vaccines through modulation of antigen presentation

and T- and B-cell growth. The success in preserving the biological activity of labile peptides such as human IFN-γ in PLGA implants is very encouraging (Eppstein *et al.* 1986).

Generally, for the production of mucosal immune response, both antigen and adjuvant should be delivered together to the organised mucosal lymphoid tissues. Thus, the same delivery vehicles should be considered for the delivery of cytokines and other regulatory molecules to target antigen to mucosal associated lymphoid tissue. Important issues for the delivery of cytokines are the doses required and possible side effects. In order to ensure a sufficient quantity of cytokine at the site of interaction, one possibility is to incorporate a gene for the cytokine into the vector along with the desired antigen as was demonstrated by Kohonen-Corish *et al.* (1990) with vaccinia virus expressing interferon-γ.

Various cytokines are involved in the regulation of the function, proliferation and induction of differentiation of immunocompetent cells (Kroemer *et al.*, 1993). Interferons, for example, enhance the phagocytosis of macrophages and the cytotoxicity of cytotoxic T lymphocytes, natural killer and killer cells and the regulation of antibody production. IL-2 and TNF also activate various kinds of immunocompetent cells and IFN, IL-2 and TNF induce enhancement of the immunological response under certain conditions.

1.4.3.1. Interferon-gamma

IFN-γ has a positive charge at neutral pH and a monomer molecular weight of 17kDa by SDS-PAGE. It exhibits biological activity in the form of a dimer (non-covalent in the human form but covalent in the murine form). Human IFN-γ (HuIFN-γ) is a homodimer of 143 residues of amino acids, is highly α-helical and unrelated in sequence to the other IFNs. The quaternary structure of the molecule explains its sensitivity to extremes of conditions, being denatured at temperatures above 56°C and at pHs below 4.0 and above 9.0 (Farrar & Schreiber, 1993).

IFN-γ has been successfully incorporated into microspheres using trehalose as a stabilising agent with retention of bioactivity and native conformation (Cleland & Jones, 1995). The outcome of including IFN-γ in an adjuvant preparation is difficult to predict. Synthesised in T cells or natural killer cells, IFN-γ activates antigen presenting cells, enhancing the recruitment of leukocytes and expression of class I and II MHC molecules. This, together with IL-1 would increase antigen driven stimulation of

responder T cells. The anti-proliferative capabilities of IFN-γ, however, would serve to minimise the proliferative effects of IL-1 on the surrounding connective tissues. This could prove advantageous in reducing scar tissue development at the site of inoculation. Although it remains to be seen if a cytokine cocktail can provide a useful adjuvant, from what is known about their biological effects, it does appear to be a rational approach to enhance response to inactivated vaccines. The combination of cytokines with other adjuvants, in this case microspheres, or other cytokines has not been extensively investigated and much more work is required in this area.

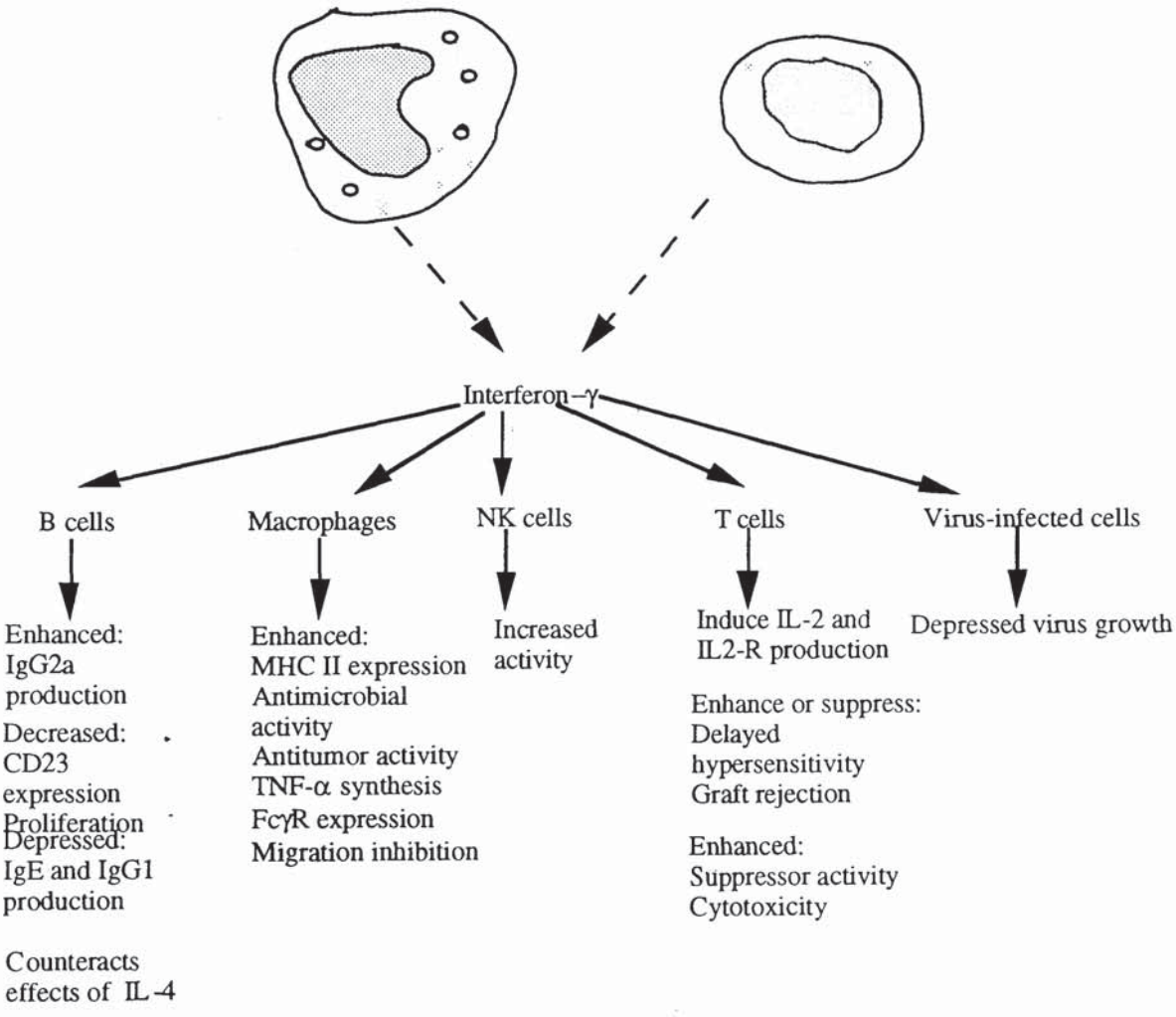


Figure 1.10. The origins and targets of interferon-gamma (adapted from Tizard, 1995)

Czerkinsky *et al.* (1993) found cholera vaccine to be a very potent inducer of local production of IFN-γ, an observation that may have important implications concerning

the role of the cytokine in immune protection at intestinal surfaces. Recent *in vitro* studies suggest that IFN-γ may influence the efferent phase of a secretory antibody response by increasing epithelial cell surface expression of the secretory component and thus increasing the transport of potentially protective secretory antibodies. It may also directly modulate such basic physiological properties of epithelia as barrier function and permeability to electrolytes. A summary of some of the actions of this cytokine are outlined in figure 1.10.

Heath & Playfair (1990) found IFN-γ to be an effective adjuvant, enhancing antibody, T-helper cell and delayed type sensitivity reactions. It also increases the protection afforded by the vaccine against a live challenge infection. To be at its most effective as an adjuvant, it must contact the same presenting cell as the antigen. Coupling of IFN-γ to the antigen may target the optimal dose to cells contacting antigen with no spillover to irrelevant cells. The mode of action appears to be by enhancement of the efficiency of antigen presentation by increasing MHC class II expression on antigen presenting cells. An excess of free IFN-γ could enhance antigen presentation by cells that have not contacted the immunising antigen, leading to a negative effect on immunogenicity and injection into a site different from antigen injection could actually suppress the response and the adjuvanticity of the cytokine fell as the temporal spacing between the two injections was increased (Heath, 1995). These experiments have yet to be extended to see whether the observation is the same for different situations and vaccines but IFN-γ is effective over a wide dose range and has been effective in clinical trials (2 million units) administered s.c. with an antigen against hepatitis B (Quiroga *et al.*, 1990) increasing the response rate and antibody titres to the recombinant antigen.

A typical s.c. dose is approximately 5000 units of interferon-gamma to give optimal protection. IL-2 must be administered for a continuous period of 5 days following vaccination or must be emulsified with the vaccine. IL-1 is at its most effective 2h post-vaccination whereas IFN-γ is most effective when administered with the antigen. Intra-nasal administration to mice of inactivated influenza virus with muIFN-γ resulted in increased survival rate after challenge compared with vaccine alone (Cao *et al.*, 1992). The increased IgA and IgG levels in lung washes and serum were attributed to the adjuvant effects of IFN-γ.

Human trials of IFN-γ with a synthetic peptide malaria sporozoite vaccine failed to enhance antibody production, contrary to the effects of IFN-α (Sturchler *et al.*, 1989). Genetic make-up of the host, antigen dose, timing and route of administration were suggested explanations for the lack of adjuvanticity. In the early stages of infection,

antibody production is enhanced with IFN-γ. In the late stages, antibody production may decrease due to the more efficient neutralisation of the antigen at the local site. The site and timing of antigen and tissue-specific cytokine delivery are important to induce optimal immune responses and to avoid unwanted side-effects. Playfair & DeSouza (1987) reported a strong adjuvant effect against blood stage malaria in a murine model of vaccination when administered with the vaccine by subcutaneous or i.p. routes. Also, by using delivery vehicles, such as microspheres or liposomes, it is possible to selectively deliver cytokines and antigens to the desired site of action, localising the effect of cytokines within the same cell.

Regardless of which cytokine is used to enhance antigen presentation of T- and B-cell development and expansion, a controlled and selective cytokine delivery with controlled timing may reduce toxicity of these compounds and improve their therapeutic and practical value in providing a safe and effective novel class of adjuvants.

1.5 AIMS AND OBJECTIVES OF THE STUDY

Conventional vaccine delivery requires multiple injections and is associated with high drop-out rates, especially in developing countries. Aluminium preparations are the only adjuvants approved for use in humans. Manufacturing difficulties and a specificity of action, limit its potential in vaccine delivery.

Microencapsulation involves the coating of a bioactive agent in a protective polymer coating. Three important advantages of vaccine delivery in microspheres are (a) a substantial potentiation of the antibody response (b) the ability to construct formulations that release multiple discrete pulses of vaccine mimicking booster immunisations and (c) the ability to effectively deliver vaccines to the mucosal immune inductive tissues. Results indicate that biodegradable microspheres have enormous potential in the generation of antibodies in the secretory immune system. While polylactides and glycolides may remain the polymers of choice for vaccine delivery for some time, other polymers should be considered to increase the uptake of microspheres, particularly more hydrophobic ones.

Following the line of investigation already carried out in our laboratory (e.g. Alpar *et al.*, 1989; Almeida *et al.*, 1993; Alpar & Almeida, 1994), this project was designed to study the physico-chemical properties of biodegradable microspheres and their influence on the immune response generated. Particles were designed with properties

which would promote the enhancement of immune response following both systemic and/or mucosal delivery. The co-encapsulation of different proteins within the one preparation, whether to enhance release rate or for an adjuvant effect, will affect the properties of the microspheres generated. The immune response generated would also be affected by these properties and full characteristion is essential to enable interpretation of *in vivo* results. A number of biodegradable polymers, with different properties, were used for encapsulation of model drugs, antigens and adjuvants to enable optimastion of dosing schedules.

2.0 METHODS FOR MICROSPHERE PREPARATION AND ANALYSIS

2.1 MICROSPHERE PREPARATION

All chemical reagents not specified in the text were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.) and Fisons (Loughborough, Leics., U.K.) and were of Analar grade or equivalent

2.1.1 Preparation of double emulsion (w/o/w) microspheres

The primary emulsion was prepared using an aqueous solution containing the protein (2.5-100mg) and emulsifying agents and an organic solution containing the polymer. Unless otherwise stated, the emulsifiers were 0.05% w/v methylcellulose (400cps) (Aldrich Chemical Co., Gillingham, Dorset, U.K.) and 0.5% w/v polyvinyl alcohol (Mw 13-23kDa, 88% hydrolysed) or 0.5% w/v polyvinylpyrrolidone (Mw 10kDa) (Aldrich Chemical Co., Gillingham, Dorset, U.K.). The organic solution consisted of 250mg of the polymer in 5ml of dichloromethane (HPLC grade). This had been previously cooled in an ice bath for one hour. 1.25ml of aqueous phase was mixed at high speed using a Silverson homogeniser STD2 (Silverson Machines, Chesham, Bucks., U.K.) with a 3/8" mini-micro probe (Silverson Machines, Chesham, Bucks. U.K.). Unless otherwise stated, a stirring speed of ≥16000rpm was used for 4 minutes.

The resultant emulsion was then emulsified into 75ml of an aqueous 1.5% w/v solution of polyvinyl alcohol (as above) for 8 minutes at 16000rpm using a 1" tubular probe (Silverson Machines, Chesham, Bucks., U.K.). The w/o/w emulsion was stirred, on a stirring plate, for up to 18 hours (overnight) to allow the solvent to evaporate. The particles were collected by centrifugation at 14000rpm for 40 min (JA-14 rotor, Beckman Centrifuge; Beckman Instruments Ltd., Bucks., U.K.) and washed three times in double-distilled water. The microspheres were freeze-dried (Edwards Modylo freeze drier; BOC Ltd., Sussex, U.K.) and stored in a desiccator at room temperature,

or at 4°C as appropriate, before use. The yield was calculated form the ratio of the weight of microspheres obtained to the total amount of drug and polymer used in the preparation (see section 2.2).

Unless otherwise stated, all formulations were prepared using this or a scaled down version of this formula.

2.1.2 Preparation of single emulsion (o/w) microspheres

The protein (2.5-50mg) was suspended in a solution containing 250mg of polymer and 5ml of dichloromethane. This was added, drop by drop, to 75ml of an aqueous solution containing 1.5% PVA w/v (as before) and homogenised for 8 minutes at 16000rpm. Both solutions had previously been cooled in an ice bath for one hour. The final steps are as above.

Unless otherwise stated, all formulations were prepared using this or a scaled down version of this formula.

2.1.3 Preparation of microspheres using solvent extraction

This process is not often used for the production of PLA microspheres (Conti *et al.*, 1992) and can be considered an adaptation of the solvent evaporation method. Formation of the emulsion was as for solvent evaporation (sections 2.1.1 and 2.1.2) and the emulsion was rapidly added, with mixing, to a solution of 50% v/v ethanol in water, a solution which is immiscible with the polymer. The mixture was stirred for one hour to extract all the solvent and allow precipitation of the spheres. They were collected and washed as before. The preparation procedure is affected by the same factors that affect solvent evaporation but according to some authors, it can produce more porous microspheres due to the rapid removal of the solvent (Pavanetto *et al.*, 1990; Sato *et al.*, 1988).

2.1.4 Adsorption of proteins to the surface of microspheres

50mg of blank particles were incubated in a shaking waterbath overnight (100 cycles *per* minute) at 25°C in a solution of protein (250-500mg) in 2ml saline or phosphate buffered saline (PBS). Controls were run using protein solutions in the same quick fit tubes incubated under the same conditions. The particles were centrifuged at 15000rpm for 35 minutes (JA-14 rotor, Beckman Centrifuge, as before) and the supernatant

carefully decanted. The particles were dried in a drying piston at room temperature and under a pressure of 1 atmosphere. The supernatants were analysed for protein content using a BCA method accounting for the protein adsorbed to the container surface (see section 2.7).

2.2 DETERMINATION OF THE PROTEIN CONTENT IN MICROSPHERES

(a) The percentage of protein encapsulated *per* unit weight of spheres was determined by a method similar to that described by Hora *et al.* (1990). 1mg of spheres was incubated *per* ml of 0.1M NaOH, (as a strong base to attack ester linkages of the polymeric backbone) containing 5% w/v sodium dodecyl sulphate (SDS), to aid protein solubilisation, for at least 3 hours, until the medium lost all turbidity. The solution was then neutralised to pH 7 with addition of 1M HCl. The resultant solution was analysed for protein using a BCA assay procedure against a series of protein standards prepared in 0.1M NaOH containing 5% w/v SDS. All samples were assayed in triplicate and results are the mean of three determinations.

(b) For slower degrading polymers, polyhydroxybutyrate and poly(DTH carbonate), this hydrolysis was not rapid enough for our purposes and loading was determined by dissolving approximately 1mg of spheres *per* ml DCM. The protein was then extracted with 3x3ml washes with double-distilled water and a BCA assay used to determine protein concentration against a series of standards prepared in double-distilled water. All samples were assayed in triplicate and results are the mean of three determinations.

From these results, the % w/w protein encapsulated *per* dry weight of microspheres could be determined and the encapsulation efficiency was determined.

$$\text{Encapsulation efficiency} = \frac{\text{Actual drug loading \% w / w}}{\text{Theoretical drug loading \% w / w}} \times 100$$

2.3 *IN VITRO* RELEASE STUDIES

2.3.1 Release in phosphate buffer

The release of protein from microspheres was performed by incubation of 1mg of spheres *per* ml (generally 10mg/10ml) of 20mM phosphate buffer (pH 7.5) containing 0.02% w/v sodium azide, in glass sample tubes. These were incubated at 37°C in a

shaking water bath. Suitable aliquots of solution (typically 5%) were removed at periodic intervals and centrifuged at 13000rpm (Micro Centaur bench top centrifuge) in ependorff tubes. A sample of the supernatant was removed for analysis and the sediment resuspended in an equivalent volume of fresh buffer and returned with washing to the dissolution vessel. Dilution of the dissolution medium was corrected for in all calculations. All release studies were performed in triplicate and results are the mean of three samples (±s.d.).

After completion of the release studies, the remaining polymer was dried and digested using the method described previously. The amount of protein present was determined using a BCA protein assay. Protein recovery from the remaining spheres was good with all batches having recovery levels between 90 and 105%. Most were in the region of 95-100% with low loaded batches showing the least reproducibility, possibly due to the significantly lower levels of protein involved.

2.3.2 Release in simulated gastric media

The release of protein from microspheres was evaluated in simulated gastric media, USP XXI (table 2.1).

Table 2.1. Simulated gastric media, USP XXI

Ingredient	Quantity
NaCl	2.0g
pepsin	3.2g
HCl	7ml
double-distilled water	to 1 litre

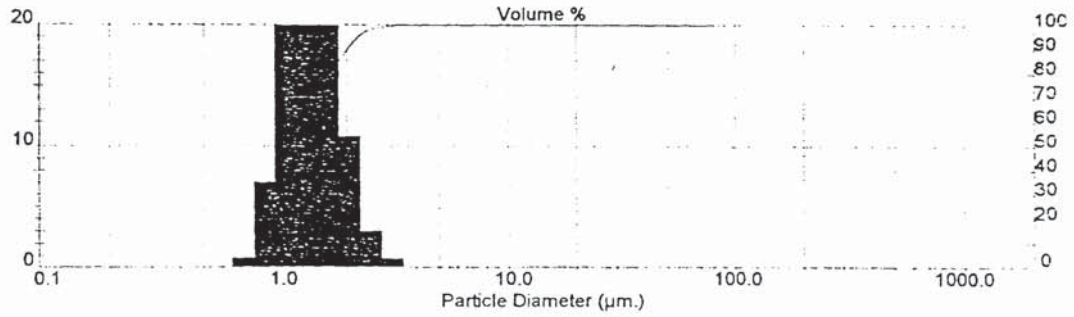
The resultant pH of the solution was ~1.2 and it was prepared immediately before use. 10mg of the microspheres were incubated in the medium for up to 3 hours at 37°C on a shaking water bath. The supernatant was analysed for protein content using ^{125}I-labelled proteins and the protein released expressed as a percentage of the loading. All release studies were performed in triplicate and results are the mean of three samples (±s.d.).

2.4 SCANNING ELECTRON MICROSCOPY

The surface morphology of the particles was studied using scanning electron microscopy. Samples were prepared by mounting on aluminium stubs using carbon tape and coated with gold for 1.5 minutes under an argon atmosphere (Emscope SC500). Under magnification using a Cambridge Instruments Stereoscan 90B, the surface of the particles could be studied. Photomicrographs were taken with a Nikon F301 35 mm camera.

2.5 PARTICLE SIZE DETERMINATION

The lyophilised particles were redispersed in double distilled, 0.22μm filtered water and sized by laser diffractometry using a Malvern MasterSizer/E (Malvern Instruments, Malvern, U.K.). The instrument was fitted with a 45mm angle lens and a flow cell and the presentation used was for polystyrene in water (2PAD) and an example of a typical printout and statistics are illustrated in figure 2.1.



Malvern Instruments Ltd.
Malvern, U.K.

MasterSizer E Ver. 1.2a
Serial No. 7241

01 Dec 94

	Mean	Stan. Dev.	Skew	Kurtosis
Volume	1.47	0.410	1.0593	1.7257
Surface	1.37	0.372	1.0836	2.0042
Length	1.28	0.337	1.0858	2.1153
Number	1.21	0.306	1.0815	2.1253

D [4, 3] = 1.47 um
D [4, 2] = 1.42 um
D [4, 1] = 1.37 um
D [4, 0] = 1.33 um

D [3, 2] = 1.37 um
D [3, 1] = 1.33 um
D [3, 0] = 1.29 um

D [2, 1] = 1.28 um
D [2, 0] = 1.24 um

D [1, 0] = 1.21 um

Percent	Size (um)
10.0	1.03
20.0	1.14
50.0	1.41
80.0	1.75
90.0	2.01

Mode (um)
1.40

Figure 2.1. Typical printout from Malvern MasterSizer/E for PLA microspheres

2.6 DIFFERENTIAL SCANNING CALORIMETRY

Thermograms of drug, polymers and microspheres were produced using a computer-interfaced Perkin-Elmer System 4 differential scanning calorimeter. Temperature calibration was carried out using an Indium standard. Samples (4-8mg) were sealed into aluminium pans and DSC measurements were run from 25° to 400°C against an empty pan. All tests were run under a nitrogen atmosphere at rates of 10°C/min for heating and at 320°C for cooling. For quench cooled samples, samples were heated to 200°C, then rapidly cooled to -40°C and heated from -40°C to 250°C. Heat, cool and hold functions were controlled by the computer and routine analysis carried out using the computer software. The glass transition temperature was taken as the midpoint of the transition curve.

2.7 BICINCHONINIC ACID PROTEIN ASSAY

For accurate determinations of low protein content, a bicinchoninic acid (BCA) assay procedure was followed (Smith *et al.*, 1985). Proteins react with alkaline copper II to produce copper I. Two molecules of the BCA reagent (figure 2.2) react with Cu^{1+} to form a copper/peptide chelate.

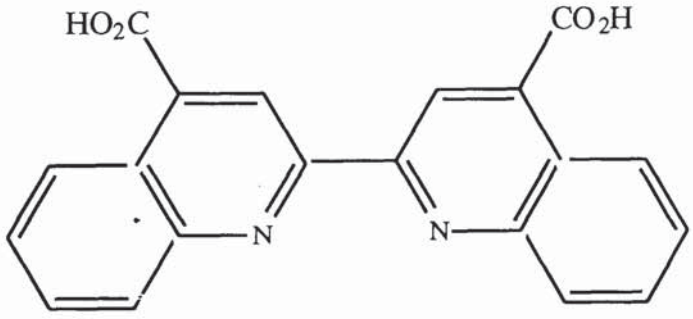


Figure 2.2. Structure of bicinchoninic acid

The product of the reaction (figure 2.3) is water-soluble and has an intense purple colour.

500ml of Reagent A was prepared, the solution being stable for up to 6 months at room temperature (table 2.2)

Table 2.2. Formula for BCA reagent A

Ingredient	Quantity
BCA disodium salt	5.00g
$Na_2CO_3 \cdot H_2O$	10.00g
Na_2 tartrate	0.80g
NaOH	2.00g
$NaHCO_3$	4.75g
double-distilled water	to 500m1

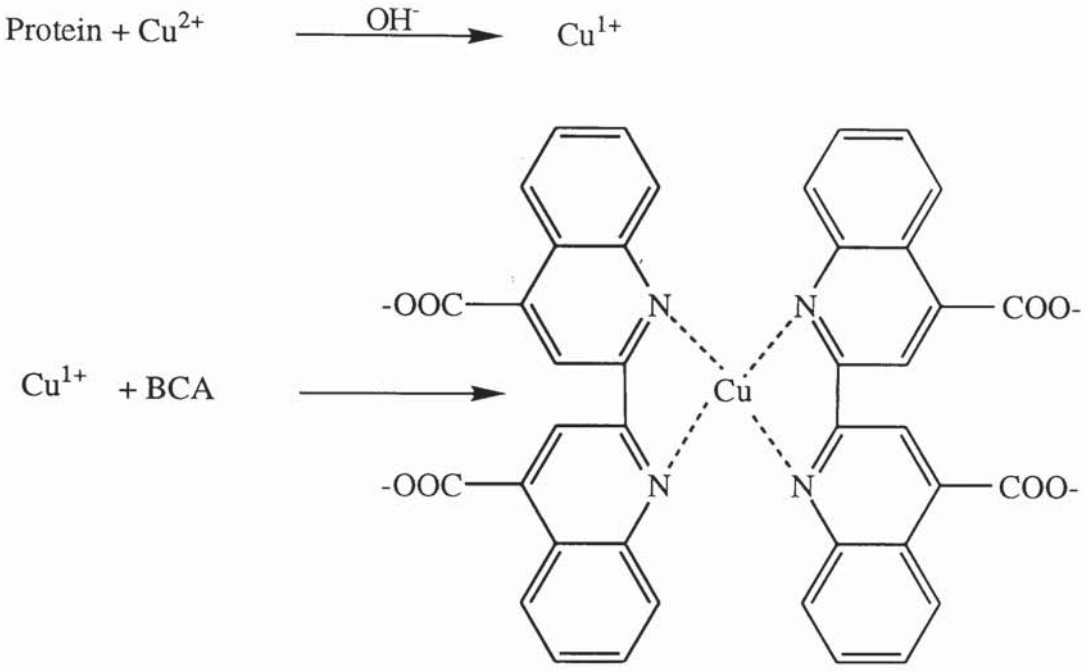


Figure 2.3. Formation of purple complex with BCA and cuprous ion

The pH of the solution was adjusted to 11.25 with stepwise addition of 50% w/v NaOH or $NaHCO_3$ for maximum rate of colour development. Reagent B consists of 4% $CuSO_4 \cdot 5H_2O$. These reagents are stable for up to six months at room temperature. To prepare the working reagent 50 parts of reagent A are added to 1 part of reagent B. This solution is initially apple green but develops the purple colour on standing.

200µl of the working reagent was added to 10µl of the protein sample on a 96 well microtitre plate (Fisons, Loughborough, U.K.). The solutions were mixed for 1 minute and incubated at 60°C for one hour. The sensitivity of the assay was heightened by extending the incubation period from 30 minutes recommended to one hour (Smith *et al.*, 1985).

A series of protein standards was prepared in the same solution as the sample for each assay run. The concentrations of protein used were in the range of 10-250µg/ml. After cooling to room temperature, the absorbances were read using an Anthos 2000 Plate Reader (Anthos Labtec Instruments, Austria) at 570nm - the recommended value of 562nm could not be produced with this instrument. Blanks were run under the same conditions and these values were subtracted from the absorbance of the standard or the unknown. Calibration curves were constructed by plotting net absorbance at 570nm versus protein concentration and the concentrations of the unknowns were determined. To ensure accuracy at such low protein levels, each absorbance is the average of at least 4 readings.

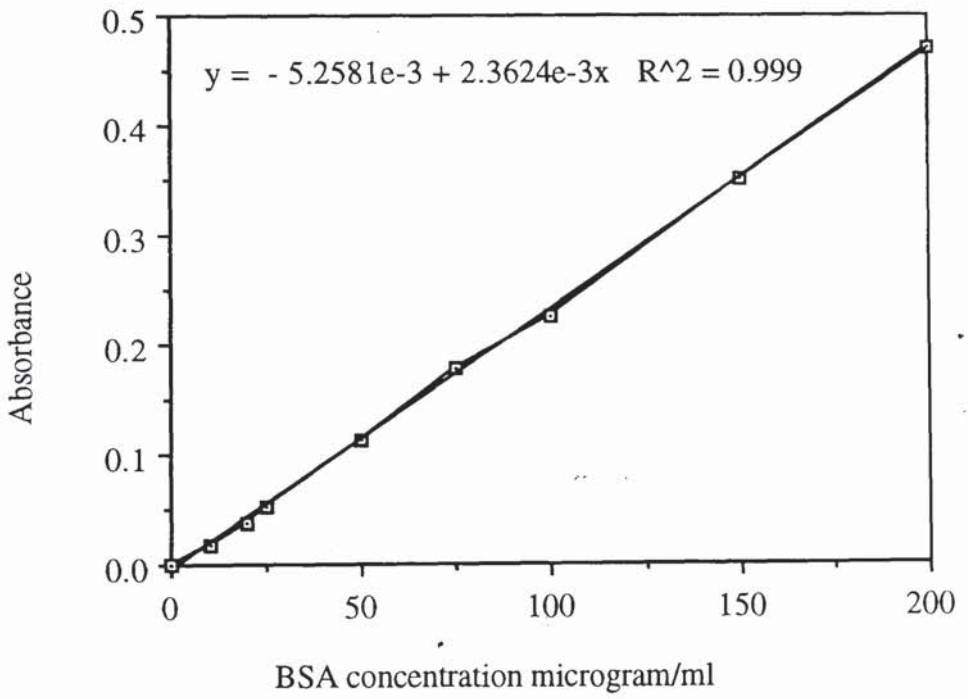


Figure 2.4. Calibration curve for the estimation of proteins by a bicinchoninic acid assay

2.8 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis is the movement of colloidal particle with respect to the liquid when a potential difference is maintained across the solution. Electrophoresis techniques are based on the differences in the electrically induced migration of a protein in a sieve-like gel based on the molecule's size and/or net charge. Proteins contain both basic and acidic groups in the molecule and in alkaline solution the acidic group COOH will ionise to COO^- so that the protein carries a negative charge. In acid solution the basic NH_2 will ionise to NH_3^+ so the protein carries a positive charge. At a certain definite pH, the isoelectric point, the total number of positive charges will equal the total number of negative charges and the net charge is zero. The protein is least soluble at this pH and is readily precipitated.

The sample is dissolved or suspended in buffer and the supporting medium is saturated with the same buffer. If a constant state of ionisation is maintained then a uniform charge mass ratio is produced and separation is achieved solely on molecular size. The proteins were boiled in an equal volume of buffer for ten minutes prior to electrophoresis to break any disulphide bonds. The process also attaches an ionisable group at regular intervals and aids solubilisation of the protein (Anwar *et al.*, 1983). All samples and reference standards were run under the same conditions. All chemicals used were of electrophoresis grade.

Table 2.3. Sample buffer formulation for SDS-PAGE

Sample buffer	Quantity
10% w/v SDS	6ml
50% v/v glycerol	4ml
double-distilled water	8.72ml
0.062M tris(hydroxymethyl)-aminomethane (Tris.) (pH 6.8)	1.24ml
5% w/v bromophenol blue	40µl

75µl of mercaptoethanol was added to 450µl of sample buffer and gels were prepared by a modification of the method described by Lugtenberg *et al.* (1975). A 7.5%, 10% or 15% running gel was used on Biorad Mini-Protean apparatus (Biorad, Hertfordshire, U.K.) (table 2.4).

Table 2.4. Running gel formulations for SDS-PAGE

Ingredients	% gel (ml)		
	7.5	10	15
1M Tris. (pH 8.7)	10.00	14.93	20.00
2%w/v bis-acrylamide	3.57	2.67	1.73
double-distilled water	14.93	8.87	14.93
30% w/v acrylamide	11.00	13.33	3.13

To 10ml of the above solution was added:

Ingredients	% gel (ml)		
	7.5	10	15
20% w/v SDS	50µl	50µl	50µl
ammonium persulphate (AMPS)	10mg	10mg	10mg
N,N,N,'N'- Tetramethylethylene diamine (TEMED)	5µl	5µl	5µl

A stacking gel was used to concentrate the proteins (table 2.5).

AMPS solutions must be freshly prepared and added simultaneously with the TEMED to initiate polymerisation. The solutions were stored at 4°C protected from light for up to two weeks and were degassed in a vacuum desiccator prior to use.

The glass plates were separated with 0.5mm spacers and the running gel poured between the plates. The gel was protected from air by addition of a small amount of water. This was left to set for about 20 minutes, the water removed and the top of the gel was rinsed. The stacking gel was cast and a teflon comb inserted to provide the

wells. The comb was removed when the gel was set and electrode buffer placed in the wells until the gel was ready for use.

Table 2.5. Stacking gel formulation for SDS-PAGE

Ingredients	Quantities
1.0M Tris. (pH 6.9)	1.25ml
30% w/v acrylamide	1.7ml
20% w/v SDS	50µl
double-distilled water	6.35ml
AMPS	10mg
TEMED	3µl
2% w/v bis-acrylamide	0.7ml

5x Laemmli buffer was prepared and stored at 4°C prior to use (table 2.6).

Table 2.6. Five times concentrated Laemmli buffer

Ingredients	Quantity (g)
Tris.	30.0
glycine	144.0
SDS	5.0
double-distilled water	to 1 litre

The integrity and stability of the protein encapsulated in the microparticles was analysed by SDS-PAGE. Protein that was released from the microspheres was collected from the supernatant of the samples used for *in vitro* release experiments. Where appropriate, the samples were concentrated prior to analysis. Samples of released proteins, unencapsulated (native) proteins and molecular weight standards (Sigma) were solubilised using Laemmli sample buffer.

5µl of sample or reference, either free protein or molecular weight standards, was applied *per* well and a constant current of 15mA was applied into the separating gel which was increased to 30mA through the running gel until the tracking dye approached the bottom of the plate. The gel was removed and stained for two hours in a solution of 0.1% w/v Coomassie blue in 50% v/v methanol in water containing 10% v/v glacial acetic acid. The gel was destained in 50% v/v methanol in water containing 10% v/v glacial acetic acid, washed and dried onto filter paper using a gel drier (Biorad Gel Drier, Model 583).

2.9 DETERMINATION OF pH CHANGES IN PHOSPHATE BUFFER DURING POLYMER DEGRADATION

The pH changes in a 20mM phosphate buffer (pH 7.5) were monitored for 40 days after incubation with various microsphere formulations. Microspheres (10mg) were incubated in phosphate buffer, containing 0.02% w/v sodium azide, at 37°C for 40 days in a shaking water bath. pH changes were monitored over this period using a digital pH meter (WPA CD 660 Digital pH Meter; WPA Linton, Cambridge, England) appropriately calibrated with ColourKey Buffer Solutions (BDH Ltd., Poole, Dorset, U.K.). Batches of microspheres were manufactured to produce similar sizes and loading to minimise any effects due to drug loading and particle size.

2.10 DETERMINATION OF RESIDUAL DICHLOROMETHANE CONTENT IN MICROSPHERE FORMULATIONS

Approximately 20mg of microspheres was accurately weighed and dissolved in 1ml of HPLC grade chloroform. This was prepared immediately prior to injection in the gas chromatograph (Pye Unicam Series 304). The injection volume was 2µl and the column used for dichloromethane detection was a 1 metre 0.8% THED on Carbopack C 80/100 mesh and conditions used were as detailed in table 2.7.

At a flame ionisation amplitude of $1x10^2$ attenuation, standards of DCM in chloroform were used to construct a calibration curve for the instrument in the required region (see section 3.3.9). Samples were run under the same conditions and the chromatograms obtained were plotted using an Omniscribe Series D5000 Recorder at a chart speed of 1cm/min.

Table 2.7. GC settings for detection of DCM in chloroform

Parameter	Temperature °C
Column	60
Injector	70
Detector	120

2.11 HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

The distinctive nature of proteins having hydrophobic and hydrophilic areas enables separation using HIC techniques. It has high resolving power and is usually used with a decreasing ionic salt concentration gradient (Shaltiel, 1974). HIC makes use of the hydrophobic areas with the affinity of the proteins for the hydrophobic matrices proportional to the chain length of the derivative. Using an adaptation of procedures developed for determining the hydrophobic surface characteristics of bacteria (Mozes & Rouxhet, 1987; Smyth *et al.*, 1978), it is possible study the hydrophobicity of microsphere surfaces. The main work on determination of microparticle surface hydrophobicity has been carried out by Müller (1991). The procedure employed was based on this method (Alpar & Almeida, 1994) using a series of agarose derivatives to distinguish between batches of slightly differing hydrophobicities. The stationary phases used were agarose (sepharose), propyl-agarose, pentyl-agarose, hexyl-agarose and octyl-agarose (Sigma). These are neutral gels, manufactured to minimise any electrostatic interactions, maximising the contribution of hydrophobic interactions (Hjertén *et al.*, 1974). The gels were washed several times in double-distilled water, suspended in 0.6M NaCl and degassed before packing. The pH of the salt solution was adjusted to 7.4 using dilute NaOH to allow maximal differences in retention between the least hydrophobic (sepharose or agarose) and the most hydrophobic (octyl-agarose) phases for a PLA preparation (Almeida, 1993).

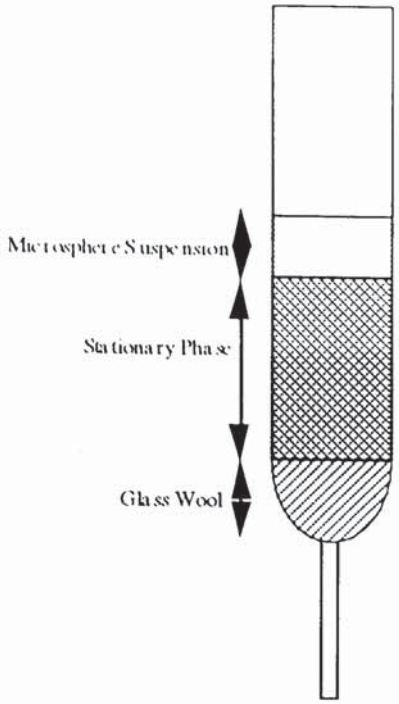


Figure 2.3. Diagram of a HIC column adapted from a glass Pasteur pipette

The columns were prepared by layering 30mm of the gel onto a glass wool sinter in a Pasteur pipette (figure 2.3). This is equivalent to approximately 1ml packed gel volume. The columns were washed with double-distilled water and then with 10ml of 0.6M NaCl solution. A suspension of the microspheres was prepared in 0.6M NaCl (pH 7.4) and adjusted to an OD_{600} of 0.5 (Phillips UV/Vis spectrometer PU 8730) and 1ml volumes loaded onto the columns. Three identical columns were run for each microsphere sample on each type of agarose used. The eluent was collected in 1ml plastic cuvettes (Fisons) and the columns washed with 2 x 1ml of 0.6M NaCl and 2 x 1ml volumes of 0.1% Triton X-100 (Sigma) in double-distilled water. The OD_{600} of the five 1ml fractions were compared to the OD_{600} of the original 1ml suspension and expressed as percentage decrease in OD_{600}. Graph of cumulative percentage eluted were plotted and final retention values calculated. The hydrophobicity of the particles was compared to surfactant-free polystyrene latex controls of similar sizes (Bangs Laboratories) and all results are comparative values between samples run under the same conditions.

2.12 DETERMINATION OF RESIDUAL PVA CONTENT IN MICROSPHERES

Residual PVA in microsphere formulations was determined using a modification of a method described by Alléman *et al.* (1993). Iodine complexes with PVA in solution in the presence of boric acid to form a stable coloured complex which has an absorbance maximum at 690nm (Finley, 1961).

Approximately 40mg of spheres was accurately weighed and dissolved in 5ml of chloroform (HPLC grade). This was sonicated for 10 minutes and filtered on a cellulose filter (3.0μm pore size, Whatman). The filter was washed with a further 10ml of chloroform and dried. To dissolve the PVA on the filter, it was boiled in 20ml distilled water. A 1ml aliquot of this PVA solution was added to 3ml boric acid (4%w/v) and 0.6ml of a iodine solution (containing 1.27% w/v iodine and 2.5% w/v potassium iodide). The volume was adjusted to 10ml with double-distilled water. The absorbance was measured at 690nm (Phillips UV/Vis spectrometer PU 8730) and the percentage of PVA remaining calculated from a calibration curve. Values given are the mean of three assays (±s.d.).

2.13 ZETA POTENTIAL DETERMINATION

The relationship between particle surface charge and phagocytosis is complicated but it is generally accepted that uncharged microspheres display reduced uptake by the RES (Tabata & Ikada, 1990). The charge carried by the microspheres can be determined by measuring the particle mobility in an electric field where

$$\text{Electrophoretic mobility } (\mu_E) = \frac{\text{Particle mobility (V)}}{\text{Field strength (H)}}$$

The electrophoretic mobility can be converted to a zeta potential using the Debye-Huckel parameter (k) which is dependant on the electrolyte concentration and the particle radius (a). At large values of ka the Smoluchowski equation can be used and electrokinetic or zeta potential (ζ) can be measured on the basis of electrophoretic mobility and calculated using the following equation:

$$\zeta = \mu_E \frac{4\pi\eta}{\varepsilon}$$

where η is the viscosity and ε is the respective dielectric constant. Zeta potential measurements can be taken as an indirect measure of the surface charge (Müller *et al.*, 1986).

Zeta potential measurements were analysed by laser Doppler anemometry using a Zetameter (Malvern Zetamaster, Malvern Instruments Ltd., Malvern, England) provided with a photon correlation spectrometer. The microsphere preparations were dispersed in two buffer systems, 10mM phosphate buffer and 10mM phosphate/citrate buffer (pH 7.0) and each value is the mean of five determinations (±s.d.). It must be remembered that zeta potential is strongly pH dependant and represents an overall measurement of surface interactions with the medium, rather than the surface charge. Therefore it is important to report the solution in which the microspheres were suspended.

2.14 GEL PERMEATION CHROMATOGRAPHY (GPC)

The molecular weights of the PLA raw materials and microspheres during degradation were studied using gel permeation chromatography. An Altex model 110A adjustable flow rate pump preceded by a sintered metal frit was used to pump GPC grade chloroform at 1ml/minute around the system. Two 300 x 7.5mm, 500Å pore size, 5µm mixed pore highly cross-linked spherical macroporous polystyrene-divinylbenzene matrix (PLGel) columns (Polymer Laboratories Ltd, Shropshire, U.K.) were used in series and were protected by a 50 x 7.5mm 10 µm mixed pore guard column (PLGel) (Polymer Laboratories Ltd, Shropshire, U.K.). A Pye Unicam LC3 UV detector was tuned at a wavelength of 238nm and was connected in parallel to a Gallenkemp Euroscribe chart recorder and a Hewlett Packard 3390 A integrator. Detector sensitivity was set at a range value of 0.08 (arbitrary units). Microspheres of PLLA, PDLA and PLGA were incubated in 20mM phosphate buffer for 3, 7 and 28 days. They were maintained in a shaking water bath at 37°C. The particles were freeze-dried (Edwards Modylo freeze drier; BOC Ltd., Sussex, U.K.) and stored in a desiccator until analysed. The samples were dissolved in chloroform, filtered and injected using a 100µl sample size through a Rheodyne injector valve (Waters, CA, U.S.A. 7125).

Standardisation of the GPC system was achieved using narrow-MW polystyrene standards (EasiCal, Polymer Laboratories Ltd, Shropshire UK 2010-0501) inert PTFE strips coated with polystyrene (~5 mg) were immersed in 50ml of chloroform to give a polystyrene concentration of 0.010 % w/v. The kit contained two types of strips (A and B) each representing Mw values of 640, 9375, 66000, 321000, 3066000 and 3250,

28720, 158180, 1028500, 8520000 respectively. 100µl aliquots were injected into the GPC system to elucidate the retention time for each Mw value and a calibration curve was obtained.

GPC is in simplest terms a mechanism of solute separation with molecular size as the discriminating factor. Sample molecules permeate the stationary phase to different degrees and are thus retained within the column for periods of time proportional to their molecular size. Columns are tightly packed with a gel or some other porous material and completely filled with solvent (the mobile phase). The same solvent is used to dissolve the sample before introducing it into the column. Within the column the pore size of the packing particles determines the molecular size range within which separation occurs. Using the appropriate type of packing material it is possible to separate soluble molecules with molecular weights ranging from 100 to several millions. Traditionally, GPC has been used for the analysis of molecular weight distributions of synthetic polymers. Data treatment can involve the calculation of molecular weight averages imparting information about chain length and the extent of cross linking within a polymer. The molecular weight averages (Mn, Mw) indicate the number and length (or weight) of the polymeric chains formed during manufacture. Mn is the number-average molecular weight, which is the molecular weight of the average chain length in a polymer sample. Mw refers to the molecular weight equal to the modal molecular weight of polymer chains, known as the weight average molecular weight. As Mn represents the molecular weight of the average chain length in a polymer sample, and Mw refers to the molecular weight equal to the modal molecular weight of the polymer chains the value of Mw is always larger than Mn except in the case of a truly monodisperse system where the values are identical. Manual calculation of Mn and Mw can be accomplished by digitisation of chromatograms (Jani *et al*., 1990).

The system used here for the detection of PLA polymers, however, was not sensitive at the low molecular weights involved to allow detailed analysis of the results. It does allow the study of the molecular weight distribution pattern and changes that occur over time but not the calculation of accurate values as calibration was not possible over the desired range.

3.0 MICROENCAPSULATION OF A MODEL ANTIGEN IN PLA MICROSPHERES

3.1 INTRODUCTION

As BSA has been a model protein for many physical chemistry studies, it is well characterised (Peters, 1975). It has a molecular weight of 66kDa and consists of 581 amino acid residues. Albumin is characterised by a relatively small size but good solubility. It is ideal as a model antigen, producing good titres and its stability, flexibility and charge have been extensively examined. It was selected as a model antigen in order to study microsphere formation using a number of techniques and polymers. PLA and PLGA polymers are predominately used for the preparation of microspheres by solvent evaporation techniques and this chapter presents a methodological summary of the effects of process and manufacturing parameters on microsphere formation.

Due to the ability of BSA to interact with the organic polymer phase to form a semisolid interfacial film, stabilising the emulsion (Nihant *et al.*, 1994), results cannot be directly extrapolated to other proteins with different physicochemical properties. However, formulation studies regarding physicochemical properties of the polymer are still useful.

3.2 MATERIALS AND METHODS

The biodegradable PLA polymers used in these experiments were poly(L)lactide, Mw 2000 (Resomer L104), poly(DL)lactide, Mw 2000 (Resomer R104) and poly(DL)lactide-co-glycolide 50:50 and were purchased from Boehringer Ingelheim *via* Alpha Chemicals (Berkshire, U.K.). Bovine serum albumin (BSA), (fraction V powder, 98-99% pure) was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). ^{125}I-bovine serum albumin was supplied by ICN Biomedicals Inc. (High Wycombe, Bucks., U.K.) in a 0.1M potassium phosphate buffer, pH7.5 and was stored at -20°C until use, in accordance with supplier's specifications. Surfactant-free polystyrene particles for surface characterisation studies were obtained from

Brookhaven Instruments Ltd. (Worcestershire, U.K.). All other chemical reagents not specified in the text were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.) and Fisons (Loughborough, Leics., U.K.) and were of Analar grade or equivalent. Methods for preparation and analysis of microspheres are detailed in chapter 2.

3.3 RESULTS AND DISCUSSION

3.3.1 Microsphere formulation and loading

3.3.1.1 The effect of stirring rate on microsphere formation

Throughout this study, the aim was to produce microspheres of approximately 1μm in size, promoting maximal absorption across the GI tract (Eldridge *et al.*, 1990; Jani *et al.*, 1989). A number of parameters influence particle size (Jeffrey *et al.*, 1991). Mixing speeds has a great influence on particle size formation and generally decreases with increased mixing speed. This is associated with a narrowing of the particle size distribution (Benita *et al.*, 1984). Using a Silverson homogeniser at top speed (≥16000) allows production of smaller droplets in the internal phase of the emulsion. When observed under the electron microscope, the size frequency histograms demonstrate a log normal distribution (figure 3.1).

Under the conditions employed, formation of smooth, spherical microspheres was possible (figure 3.2). The successful entrapment of BSA into these particles enabled extensive investigation into a variety of microsphere characteristics.

3.3.1.2 The effect of polymer molecular weight on microsphere formation

Particle size increases with an increase in polymer molecular weight (table 3.1). An increase in polymer molecular weight increases the viscosity of the polymer dissolved in the organic phase which may result in an increased droplet size and consequently in a larger particle diameter. Similarly, an increase in polymer concentration of the organic solution would impede mixing efficiency by increasing viscosity and result in a larger particle diameter (Watts *et al.*, 1990). Both molecular weights of polymer produce particles with a spherical shape and smooth surface.

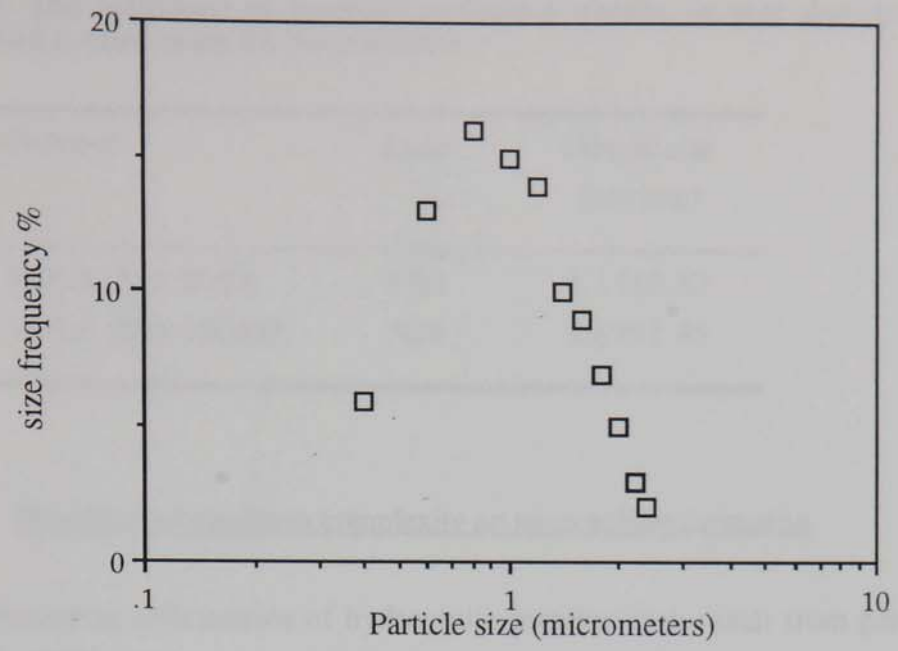


Figure 3.1. Particle size distribution of PLLA microspheres formed using a single emulsion method (n>100)

Figure 3.2. Scanning electron micrograph of PLLA microspheres loaded with 10.2% w/w BSA formed by a single emulsion method

Table 3.1. The influence of polymer molecular weight on size distributions of microparticles formed using the same method

Polymer	Load	Particle size (μm±σg)
PDLA (Mw 2000)	5.09	1.15±0.52
PDLA (Mw 156000)	5.26	2.67±1.73

3.3.1.3 The effect of emulsion complexity on microsphere formation

Low encapsulation efficiencies of hydrophilic proteins may result from partitioning from the hydrophobic organic phase into the external aqueous phase (Bodmeier & McGinity, 1987). Therefore, low molecular weight, water-soluble molecules could not be successfully loaded into microspheres formed using an o/w emulsion, solvent evaporation technique. Encapsulation of water soluble drugs using a double emulsion technique may lead to increased drug entrapment (Ogawa *et al.*, 1988).

Microspheres prepared by a w/o/w emulsion method are generally only slightly larger than those prepared by the o/w method due to the presence of a more complex structure during emulsion preparation (figure 3.3). The particles produced are spherical with a smooth surface (figure 3.4). It is known that the rate of solvent diffusion has a determining effect on the surface morphology of the microspheres (Arshady, 1991). Cooling the organic and external aqueous phases prior to homogenisation increases the viscosity of the emulsion, promoting stability and reducing the evaporation rate of DCM. The procedure used allows a slow evaporation of the solvent leading to the formation of microspheres with a smooth surface morphology. The internal aqueous phase is maintained at room temperature in an effort to minimise the formation of larger droplets.

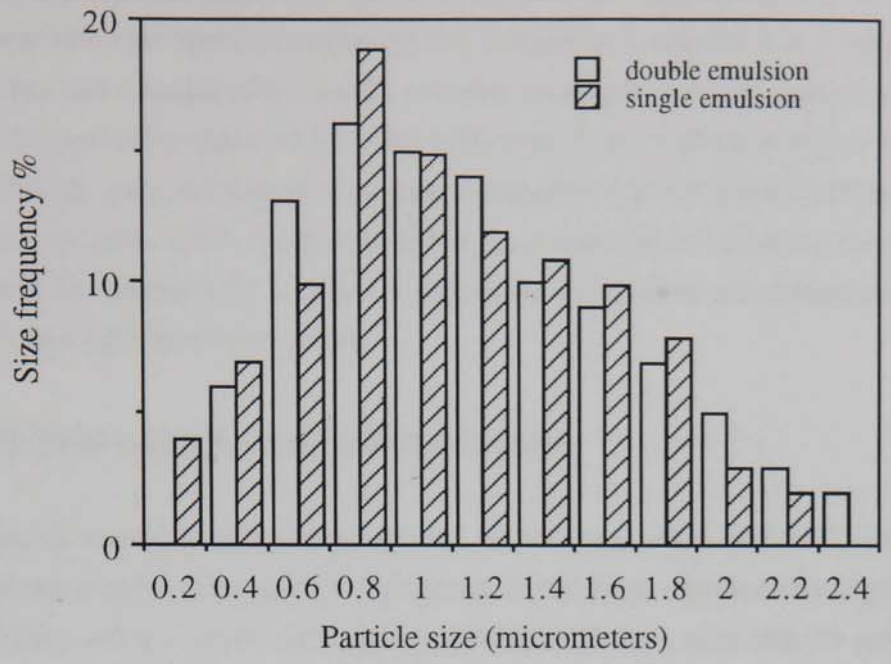


Figure 3.3. Particle size frequency distributions for PLLA microspheres prepared using single or double emulsion techniques

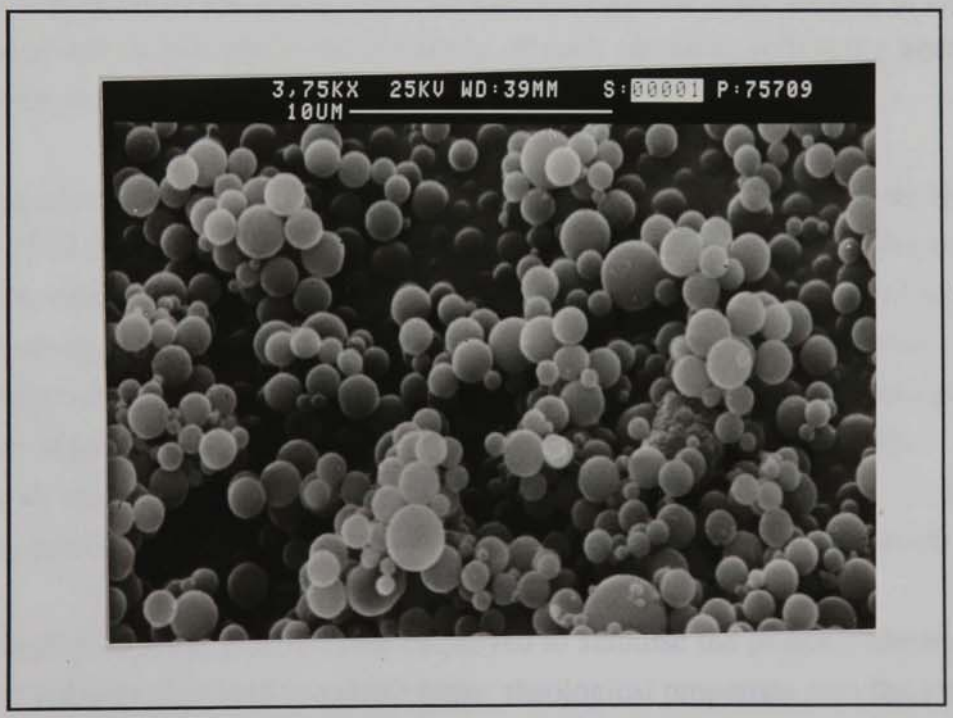


Figure 3.4. Scanning electron micrograph of PLLA microspheres loaded with 10.7% w/w BSA formed by a double emulsion method

Contrary to many reports, Sah *et al.* (1995) found that the increase in homogenisation speed (or shear rate) involved in preparing the primary w/o emulsion had no effect on particle size but had a major effect on the internal morphology of the particles formed. Increased homogenisation rates leads to the formation of microspheres with a decreased porosity within the polymer matrix allowing an extension of the protein release period and a decrease in burst effect. Homogenisation techniques seem to have a large impact on determining the distribution of BSA within the microspheres and consequently, the release behaviour (Sah & Chien, 1993).

3.3.1.4 Polyvinyl alcohol in microsphere formation

The presence of viscosity enhancing agents, in this case PVA and MC, was found necessary for successful microsphere formation. It has been reported that high protein entrapment ratios using a w/o/w solvent evaporation technique, may only be achieved in the presence of a viscous internal aqueous phase (Reich, 1995). Gelatin, successfully used in the primary aqueous phase of a double emulsion formulation (Ogawa *et al.*, 1988), increases the primary emulsion viscosity. The increased mechanical strength resulting from the relatively high viscosity prevents the migration of the inner water phase to the external phase by local demulsification due to vigorous stirring. This reduces partitioning of the antigen into the external aqueous phase leading to increased entrapment and in this study, the presence of such viscosity enhancing agents was essential for successful microsphere formation.

It has been reported that there is an increase in particle size associated with the inclusion of gelatin, (Jeffrey *et al.*, 1993) but at low concentrations used (0.8% w/v) it did not adequately stabilise the system and caused aggregation at concentrations of above 1% w/v. These observations have been reported by other groups (Rosilio *et al.*, 1991), with gelatin not displaying as marked stabilising properties as PVA and restricting formation of spheres with a small particle size (Leroux *et al.*, 1995). For these reasons and, due to interferences with the BCA assay method and its potential for immune reactions, gelatin was not used to enhance the viscosity of the primary emulsion.

A combination of PVA and MC was employed to stabilise the primary emulsion. The combined colloids appeared to exhibit better rheological properties than the individual colloids on their own. Low concentrations of high viscosity methylcellulose (400cps) permitted formation of spheres but the surfaces were pitted with large pores visible. Methylcellulose, alone, may allow the formation of a stable emulsion can cause formation of distorted ovoid microspheres, possibly due to the high molecular weight

or solution viscosity of the colloid (400cps) (Cavalier *et al.*, 1986). This was also shown by Spenlehauer *et al.* (1986) who found collapsed microspheres when using methylcellulose alone but spherical, non-aggregated microspheres with 0.005% w/v methylcellulose in combination with a range of PVA concentrations. The most successful system developed, producing smooth, spherical particles with high loading, involved a combination of partially hydrolysed PVA (88%) with a relatively low molecular weight (13-23kDa) and methylcellulose or low molecular weight PVP (10kDa) with methylcellulose.

PVA has long polymeric chains providing steric repulsion (figure 3.5) and is available with a number of degrees of hydrolysis. The many hydroxyl groups have a high affinity to water and strong inter- and intra-molecular hydrogen bonding restricts its water solubility. However, the presence of hydrophobic residual acetate groups weakens these bonds and increases the water solubility (Okaya, 1992).

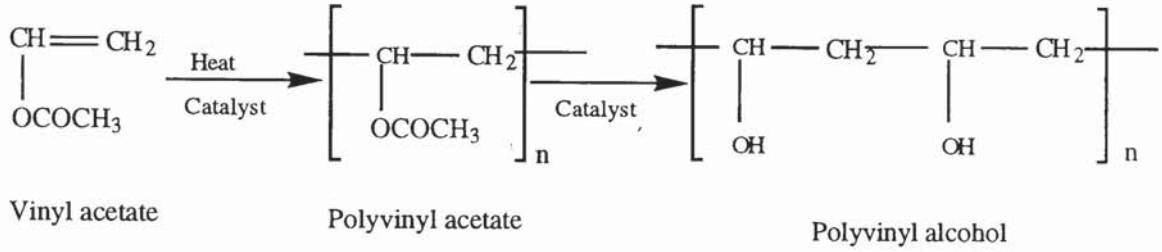


Figure 3.5. The structure and synthesis of PVA

Extensively hydrolysed PVA (98%) was not successful in the production of microspheres by this method. After adsorption at the interface, polymers such as methylcellulose, PVA and gelatin reduce interfacial energy causing an interfacial tension gradient to be produced. The emulsions formed demonstrate considerable resistance to coalescence. Studies by Lankveld & Lyklema (1972) show that the higher the percentage of acetate groups, the better the emulsifying capabilities of PVA. This is due to differences in flexibility at the oil/water interface.

Changes in the concentration and volume of the external aqueous phase were also investigated. Increasing the volume led to increased particle size and a decreased load. The increase in particle size is due to an increase in the size of the droplet formed and the fall in load is due to decreased efficiency of the mixing process. Entrapment

efficiency was increased with increasing PVA concentration in the external phase. At low PVA concentrations, the droplets are less stable and therefore, less resistant to coalescence.

As the PVA concentration of the external aqueous phase is increased, there is a corresponding reduction in particle size. Loading efficiency was not affected. The increased presence of surfactant results in an increased protection from coalescence of the particles during the hardening process. An increased volume external aqueous phase results in a decreased mixing efficiency, leading to a larger particle size and loading (Jeffrey *et al.*, 1993). For production of small particles, a small volume of external phase containing a high percentage of PVA is optimal. Optimal ratios were established for the microsphere formation using single and double emulsion techniques and these are outlined in section 2.1.

For single emulsions, a decrease in drug loading and yield was produced with increasing PVA concentration. This was due to the increased viscosity of the PVA solution causing a retardation of polymer precipitation at the interface and more drug being lost to the aqueous phase prior to polymer solidification at the phase boundary.

3.3.1.5 The effect of drug loading on microsphere formation

Encapsulation efficiency is increased with increasing primary emulsion viscosity as the mechanical strength resulting from the relatively high viscosity prevents the migration of the inner water phase to the external phase by local demulsification due to vigorous stirring (Ogawa *et al.*, 1988; Uchida *et al.*, 1994). Bodmeier & McGinity (1988) found that diffusion and drug loss across the interface of the droplet occurred only during the first minutes after emulsification, and rapid polymer precipitation should therefore prevent the loss of more drug. However, in the w/o/w technique, drug loss into the external aqueous phase during preparation is minimised by the organic barrier. Alex & Bodmeier (1990) described a loss of water soluble active agent from a complex emulsification technique caused by diffusion of the drug through pores or through the polymeric matrix after solvent evaporation and a fall in drug content with increased stirring time. As loadings from both methods are similar, leakage of BSA in this manner does not appear to be a problem but stirring time is kept to a minimum during all procedures.

High percentages of protein relative to polymer causes formation of collapsed and pitted microspheres (Jeffrey *et al.*, 1993) and microsphere formation was not possible for

particles with a theoretical load of 30% w/w BSA, or greater. This may be because there is not sufficient polymer to coat the protein.

Table 3.2. The effect of BSA theoretical load on PLLA particle size, yield and encapsulation efficiency

Theoretical load (% w/w)	Yield efficiency (%)	Actual load (% w/w)	Encapsulation efficiency (%)	Particle size (μm±σg)
10	77.0	8.02	80.2	1.06±0.42
15	72.9	9.54	63.6	1.14±0.53
20	70.7	10.22	51.1	1.54±0.70
25	61.2	10.68	42.7	1.11±0.43

Yields are in the range of 60-80% depending on the theoretical load, the process being most efficient at 10% w/w theoretical load and decreasing with increasing load (table 3.2). Theoretical load describes the initial amount of drug as a percentage of polymer while the encapsulation efficiency expresses the actual amount of drug loaded as a percentage of this theoretical value. An increased theoretical load leads to an increased actual load up to a value of 25% w/w. Above this level, formation of particles was not possible. The drug to polymer ratio is increased to such a level where insufficient polymer is present to encapsulate the BSA. It has also been suggested that high ratios of protein disturb the stability of the w/o/w emulsion and disrupt successful microsphere formation (Heya *et al.*, 1991a). The encapsulation efficiency falls as the drug level increases and there is increased diffusion of the drug into the external aqueous phase.

The particle size remains around 1μm for all loadings. Although the ratio of drug to polymer is increased, all other preparative conditions are kept constant, and the mixing efficiency remains consistent for all the preparations described. The particle size is controlled mainly by the speed of mixing which is maintained at ≥16000rpm for all procedures. Using this method, high BSA loadings (15% w/w) can be achieved and efficiency is maximal at theoretical loads of 10% w/w or below with over 90% of the protein being successfully incorporated into the particles.

Other process parameters were studied to ensure preparation of the microspheres under optimal conditions. Decreasing the volume of organic phase led to a decrease in loading

efficiency from over 60% to 47% for PDLA particles with a theoretical load of 10% w/w. An increase in internal aqueous phase volume can result in large microspheres with obvious pores and cracks (Eparza & Kissel, 1992).

3.3.1.6 The effect of different surfactants in the primary emulsion on microsphere formation

The effect of emulsifying agents employed during the formation of the primary emulsion was studied by substituting PVP for PVA at the same ratios employed for optimal loading in the w/o/w emulsion method. Differences in surfactant may cause changes in interfacial tension allowing increased solvent penetration and increased wetting of the particles (Leelarasamee et *al.*, 1988). It is essential to study the effects of different emulsifying agents individually as this may affect loading and diffusional release. Differences between the protein incorporation efficiencies were observed when the two different polymeric emulsifiers, i.e. PVA and PVP, were employed (table 3.3).

Generally, the incorporation of PVP in the microspheres resulted in higher encapsulation efficiencies at similar loadings and smaller particle size. At 20% w/w theoretical loading, the inclusion of PVP resulted in high encapsulation efficiencies (69.7 vs. 52.3%) and reduced the mean particle size by over 50% (1.54 vs. 0.63μm). These results in combination with the respective release profiles are discussed in sections 3.3.3.5 and 3.3.4.

Table 3.3. The effect of increasing theoretical load and emulsifying agents on entrapment and particle size of BSA loaded PLLA microspheres [(a)]

Theoretical Load (% w/w)	Emulsifying Agent (0.5% w/v)	Actual Load (% w/w)	Encapsulation Efficiency (%)	Particle Size (μm±σg)
20	PVA	10.45	52.3	1.54±0.70
25	PVA	11.34	45.3	1.11±0.43
20	PVP	13.92	69.7	0.63±0.27
25	PVP	15.20	60.9	1.03±0.45

[(a)]All batches include 0.05% w/v methylcellulose in the primary emulsion and 1.5% w/v PVA in the external aqueous phase

Increasing the concentration of emulsifying agent, e.g. PVP, in the primary emulsion affects the loading efficiency of the system (table 3.4).

Table 3.4. The effect of increasing emulsifier concentration on the loading of BSA into double emulsion particles at 20% w/w theoretical load[a]

Polymer	PVP in the primary emulsion (% w/v)	Actual load (% w/w)
PLA	0.25	10.42
PLA	0.50	13.97
PLA	1.00	12.49

[a]All batches include 0.05% w/v methylcellulose in the primary emulsion and 1.5% w/v PVA in the external aqueous phase.

A similar trend was observed at different loadings and with PVA in the primary emulsion. As the surfactant concentration increases more stable polymer droplets are formed. Above a certain concentration of surfactant, it is likely that no more can be adsorbed at the interface and there is no increase in the interface surface area. The polymeric surfactant, in these cases, is no longer able to unfold to prevent coalescence and the interfacial gradient cannot persist. An optimal PVA or PVP concentration of 0.5% w/v was established for stability of the primary emulsion in the majority of these systems. Benita *et al.* (1984) found that for a constant rate of agitation, an increase in PVA concentrations also led to an increased particle size. This was due to an increase in viscosity associated with the high levels of emulsifier (5% w/v) which caused problems in removal during washing procedures leading to the formation of aggregates.

3.3.1.6 The effect of solvent removal on microsphere formation

Yields from batches produced using a solvent extraction method were slightly lower than those of solvent evaporation methods, from 60-70%. The most even, spherical particles were produced when the double emulsion was added in a 1:1 ratio, with stirring, to 50% v/v ethanol in water. The proposed porous structure of the particles was not visible under SEM and the particles were not larger than those produced by more conventional methods (figure 3.8). Loading and efficiency were similar whether the solvent was removed by slow evaporation or by extraction.

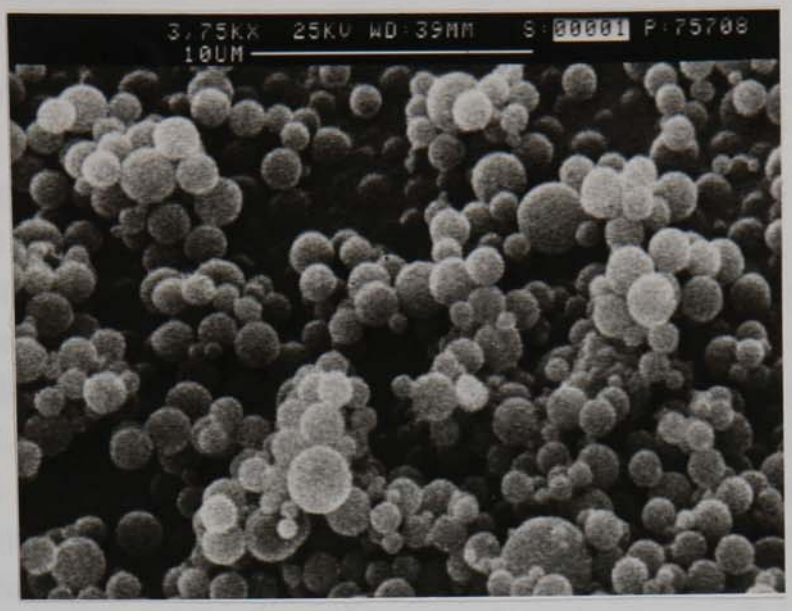


Figure 3.6. Scanning electron micrograph of PLLA microspheres loaded with 10.1% w/w BSA formed by a solvent extraction method

Microsphere formation is a phase separation process with diffusion of the organic solvent into the external phase. The external aqueous phase will diffuse into the microsphere causing precipitation of the polymer at the droplet interface. The amount of solvent and non-solvent present in the spheres at the time of polymer precipitation will affect the porosity of the product. While the droplet is in an aqueous state, solvent diffusion into the external phase will lead to a reduction in particle size but once the polymer has precipitated, there will be no further reduction in particle size. However, the particles formed by extraction procedures were of a similar size to those formed by other techniques (about 1μm). These results were confirmed by the findings of Pavanetto *et al.* (1992) and Sato *et al.* (1988) who described smaller particles resulting from extraction procedures. This could be due to a reduction in coalescence of the particles during solvent removal procedures.

Thus, the way the coating polymer is precipitated strongly affects the drug loading, porosity and the surface morphology of the microspheres (Alex & Bodmeier, 1990). Porous particles will be formed if the polymer precipitates rapidly after droplet formation as large amount of solvent will still be present in the microspheres. The extraction procedure removes the dichloromethane in under an hour and the particles

can be harvested in the normal manner. The question of porosity will be considered further in section 3.3.2.

3.3.1.7 BSA loading by adsorption techniques

Microspheres prepared by a multiple emulsion method will be more porous due to the presence of the internal aqueous phase in addition to the organic solvent (Bodmeier *et al.*, 1991) and these were used for adsorption studies. The effect on temperature on the adsorption process was investigated and the process was more efficient at 25°C rather than 37°C. Different media were studied and higher loadings were achieved using BSA in saline for PLA and BSA in PBS for PLGA co-polymer. These results may be due to differences in the surface properties of the particles prepared from the different polymers. Very low levels of BSA were adsorbed to the surface of PDLA spheres under any conditions (less than 0.9% w/w). The maximum loading achieved was 2.34% w/w. This was onto PLLA spheres formed by solvent extraction methods. Adsorption of BSA onto the surface of spheres formed by this method gave consistently higher results (15-20%) than for particles formed by evaporation methods. This may suggest that the particles formed in this way are more porous, presenting a larger surface area for BSA adsorption, though this is not obvious from the SEM images (figure 3.6).

Although the loadings are low, release studies were performed on particles prepared by this method (section 3.3.2.9).

3.3.2 *In vitro* release studies

The extent to which the antigen, both entrapped within the polymer matrix or surface located, is released is dependant on (a) the drug and loading involved, (b) the polymer employed to produce the microspheres, (c) the particle size and (d) the surface hydrophobicity of the particles. All the results shown are the mean of at least three separate determinations and the standard deviations from the mean are illustrated. Unless otherwise stated, the release medium was 20mM phosphate buffer pH 7.5 and studies were carried out at 37°C.

3.3.2.1 The effect of drug loading on release of BSA

The rate of drug release tends to increase with increased drug loading (figure 3.7). This shows good agreement with published results (Hora *et al.*, 1990a; Sah *et al.*, 1994). The release rates are partly governed by the drug: polymer ratio, i.e. a low drug load will result in a relatively higher polymer content which will retard the release rate. Huang & Ghebre-Sellasie (1989) attribute this to a thicker water-insoluble wall material or a longer path through which drug transport occurs. Channels or pores, facilitating release (see section 3.3.3.4) will form much more readily in microparticles that have higher loadings of a hydrophilic protein. An increase in the drug loading, or relative decrease in the amount of polymer produces microspheres with a less compact matrix, which might cause the movement of BSA to the outer parts of the microsphere during the drying process. When microspheres are being dried under vacuum, water escapes from the inside of the microcapsules which may facilitate the migration of BSA onto the surface. This mechanism may be responsible for the increased burst effect visible with increased drug loading (Sah *et al.*, 1994).

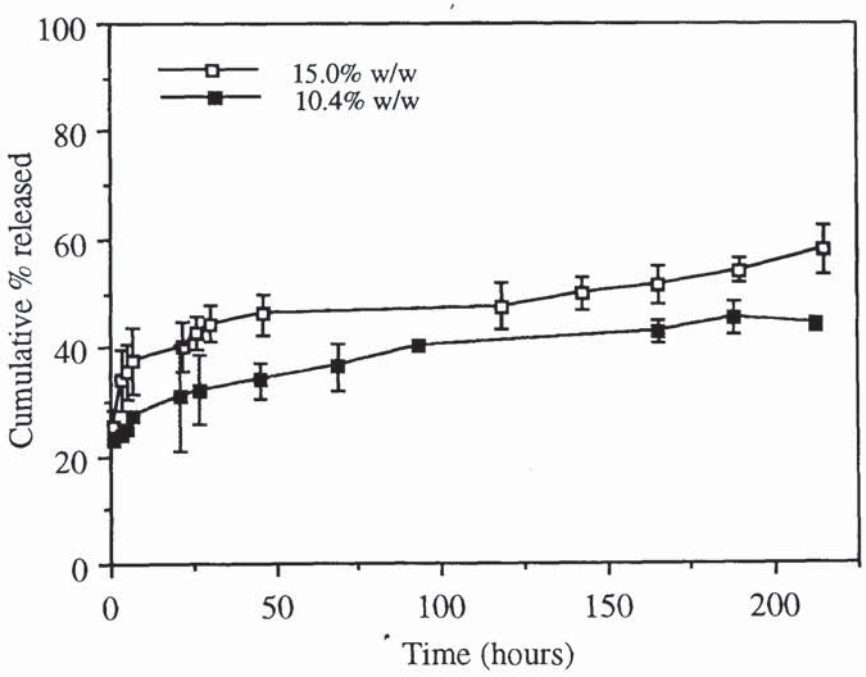


Figure 3.7. Release of BSA from two PLLA microsphere formulations of different loadings, 1μm diameter formed by a double emulsion method (n=3; mean±s.d.)

3.3.2.2 BSA release profiles from amorphous polymers

BSA release from microspheres formed by a similar method involving amorphous PLGA co-polymer and PDLA show much faster release profiles than the semi-crystalline PLLA, as expected. These particles, though of a similar size to PLLA spheres have lower loading efficiencies (approximately 50% at a 10% w/w theoretical load), possibly due to the better stability of the emulsion containing the semi-crystalline polymer (Schugens *et al.*, 1994). The faster release rate is, therefore, not due to high drug loadings or the effects of particle size (figure 3.8). Release from the amorphous polymers is almost complete for both in 300 hours (over 800 hours for PLLA spheres). The PDLA polymer exhibits slightly faster release than the 50:50 co-polymer. This may be due to the slightly lower molecular weight of the PDLA, leading to an increase in hydrophilic domains and also to a slightly smaller particle size.

Both curves show a characteristic "shoulder" which is not visible on release profiles for PLLA spheres. These results are discussed in section 3.3.2.4. The burst effect is lower for PLGA spheres than for the higher loaded PLLA particles. A similar result was described by Alonso *et al.* (1993) for PLGA and PLLA particles of the similar loadings to those used in these studies. Sanders *et al.* (1984) described drug release of macromolecules as a three part process, i.e. initial diffusion from superficial regions followed by a slower release due to polymer hydrolysis and finally release resulting from polymer erosion. This theory may apply to BSA release from PDLA and PLGA spheres with diffusion through the pores formed during polymer hydrolysis. A third phase of release is indicated after 150 hours which may be due to erosion of the amorphous polymer. This third phase is not exhibited with release from PLLA particles (figure 3.9).

Release from both amorphous and semi-crystalline microsphere systems is complete before total erosion of the polymer occurs (see section 3.3.7).

3.3.2.3 The effect of particle size on release of BSA

There are some general principles that can be applied to release from any microsphere system (Jalil & Nixon, 1990a). The release rate will increase with a decrease in particle size (figure 3.9). The same weight of particles will have a much larger surface area if the particle size is small. This will lead to a faster release rate from the surface of the spheres.

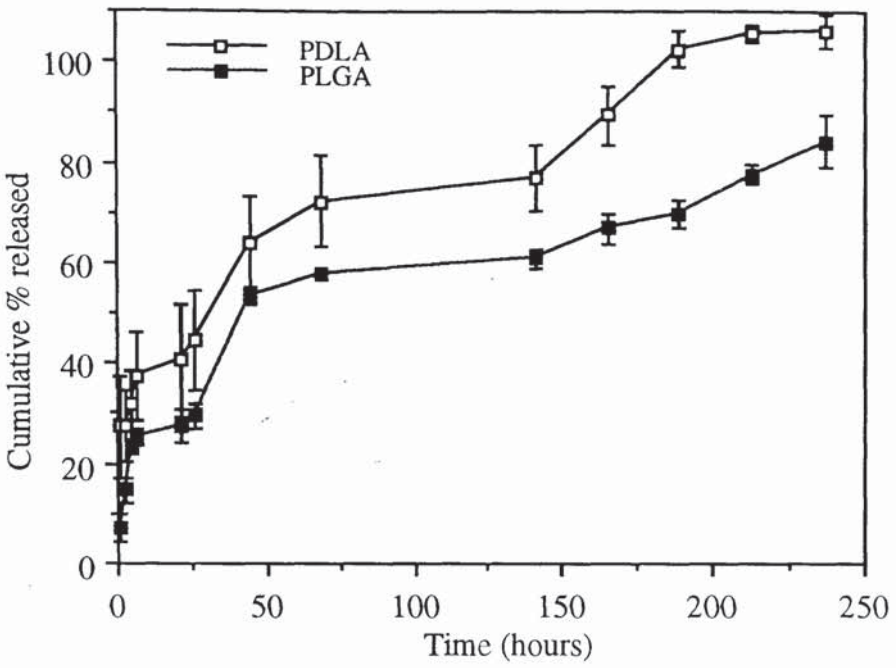


Figure 3.8. The *in vitro* release of BSA from microspheres prepared using different amorphous polymers (n=3; mean±s.d.)

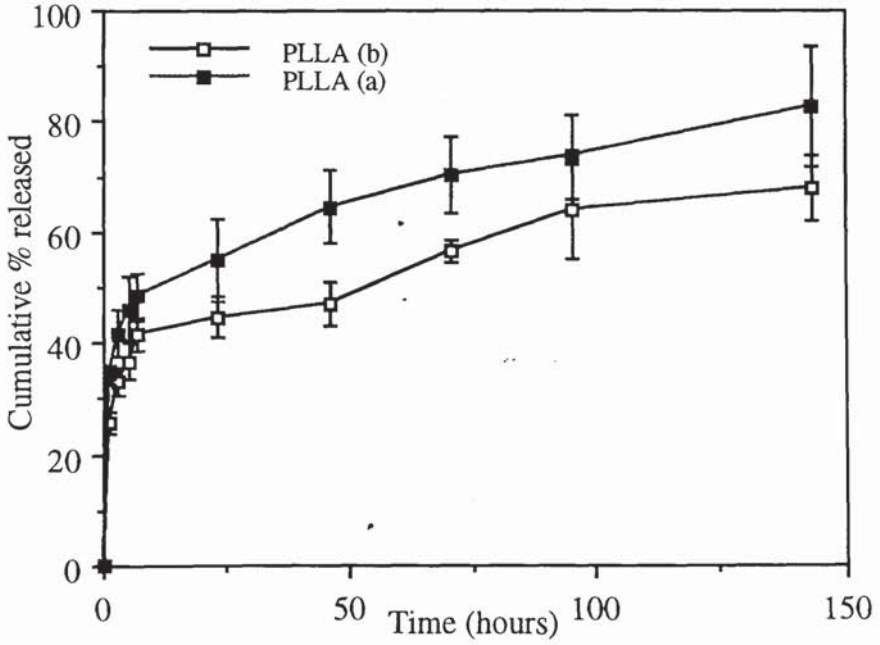


Figure 3.9. The effect of particle size on release from 10% w/w loaded spheres formed by a single emulsion method. PLLA (a) are 1.04±0.55μm and PLLA (b) are 0.87±0.32μm (n=3; mean±s.d.)

The decrease in overall release rate between microspheres formed using single or complex emulsion techniques is obvious from comparison of figures 3.7 and 3.9. The mechanisms behind release from these preparation are discussed in the following section.

3.3.2.4 Mechanisms of protein release from microspheres

Microspheres and microcapsules are complex heterogeneous systems. Many theoretical mechanisms exist for the release of drugs from polymer matrixes (monolithic systems) and from microspheres and in most cases some of these processes may co-exist. Distinction between these mechanisms is not always possible. Biodegradation of the polymer is the rate controlling step in antigen release from microspheres formed by Cohen *et al.* (1991). However, pure polymers or thick-walled core-wall microspheres could have a slow enough rate of degradation to allow the majority of the antigen to be released by diffusion (Cowsar *et al.*, 1985). During erosion, the porosity of the matrix increases and the release of the protein is facilitated by diffusion. Therefore, not only the degradation rate of the polymer but also the initial inner structure of the microspheres should be considered as important factors controlling the release process. Formation of the primary emulsion by controlling the shear forces involved can produce microspheres with reduced or negligible burst effects (Sah *et al.*, 1995). The use of high shear forces lead to the production of microspheres with reduced porosity having a matrix-like structure with an even distribution of protein throughout the microsphere (see section 1.1.4; figure 1.5).

The aqueous channels which facilitate release are generated by two mechanisms (Furr & Hutchinson, 1992). The first is the leaching of the drug from domains at or near the surface of the microsphere which is essentially a diffusion/dissolution process controlled by such parameters as drug loading, drug/polymer morphology and particle geometry. The second mechanism is the degradation of the polymer leading to the generation of microporosity in, and enhanced water uptake by, the degrading polymer and release of the drug from within the body of the polymer, a process dependant on the degradation properties of the polymer. By controlling the properties of the polymer, the two phases of release can be overlapped to give continuous release over the desired time period. Release rate is also affected by drug loading. When the loading is low the drug tends to exist as solid crystals or phase separated in the polymer matrix and are not in contact with each other. Therefore the drug molecules or the solvent must diffuse through the matrix before release can occur causing a slow release. If the loading is increased, the trapped drug will form a network with contact between the crystals and

the matrix acts as a drug reservoir. The presence of drug or any other substance within the polymer matrix may, therefore, affect degradation (Vert *et al.*, 1991) with a neutral hydrophobic substance opposing water uptake and abating degradation. It is therefore important to study the effects of different processing conditions on the release kinetics.

An alternative mechanism for protein release from PLGA particles was proposed by Csernus *et al.* (1990). They found that diffusion of peptides, a LHRH analogue and an agonist, through aqueous channels was negligible and release was primarily controlled by degradation of the polymer matrix. They claimed that the structures within the microspheres, described as pores or water filled channels, are actually protein crystals or granules. They observed the disintegration of the matrix starting from the centre of the microsphere from as early as day one after i.m. injection. These findings contradict the more widely accepted views of Langer (Langer & Moses, 1991) who states that the release of polypeptides from degradable polymers involves movement of the macromolecule through a complex porous path within the polymer matrix. As the polymer erodes, the release rate will be affected.

For all microsphere systems described in the previous sections (3.3.2.1 to 3.3.2.3), the protein burst is lower than described for other systems described in recent literature (Kwong *et al.*, 1986; Wang *et al.*, 1990; Cohen *et al.*, 1991; Park *et al.*, 1992). For example, in a similar receiving medium, i.e. 20mM phosphate buffer, Cohen *et al.* (1991) describe relatively large burst effects (up to 80% for BSA), which are attributed to cleavage of the relatively low molecular weight polymer or to incompletely incorporated drug at the polymer surface (Shah *et al.*, 1992) Faster release rates from low molecular weight polymers are attributed to their smaller size and also to the increase of hydrophilic regions in these microspheres, i.e. there is a high hydration capacity due to a high number of carboxylic groups (Pistner *et al.*, 1993). Design of a sustained release dosage form must take into consideration both the properties of the polymer and the drug that control rate of release. For proteins and polypeptides, where the drug has little solubility in the polymer, the most important properties of the drug are size, confirmation and solubility. As the molecular weight of the drug increases, the diffusion coefficient decreases and the polymer is unlikely to permit partition-dependant diffusion through the polymer.

The release mechanism of peptides and proteins from biodegradable polymers has been the subject of many studies (Alonso *et al.*, 1993; Hora *et al.*, 1991), being affected by to pore diffusion, swelling, bioerosion and other phenomena (Bodmer *et al.*, 1992). For PLGAs of various molecular weights and polymer ratios, the lactide/glycolide ratio

has a larger influence on the degradation rate than the polymer molecular weight (O'Hagan *et al.*, 1994). Biodegradation is affected by the constituent sequence of individual moieties comprising each molecular chain. Sequence can be random and consequently, it may be difficult to predict PLGA degradation. Release rate may be increased by the presence of a more basic drug in the matrix as the hydrolytic rate of PLA polymer increases in alkaline pH. The rate of PLGA degradation increases with a decrease in pH of the receiving medium (Heya *et al.*, 1994). They also found that peptide release decreases with increasing ionic strength of release medium. It is proposed that this is due to a more rapid pH reduction in the more dilute buffer caused by the degradation product of the polymer. However, Bodmer *et al.* (1992) conclude the reduction in release is due to a reduction in swelling of the polymeric backbone due to an ion-shielding effect. A reduction in the volume of DCM used (keeping the amount of polymer constant) also results in microspheres with a more dense core, decreasing the burst effect. Water penetration of a dense polymer matrix, also due to decreased loading or increased polymer molecular weight, is impeded and there may be a slower rate of polymer erosion with little protein release, i.e. generation of a lag phase (Cohen *et al.*, 1991).

Unusually in the studies of Bodmer *et al.* (1992), the release rate increases with an increase in polymer molecular weight, concluding that normal hydrophobicity considerations governing water uptake is not the most important factor controlling protein release from these polylactide microspheres. They conclude that, in their case, the increased presence of carboxylic end-groups in low molecular weight polymers causes an increased interaction between the positively charged protein (BSA) and the polymer. More normally, the release rate decreases with increasing polymer molecular weight as a higher number of carboxylic end-groups *per* unit weight leads to increased hydrophilicity of the polymer and a more rapid water uptake (Jalil & Nixon, 1990c). This would result in a more rapid drug release from low molecular weight polymers. Also, drug overloading increases the hydrophilic region producing aqueous channels. Such interactions have been considered for the retention of drugs and may provide an explanation for the results of Bodmer and emphasis the need for *in vitro* testing of each drug/polymer system.

Proteins are not appreciably soluble in the polymer and do not have adequate diffusivity through intact polymers. PLA microspheres which undergo bulk erosion changing the properties of the polymer phase, permeability of polymer to drug increases with time and the release rate is neither linear nor predicable (Sah *et al.*, 1994). Also the binding of multiple-charged proteins to the terminal carboxylic acid end groups of the polymers

is involved in their release, sometimes polymer-protein interactions resulting in the retention of the protein within the polymer matrix. A quasi-linear release of a protein can be successfully achieved using a minimal amount of protein. Using an ELISA method, and a double emulsion, solvent evaporation preparative technique, Sah *et al.* (1994) found that the binding activity of BSA toward anti-BSA antibody was not lost during the encapsulation procedure or during subsequent release. Binding of BSA to the low molecular polymers used in these studies does not seem to be a problem as the BSA is well released from most formulations (figures 3.8 and 3.9).

Bawa *et al.* (1985) propose the existence of a tortuous, interconnected pore network due to the incorporation of macromolecules into the polymer matrix and it is *via* this network that macromolecules can be released in a controlled manner. When the drug is uniformly dispersed throughout the polymer matrix and degradation occurs at the surface, there would be a one-to-one relationship between release of the drug and molecular weight loss. Although a relationship exists between these parameters, Heya *et al.* (1991b) have stated that the weight loss is much slower than peptide release. This suggests that the drug is able to diffuse through pores created as a consequence of polymer degradation. This was found to be more prominent in lower molecular weight polymers, possibly due to the relatively higher hydrophilicity. Release profile predictions based on calculations for BSA from a biodegradable polymer for other proteins, i.e. lysozyme and β-lactoglobulin show general agreement with the results, the observed differences being due to differences in the shapes of the proteins. They found a poor linearity of fractional release versus the square root of time for the peptide, indicating that there is a more complex mechanism of release than a simple pore diffusion.

After correction for dilution of the release media, a number of *in vitro* profiles for different PLA polymers containing BSA were fitted to the main models which have been proposed to describe drug release kinetics from microspheres and other polymer matrixes (Malamataris & Avgerinos, 1990).

Zero order $100 - M = k_0 t$

First order $\ln(100 - M) = k_1 t$

Cube root $\sqrt[3]{100} - \sqrt[3]{M} = k_2 t$

Square root $100 - M = k_3 \sqrt{t}$

where M = the percentage of unreleased drug; M_o = initial drug loading; k = rate constant for the corresponding best fit line and t= time in hours.

The values of the release rate constants and the corresponding correlation coefficients r^2 are shown in table 3.5.

Table 3.5. Correlation coefficients for fit of dissolution results for various microsphere systems loaded with BSA

Microsphere	Particle size (μm±σg)	Load (% w/w)	Zero order	First order	Cube root	Square root
			r^2	r^2	r^2	r^2
PLLA; PVA in 1°	0.63± 0.27	14.0	0.953	0.881	0.957	0.971
PLLA; PVP in 1°	1.23± 0.54	10.4	0.752	0.935	0.916	0.975
PLLA; PVP in 1°	1.03± 0.45	15.6	0.988	0.962	0.990	0.981
PLLA; PVA in 1°	0.87± 0.32	10.9	0.954	0.992	0.986	0.995
PLLA; PVA in 1°	1.04± 0.55	10.4	0.952	0.961	0.958	0.962
PLLA; s/e*	1.07± 0.45	13.5	0.913	0.911	0.907	0.947
PLLA; extract[a]	0.96± 0.43	13.4	0.962	0.987	0.986	0.995
PDLA; PVA in 1°	1.15± 0.52	5.1	0.961	0.970	0.969	0.975
PLGA; PVA in 1°	1.21± 0.49	4.9	0.885	0.909	0.938	0.962

All particles made using double emulsion, solvent evaporation methods except * single emulsion; [a] solvent extraction

The release profiles from microspheres with different characteristics can be best described using different kinetic models. The first phase of protein release is due to loss of superficial drug from the particle surface and so it was the second phase of release, after this burst effect, that was examined in further detail. The cube root model assumes geometric disappearance of the delivery system. SEM images of PLLA particles after dissolution for one week showed no obvious changes (see section 3.3.8) and, after one month the PLLA particle remained largely intact. The square root time plots generally showed higher r^2 values than the first-order plots. This indicates that release of BSA from the microspheres is mainly diffusion controlled with the drug leaving the matrix through pores and channels (Higuchi, 1963).

Sanders *et al.* (1984) described drug release as a three part process, i.e. initial diffusion from superficial regions followed by a slower release due to polymer hydrolysis and finally release resulting from polymer erosion. This theory may apply to BSA release from PDLA and PLGA spheres with diffusion through the pores formed during polymer hydrolysis. A third phase of release is indicated after 150 hours which may be due to erosion of the amorphous polymer. This third phase is not exhibited with release from PLLA particles and the cumulative release from these particles show best correlation with respect to the square root of time data during the diffusional phase.

The square root time plots for particles formed by solvent extraction also exhibited a good degree of linearity over the diffusional range but release was faster, with 90% of the encapsulated drug being in under 300 hours. This confirms the theory that release occurs mainly by diffusion through aqueous channels in the polymer matrix and particles with increased porosity have an increased release rate (Sato *et al.*, 1988).

The porosity of spheres formed using high shear forces, in this case ≥16000rpm, is relatively small, limiting the permeation of the large macromolecule across the microsphere. Thus, an increase in drug loading for particles of a controlled size distribution will increase the porosity of the system and reduce the amount of polymer (Spenlehauer *et al.*, 1986) increasing release rate (section 3.3.2.1). The relatively good correlation with zero-order kinetics for PLLA preparations indicate that the release rate remains constant with time, proving that by controlling manufacturing conditions, a steady release rate may be maintained.

The S-shaped dissolution profiles from PLGA and PDLA polymers (figure 3.8) may be explained by a slow release while the polymer undergoes hydrolysis. When the reduction in molecular weight becomes significant, porosity of the polymer matrix is increased and a faster release rate begins. Sanders *et al.* (1986) describe this type of profile as being typical for bulk-eroding controlled release polymers. Initially, when drug is being released from the surface of the spheres, the size of the microspheres is significant. However, as erosion of the matrix proceeds, surface area becomes less important. The penetration of water through PLLA microspheres is limited due to the presence of crystalline regions and the release of the protein is retarded. The increased complexity of the internal microsphere structure formed using double emulsion techniques may lead to a decreased diffusion rate for BSA from the microsphere matrix.

In vitro release pattern determination is important in early development of the process and remains necessary as a quality control procedure. In practical terms, the *in vitro* profile will not completely model the *in vivo* process, however leuprolide release from a w/o/w emulsion system was shown to be comparable (Ogawa *et al.*, 1988). Results show that zero- or first-order release kinetics can be achieved for BSA from PLA microspheres or that biphasic or multiphasic release characteristics can be produced depending on manufacturing and formulation parameters.

3.3.2.5 The effect of surfactant in the formulation on release rate

The differences between the protein incorporation efficiencies observed when two different polymeric emulsifiers (PVA and PVP) were employed in formation of the primary emulsion have been described in section 3.3.1. Generally, the incorporation of PVP in the microspheres resulted in higher encapsulation efficiencies at similar loadings and smaller particle size.

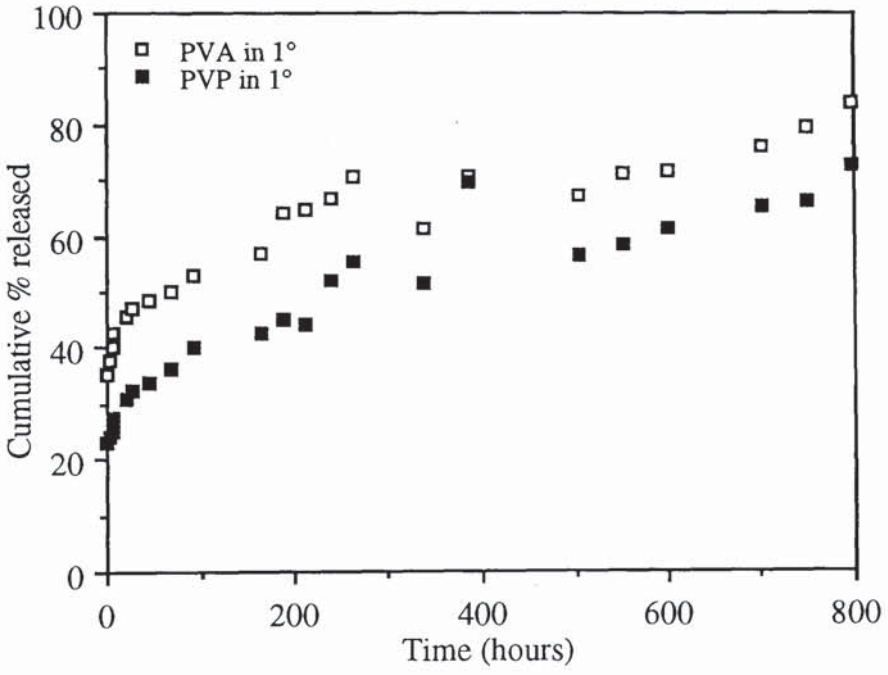


Figure 3.10. The effect of different emulsifying agents in preparation of the primary emulsion of spheres formed by the w/o/w technique. PVA in 1°; 0.63±0.27μm, 14.0% w/w BSA load with 0.5% PVA included in the primary emulsion. PVP in 1°: 1.23±0.54μm, 10.4% w/w BSA load with 0.5% PVP included in the primary emulsion (n=3; mean±s.d.)

When 0.5% w/v PVP was used to prepare the primary emulsion, a slower release pattern was observed with a corresponding decrease in burst effect (figure 3.10). It is

essential to study the effects of different emulsifying agents individually as this may affect loading and diffusional release. To determine whether these results were due to differences in particle size and/or drug loading, further investigations were carried out (figure 3.11). Although the spheres formulated with PVP in the primary emulsion, PVP1 in figure 3.11, have a correspondingly higher loading (15.6% vs. 10.4% w/w) and are of a similar size to PLLA microspheres using PVA in the formation of the primary emulsion, PVA1, they exhibit a much reduced burst effect (12% vs. 35%). Smaller spheres formulated with PVA, i.e. PVA2, having a similar load to PVA1, exhibit a slightly increased burst effect. The retardation of *in vitro* release, ~30% vs. ~80% in one week, appears to be due to the presence of PVP in the primary emulsion. This may possibly due to different properties conferred on the particles due to the emulsifying agent. To further study these differences, detailed analysis on the surface of the particles was carried out (section 3.3.3).

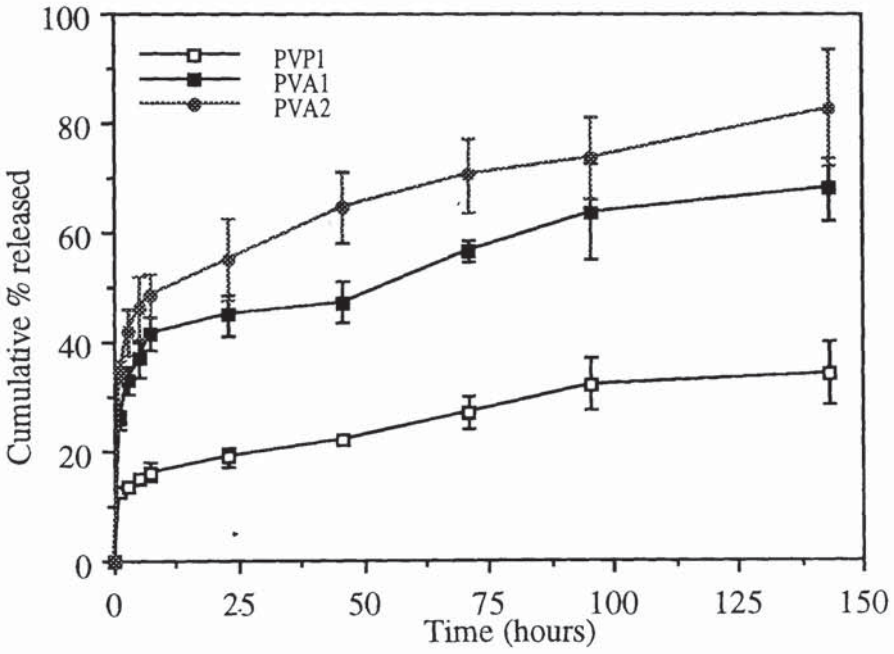


Figure 3.11. Release profiles of BSA from microspheres formed by the w/o/w emulsion method showing the influence of emulsifying agent on release where PVP1-15.6% w/w, 1.03μm; PVA1-10.4% w/w, 1.03μm; PVA2-10.9% w/w, 0.87μm (n=3; mean±s.d.)

3.3.2.6 Release of BSA in simulated gastric media

The release of BSA from formulations prepared by double emulsion solvent evaporation techniques into simulated gastric media (section 2.3.2) was carried out using ^{125}I-bovine serum albumin (ICN Biomedicals Inc., High Wycombe, Bucks..

U.K.). As exposure to acid may affect the antigenicity of the protein, a successful oral delivery system must protect against exposure to gastric acid.

The release of BSA from both formulations was enhanced (table 3.6) with up to 80% of the protein released in three hours. The hydrolysis of PLA is accelerated in alkaline pH (Makino *et al.*, 1986) with a strongly alkaline solution being used to digest particles (section 2.2). Hydrolysis is also enhanced, but to a lesser degree, in strongly acidic conditions (Makino *et al.*, 1985). Wichert & Rohdewald (1990) outline a pH dependant drug release from low molecular weight PLA (2kDa), not seen with the higher molecular weight polymers. However, for the low molecular weight drug in these studies, vinpocetine, release was decreased in acidic buffer.

Table 3.6. Release into simulated gastric media (USP XXI) of ^{125}I-BSA from 1μm spheres loaded with 20% w/w BSA formed using a solvent evaporation double emulsion technique with PVA or PVP in the 1° emulsion (n=3; mean±s.d.)

Time (hours)	Cumulative % released	
	PVA	PVP
1	56.4±12.0	45.3±4.9
2	76.0±8.6	77.4±11.8
3	67.9±4.3	78.8±9.5

The extent of the increase in release of the BSA in the acidic conditions was unexpected. Due to the low molecular weight of the polymer, degradation may be significantly enhanced, with BSA towards the outer edges of the polymer matrix being rapidly leached from the microsphere. In view of these results, lower loaded PLLA microspheres were developed for oral delivery in a effort to reduce the protein lost from the formulation in the stomach.

3.3.2.7 Release in buffer with SDS

BSA release was studied from PLLA spheres formed by a double emulsion solvent evaporation process in the presence and absence of SDS (figure 3.12). The inclusion of 0.1% SDS w/v in the release medium leads to an increase in the overall rate of protein released. Hora *et al.* (1990) include SDS in the release medium to solubilise the HSA released from the microspheres. The surfactant may also lead to an increase in the microsphere surface polarity allowing more extensive channel and pore formation inside

the polymer through which the protein can diffuse (Iguarta *et al.*, 1995). Comparison between release profiles obtained for HSA with or without SDS show little difference (Hora *et al.*, 1990). They conclude that the HSA has not been converted into a form which in insoluble due to the process of microencapsulation.

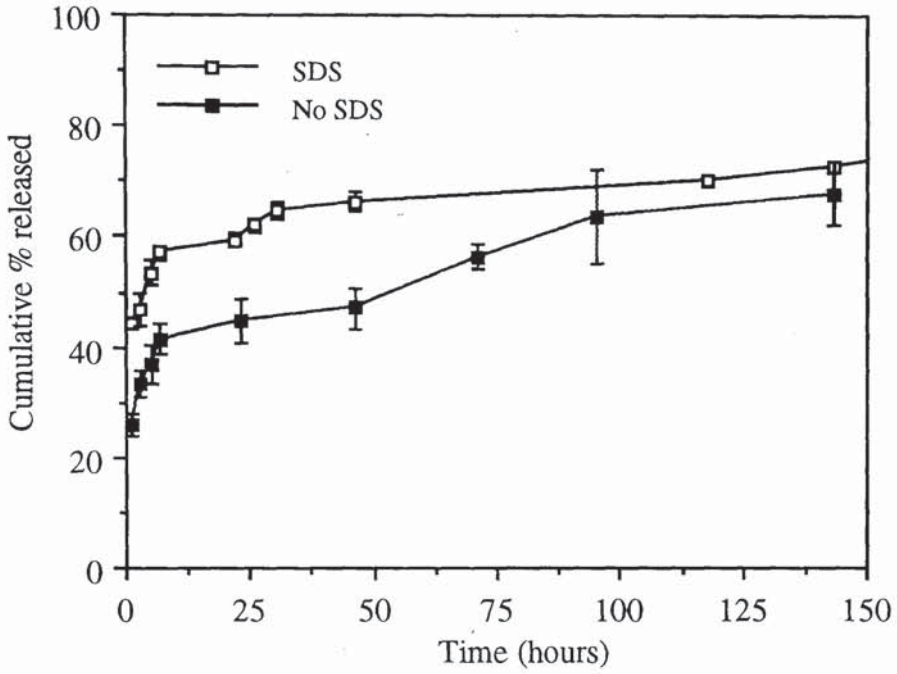


Figure 3.12. The effect of 0.1% SDS in the receiving medium on the release profiles of PLLA spheres (load 10.9% w/w).(n=3; mean±s.d.)

In the present study, release rates were increased in the presence of the surfactant (figure 3.12) up to 100 hours. In buffer containing a detergent, the surfactant adheres to the surface of the spheres making them more hydrophilic in nature and increasing the available surface area, increasing the wetting of the particles (Leelarasamee *et al.*, 1988). Also the surfactant acts as a solubilising agent for the proteins and the hydrophobic polymer fragments. All these factors could contribute to a faster drug release. After extended periods of drug release, there is little difference in the overall release rate, indicating that BSA is unaltered by the microencapsulation process. This is further confirmed by SDS-PAGE (section 3.3.10).

3.3.2.8 Release of BSA from spheres formed by solvent extraction techniques

PLLA particles formed by solvent extraction methods display a faster release profile than those formed by the solvent evaporation method, with over 85% of the protein released in under 300 hours. The size of the particles in this investigation were similar to those of spheres formed by solvent evaporation (0.96±0.43μm), as were drug loadings. The increase in release rate is probably due to increased porosity and therefore increased available surface area within the microsphere matrix (Sato *et al.*, 1988: Redmon *et al.*, 1989) associated with microspheres prepared by solvent extraction techniques.

The drug release profiles were also determined *in vitro* for particles formed by an o/w emulsion method. Although drug loadings are of a similar value to those obtained by complex emulsion techniques, after 30 hours, the release from o/w emulsion spheres is higher (figure 3.13).

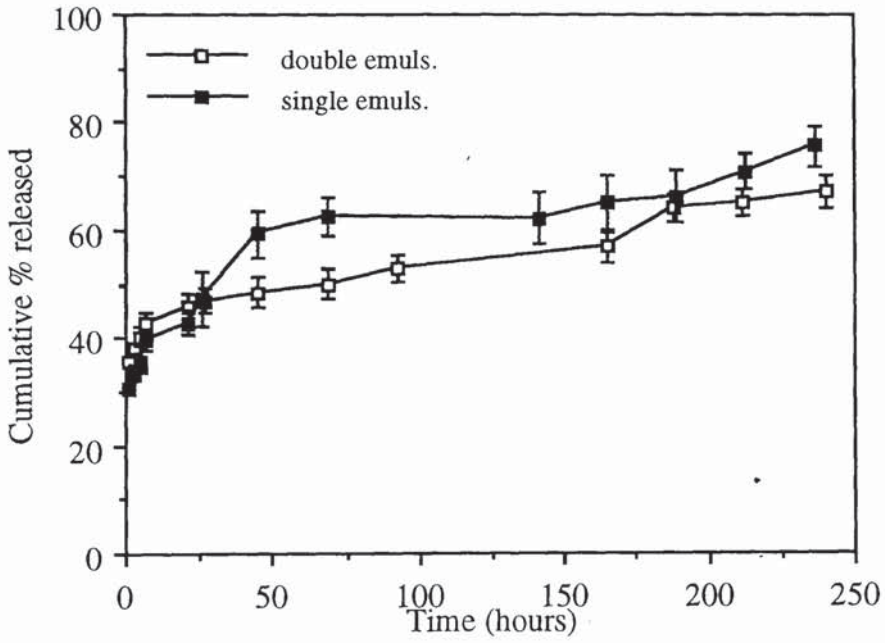


Figure 3.13. Release profiles of BSA from spheres formed by an o/w and a w/o/w solvent extraction technique. Double emuls.- 0.63±0.27μm, 14.0% w/w; single emuls.- 1.07±0.45μm, 13.4% w/w (n=3; mean±s.d.)

The larger particle size for the spheres formed with the single emulsion method must be taken into account when interpreting the results. The overall amount of BSA released from the single emulsion microspheres is greater after ~30 hours and is more erratic.

However, release rate from both microsphere preparation is similar, suggesting that BSA is being released by similar mechanisms.

3.3.2.9 Release of BSA adsorbed to the surface of microspheres

As an alternative to reported harsh encapsulation techniques, adsorption to the surface of microspheres may be a method for protein loading. Proteins were adsorbed onto empty microspheres as previously described (see section 2.1.4). The low levels of BSA adsorbed to the surface of PDLA spheres under any conditions (less than 0.9% w/w) may be due to the relative hydrophilicity of the polymer (see section 3.3.3). The maximum loading achieved using this technique was 2.34% w/w. This was onto PLLA spheres formed by solvent extraction methods. Adsorption of BSA onto the surface of spheres formed by solvent extraction gave consistently higher results (15-20%) than for particles formed by evaporation methods, confirming the particles formed in this way are more porous, presenting a larger surface area for BSA adsorption (Sato *et al.*, 1988).

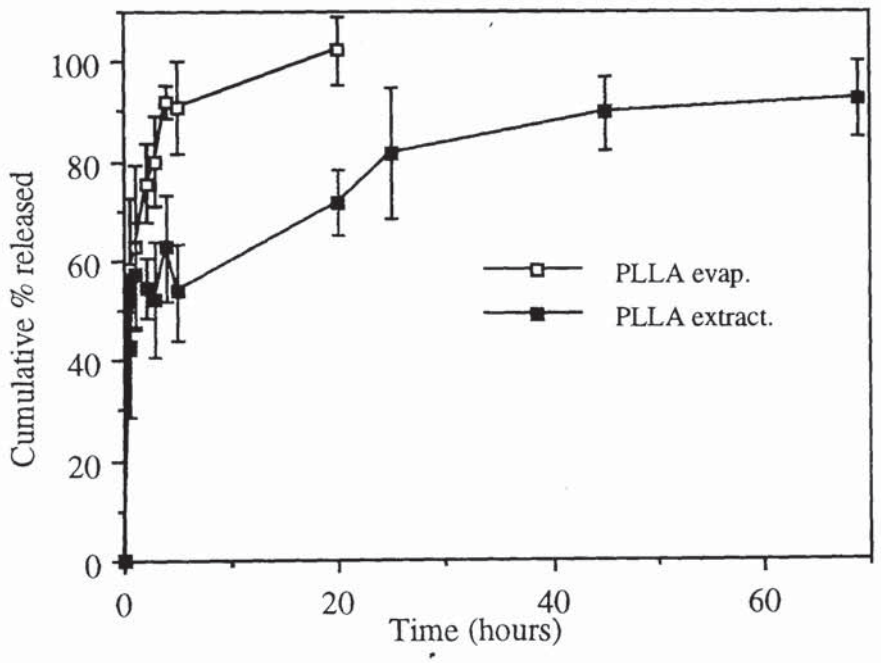


Figure 3.14. Release profiles for BSA desorption from the surface of PLLA spheres formed by solvent evaporation and extraction. PLLA evap- 1.11±0.42µm, 1.94% w/w BSA; PLLA extract.- 1.23±0.35µm, 2.34% w/w BSA (n=3; mean±s.d.)

Release profiles were generally more erratic than for particles formed by encapsulation methods. This could be due to lower amounts of protein being present and so, re-adsorption of the released drug onto the large surface presented by the particles is more significant than release from particles formed by encapsulation. The BSA adsorbed on the surface of the smoother spheres was more easily leached from the microsphere surface (figure 3.14). The slower desorption from the extracted particles may be by virtue of the increased load but some retardation of release due to the porous structure may also be involved. Similar results were seen with PLGA spheres formed by the same methods.

A comparison of protein desorption from the surface of spheres formed from PLLA, PLGA, and PDLA microspheres was also carried out. For reasons of clarity, the release up to 20 hours is shown (figure 3.15).

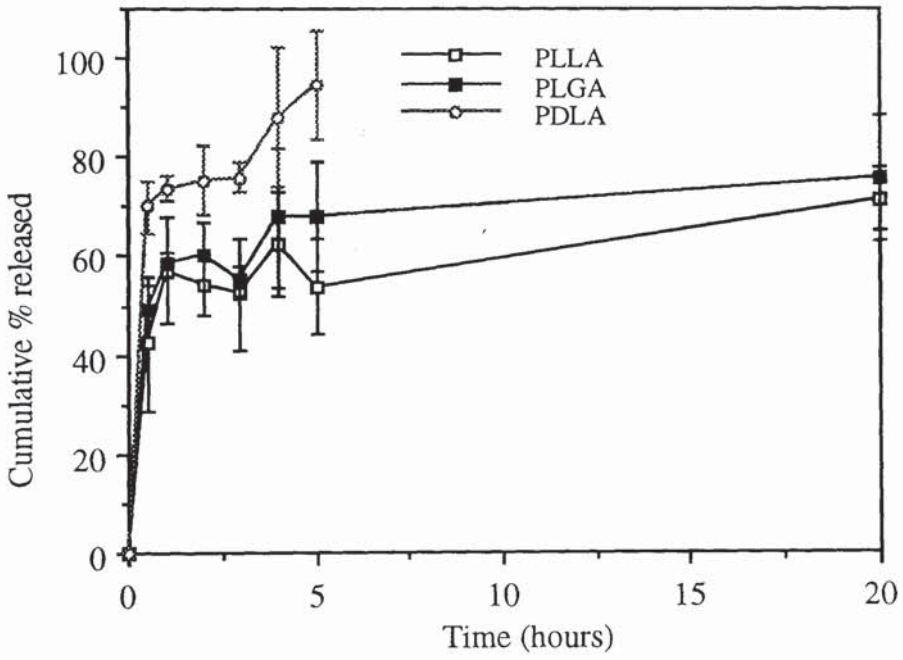


Figure 3.15. Release profiles of adsorbed BSA from the surface of spheres formed by a solvent extraction technique. PDLA- 1.11±0.42μm, 0.86% w/w load; PLGA- 0.68±0.21μm, 1.12% w/w load; PLLA- 1.11±0.42μm, 2.34% w/w load (n=3; mean±s.d.)

3.3.3 Hydrophobic interaction chromatography

Hydrophobic Interaction Chromatography (HIC) is a column chromatography which separates substances or particulates on the basis of differences in their hydrophobic

interaction with a hydrophobic gel matrix (Müller, 1991). The separation achieved is dependant on the hydrophobicity of the solute and polymer and interactions with and between the solvent water molecules. Therefore, any alterations in one of these parameters affects the degree of separation produced. Solutes bound to the matrix in a high salt concentration can be removed by reducing the salt concentration or by changing to a salt with a reduced salting-out effect (Rosengren *et al.*, 1975). As described in section 2.11, a 0.6M NaCl solution was used throughout the experiments for elution of the particles and a non-ionic surfactant (Triton X-100) was used to facilitate further elution by binding to the stationary phase causing displacement of the adhered particles (Müller, 1991).

A series of standard latexes, commercially available polystyrene standards, were used as references, having distinctly more hydrophobic surfaces than biodegradable particles (Cartensen *et al.*, 1991). These may be used as model systems for studying characterisation of hydrophobicity and there are a number of ways for display of results. A table of the final percentage eluted (after NaCl and Triton X-100 washes) is presented for a series of monodisperse polystyrene microspheres (table 3.7). Even after washing, the columns retain a large percentage of the particles, more frequent in the hydrophobic columns. The differences between commercially available microspheres from a number of sources is displayed. Only the 1.0μm particles are surfactant-free, the other being extensively dialysed prior to use. Generally, when the stationary phase is changed from propyl-agarose to pentyl-agarose, there is ~90% retention for all polystyrene batches.

Table 3.7. Final percentage of particles *per* polystyrene sample eluted *per* type of agarose (n=3; mean % eluted ± s.d.)

Formulation	Stationary Phase			
	Agarose	Propyl-	Pentyl-	Hexyl-
Latex 0.2μm	61.1±2.4	5.4±1.9	7.1±0.5	12.6±0.0
Latex 0.4μm	67.2±0.2	11.0±1.2	10.1±0.4	11.9±1.3
Latex 1.0μm	82.1±3.0	40.6±1.4	9.6±0.2	4.1±0.2
Carboxylated-Latex 1.0μm	44.2±2.4	54.2±4.7	7.1±0.7	8.0±2.2

Particles produced using PVA as the stabiliser may retain surfactant attached to the surface of the particles (Müller & Wallis, 1993). The PLA particles are washed from the

columns more readily and are therefore less hydrophobic than polystyrene particles (figure 3.16).

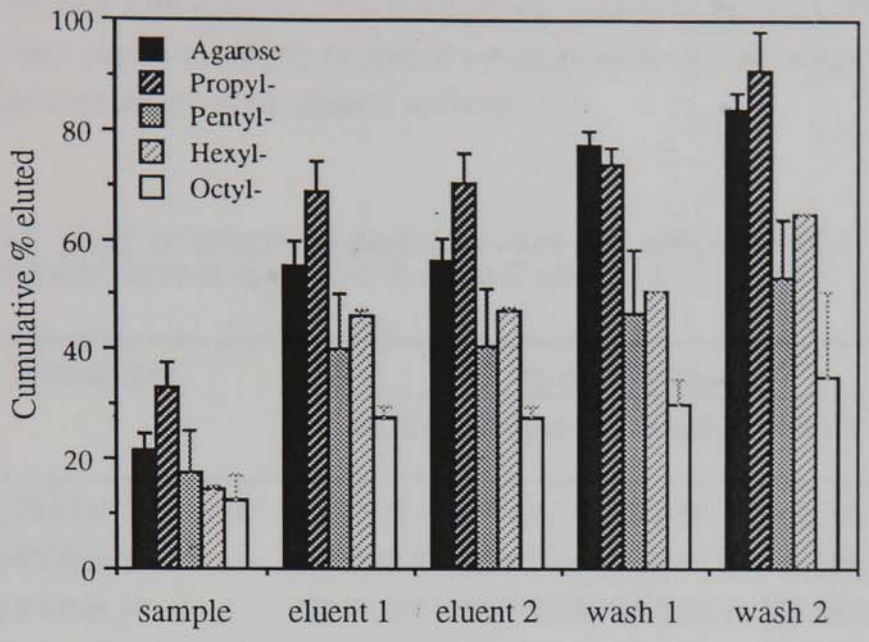


Figure 3.16. Cumulative percentage eluted from a series of columns for empty PLLA particles (1μm) formed by a double emulsion method, using PVA in the primary emulsion (n=3; mean±s.d.)

Table 3.8 lists some of the final elution percentages for a number of PLA particles. Results confirming the more hydrophilic nature of PLGA microspheres relative to PLA homo-polymer are also shown, with >80% of the microsphere suspension washed from the columns for all stationary phases studied. The technique used to form microspheres may result in some protein being deposited at the surface of the particles. When particles are loaded with BSA, the surfaces of the particles are more hydrophobic, with a higher percentage being retained on the columns following washes. This was reported previously for single emulsion microspheres (Alpar & Almeida, 1994) following incorporation of BSA and a more hydrophobic matrix was required to detect differences. This could be due to internalisation of the hydrophilic regions of the protein and exposure of the hydrophobic regions on the particle surface resulting from polar interactions between polymer and protein. As there is good correlation between helix amphiphilicty and surface activity (MacRitchie, 1978), it has been suggested that BSA adsorption at the oil-water interface is strongly influenced by its relatively high α-helix content. However, it is thought that some proteins adopt an

extended configuration at interfaces, with hydrophobic chains oriented towards the non-aqueous side and hydrophilic side chains towards the aqueous phase (MacRitchie, 1978). The globular structure of these proteins is suggested to be only partially unfolded upon adsorption (Graham & Phillips, 1979). The hydrophobic regions of the gel may interact with the accessible hydrophobic regions of the BSA. The retention in all stationary phases should be examined for an accurate overall representation of the relative hydrophobicity of the particle surfaces.

Table 3.8. Final percentage of particles eluted *per* sample of PLA microspheres (1μm) eluted *per* type of agarose (n=3; mean % eluted ± s.d.)

Formulation	Stationary Phase				
	Agarose	Propyl-	Pentyl-	Hexyl-	Octyl-
Empty PLGA* PVA in 1°	105.7±2.5	87.4±1.1	88.6±0.3	84.2±0.1	-
Empty PVA in 1°	84.1±2.8	90.9±7.3	53.3±0.6	64.7±9.8	35.0±1.6
Empty PVP in 1°	92.7±0.9	67.7±2.4	36.0±8.1	32.2±2.4	36.1±9.7
8%w/w BSA PVA in 1°	49.7±2.0	43.8±7.3	32.4±8.1	30.6±9.1	2.3±0.1
8%w/w BSA PVP in 1°	38.1±7.9	24.0±3.2	10.6±0.5	14.9±4.7	3.8±2.3
Latex control	86.9±4.7	85.1±0.4	16.7±0.9	7.0±1.1	2.2±1.3

All microspheres prepared using PLLA except * PLGA

Results have indicated that particles formed using PVP in the primary emulsion have a more hydrophobic surface than those formed using PVA. This effect is most significant for empty particles with propyl- to hexyl-agarose gels and the full profiles for propyl- and pentyl-agarose gels are illustrated in figure 3.20. As the surface hydrophobicity of the column is increased, the more hydrophobic matrix is able to bind the particles due to increased hydrophobic interaction and less particles are eluted from the column. The particles containing 0.5% w/v PVA in the primary emulsion could be eluted from the column more easily and can therefore be said to be relatively more hydrophilic than the particles containing PVP.

This effect is also demonstrated with loaded particles, though a hexyl-agarose column most clearly indicates these differences (figure 3.18). Differences in surfactant employed for the manufacture of microspheres may cause changes in wettability allowing increased or decreased solvent penetration. A more hydrophobic surface may

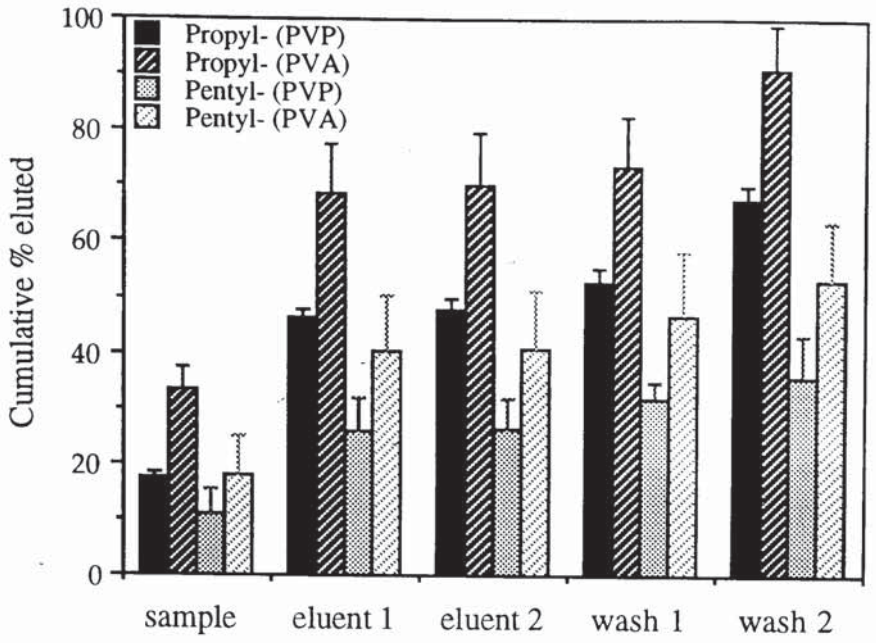


Figure 3.17. Cumulative percentage eluted from propyl- and pentyl-agarose for two microsphere batches made with different emulsifying agents in the primary emulsion (n=3; mean±s.d.)

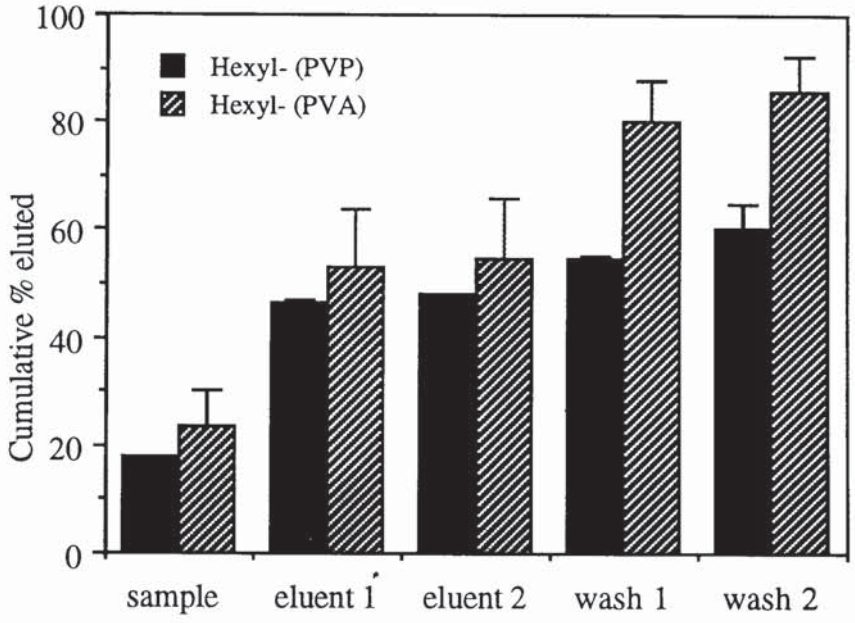


Figure 3.18. Cumulative percentage eluted from hexyl-agarose for two microsphere batches made with different emulsifying agents in the primary emulsion and with 8% w/w BSA load (n=3; mean±s.d.)

decrease the wettability of the surface, reducing penetration of the dissolution medium and resulting the smaller burst effect and overall reduction in the amount of protein released at a given time (section 3.3.2.4). In general, an increase in the hydrophobicity of a particle surface leads to an enhanced uptake by macrophages. unless the surface is very strongly hydrophobic (Tabata & Ikada, 1990). An increase in hydrophobicity may therefore lead to increased uptake at mucosal sites with subsequent translocation to the lymphatics or bloodstream by the macrophages (section 1.3.1).

3.3.4 Surface charge determination

The fate of colloidal systems in the body is determined by a number of factors, e.g. particle size, hydrophobicity and charge. Using *in vitro* cell culture techniques, it has been shown that phagocytosis takes place more readily with increasing negative zeta potentials of the microsphere surface (Tabata & Ikada, 1989). The zeta potential of microspheres is affected by the pH and ionic strength of the medium (Makino *et al.*, 1986) and therefore, only general trends regarding the surface charge can be examined. Analysis of surface charges and the assessment of changes brought about by the microencapsulation technique were carried out by studying the zeta potential of microsphere suspensions. High electric repulsion forces brought about by high zeta potentials will result in stability of the microsphere suspension. Zeta potentials were determined in two dilute buffer systems at neutral pHs to allow some comparison with the results of Müller (1991).

Table 3.9. Surface charges of various polystyrene latex formulations measured as zeta potentials (mean±s.d., n=5) in 10mM phosphate-citrate buffer

Formulation	Zeta Potential (mV)
Latex 0.2µm	-51.0±0.9
Latex 0.5µm	-54.5±0.6
Latex 1.0µm	-52.5±1.2
Latex (carboxylated) 1.1µm	-48.0±1.6

Biodegradable PLA microspheres do not show any consistent trend in zeta potentials are all less negatively charged than the polystyrene standards (table 3.9 and 3.10).

The latex spheres in table 3.10 are from a different manufacturer than those in table 3.9. and are supplied surfactant-free and the effect of the method of preparation on the surface charge can be demonstrated.

Table 3.10. Zeta potentials of a number of empty and BSA loaded formulations in two buffer systems (10mM phosphate, pH 7.0 and 10mM phosphate-citrate buffer, pH 7.0)

Formulation*	Zeta Potential (mV)	
	10mM phos. buffer	10mM phos/cit. buffer
latex	-81.9±2.3	-97.7±2.2
PLLA (PVA1)	-1.3±0.7	-1.2±0.2
PLLA (PVA2)	-7.1±0.4	-5.4±1.6
PLLA (PVP1)	0.0±0.1	1.0±0.1
PLLA (PVP2)	-6.0±0.5	-2.2±0.3
8% w/w PLLA (PVA1)	-2.3±0.5	-6.5±0.3
8% w/w PLLA (PVP1)	-4.1±0.5	-5.3±0.3
PLGA1	1.2±0.9	0.0±0.6
PLGA2	1.5±0.2	0.2±0.5

* All biodegradable spheres are prepared using a double emulsion technique using PVA in the primary emulsion, unless stated otherwise (PLLA, PVP). All preparations with the number 2 are hardened using solvent extraction whereas those with number 1 are produced by solvent evaporation.

Spheres containing encapsulated BSA are not significantly more charged than empty particles. The charges on the surface of the biodegradable polymer microspheres are therefore much lower than latex particles examined under similar conditions (table 3.10) and micronised PLLA itself which had a zeta potential of -37.5±2.9mV in 10mM phosphate buffer. The process of microsphere formation reduces the zeta potential of the polymer, possibly due to residual surfactant and adsorbed protein. Particles containing BSA were slightly more charged than corresponding empty spheres but there was no significant difference between emulsifying agents used for formation of the primary emulsion. Residual PVA may reduce the absolute negative value of the microsphere surface (Yamaoka *et al.*, 1993).

Particles with different surface charges will show, after incubation in serum, a similar negative charge (-11mV to -18mV) due to adsorption of serum components (Müller *et al.*, 1986). In this situation, hydrophobicity will play the major role in controlling uptake by the RES. This study was carried out in dilute buffer to allow comparison between formulations and it was seen that the charges were close to zero, indicating a reduced tendency for phagocytosis. Generally, it is the hydrophobicity of the particle surface which determines the extent of macrophage uptake (Tabata & Ikada, 1990).

3.3.5 Differential Scanning Calorimetry

Differential scanning calorimetry may provide insight into the distribution of drug within the polymer matrix and the effects of drug loading and other manufacturing parameters on the properties of the polymers employed to form the microparticles (section 1.1.5).

The poly(L)lactide polymer, prior to formulation, exhibits only a melt endotherm (T_m) indicating the powder is completely crystalline (figure 3.19). When PLLA is formulated into microspheres, it becomes semi-crystalline, having amorphous and crystalline regions (figure 3.20), and altering its thermal properties. The PLLA thermal behaviour illustrates that of a semi-crystalline solid with three separate and well-defined transitions corresponding to the glass transition (T_g), crystallisation (T_c) and melting (T_m) of the polymer. The operative process of microsphere production may modify the organisation of the polymer chains in the solid state, consequently leading to alterations in T_g and polymer crystallinity. The problems described by Jalil & Nixon (1989) are not encountered using this method of microsphere preparation. They observed large variations in particle size and an increased tendency for aggregates to form when using low molecular weight PLLA polymer. Although the T_g is not directly related to polymer molecular weight, lower molecular weight polymers tend to have a lower T_g. This may contribute to the degree of aggregation if particles are prepared close to the T_g of the polymers. Using 25°C or lower temperatures throughout the procedure minimises this problem.

The endothermic peak arising at the glass transition temperature (3.20) represents the increased energy required to over come the ordered microstructure developed during microsphere formation (Bodmeier *et al.*, 1989). This situation becomes more complicated when a drug is encapsulated into the polymer matrix. The presence of solvent may be assessed by an evaporation endotherm around 60-80°C. The absence of this broad endotherm corresponding to the vaporisation of the solvent indicates that the

drying process was effective in its removal of the solvent (Rosilio *et al.*, 1991). Reported glass transition temperatures (T_g values) are peak maxima as calculated by the computer software.

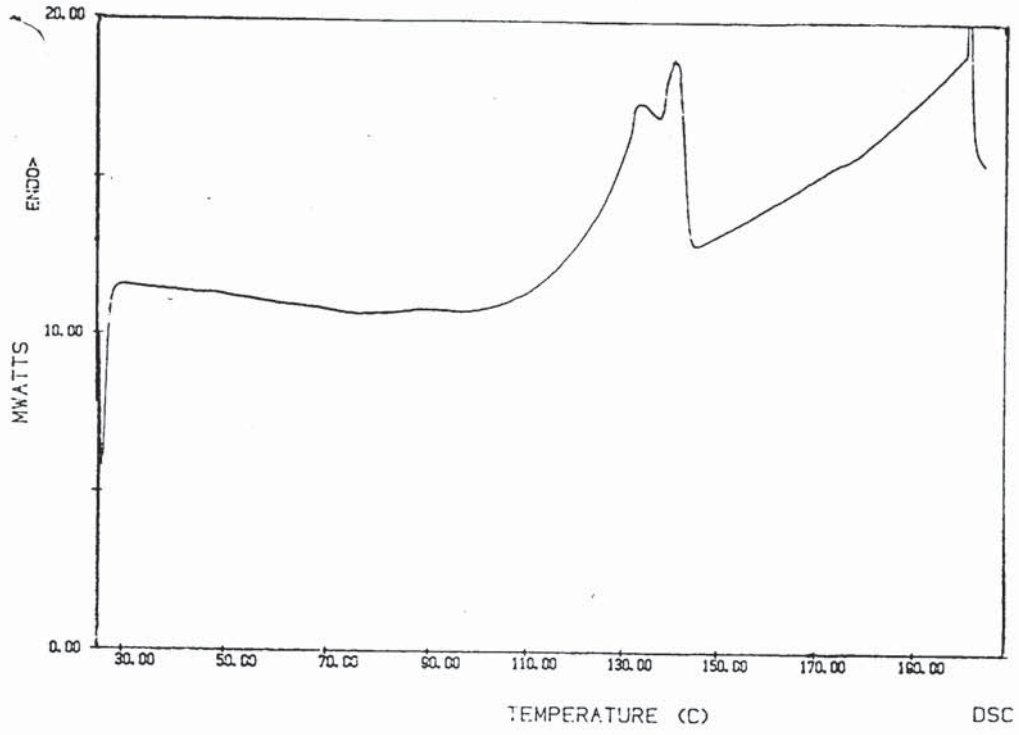


Figure 3.19. Thermal profile for PLLA polymer (Mw 2000)

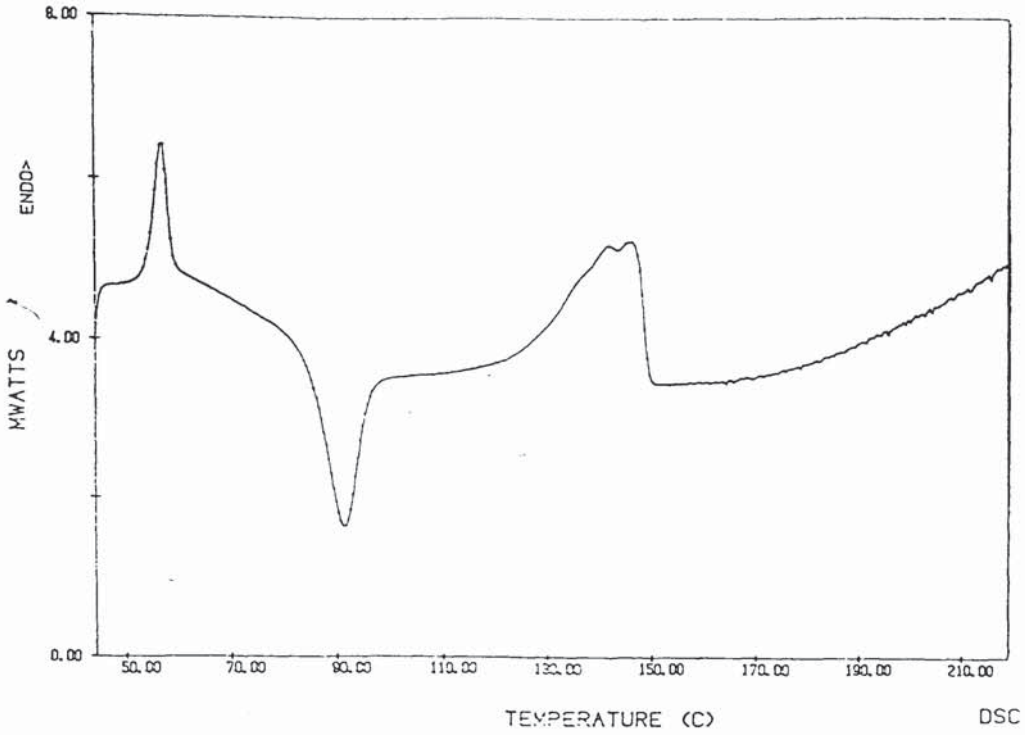


Figure 3.20. Thermal profile for empty PLLA microspheres

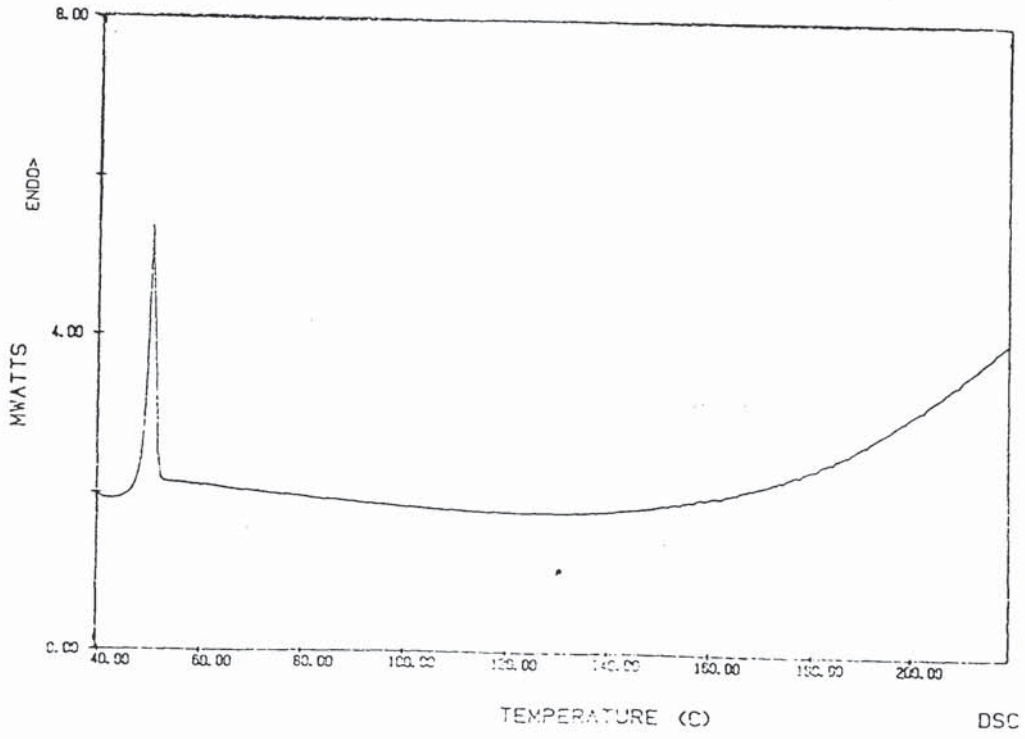


Figure 3.21. Thermal profile for empty PLGA (50:50) microspheres

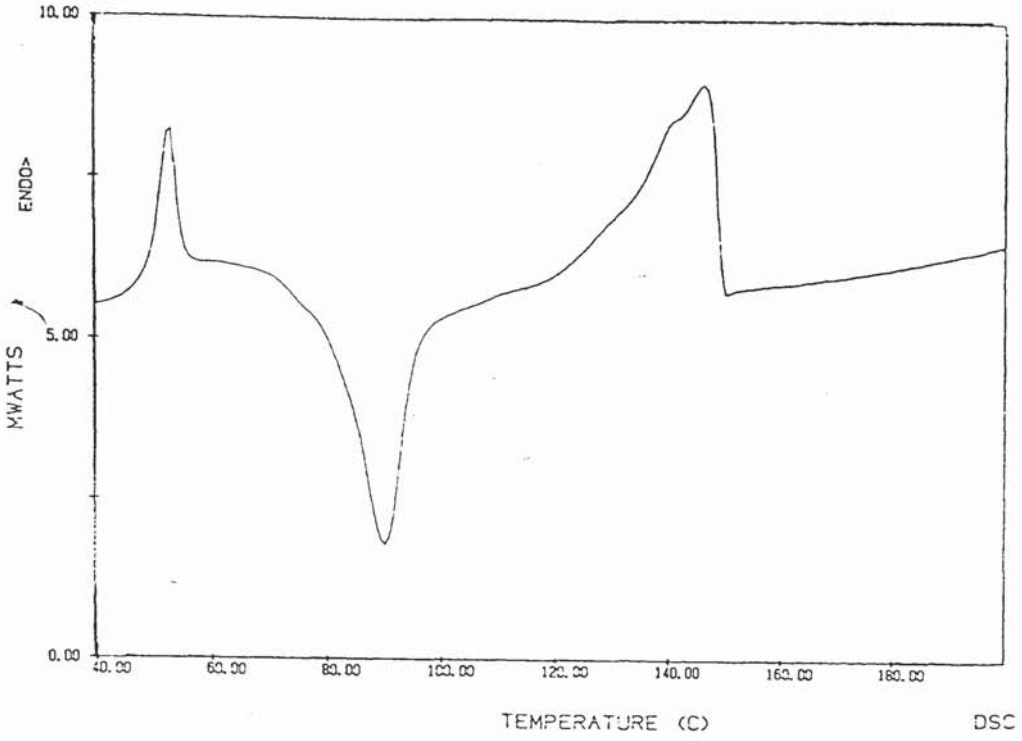


Figure 3.22. Thermal profile for PLLA microspheres loaded with 12.4% w/w BSA

For PLGA co-polymers, the T_g gradually decreases with increasing glycolide content (Asano *et al.*, 1989). Low molecular weight PDLA and 50:50 PLGA co-polymer (figure 3.21) are amorphous, exhibiting only glass transition temperatures (table 3.11). The effect of encapsulation of BSA on the glass transition temperature is also shown.

Table 3.11. Glass transition temperatures for amorphous PLA microspheres (results are the mean of three separate determinations)

Description	T_g °C
PLGA empty spheres	50.7
PLGA loaded spheres (~5% w/w)	47.7
PDLA empty spheres	48.8
PDLA loaded spheres (~5% w/w)	46.4

Drug with no affinity for the polymer will have no effect on the T_g of the polymer. If the drug interacts with the polymer to form a true or partial solution, it will plasticise the

polymer and reduce the T_g. If there is little drug-polymer affinity then the drug will tend to form crystals due to phase separation whereas drug in a solution will be thermodynamically stable and remain homogeneously dispersed. The effects of loading of active drug on the thermal behaviour of PLLA was studied using single emulsion and double emulsion techniques with both PVA and PVP in the primary emulsion (table 3.12). The presence of BSA within the microsphere reduces the T_g for the PLLA polymer (figure 3.22) but the DSC profile is otherwise unchanged. This reduction also occurs when microspheres have been formed with the amorphous PDLA and PLGA polymers (table 3.11). The T_g falls further as the loading is increased. This could be due to a small amount of water associated with the BSA. In other studies, a corresponding fall in melting transitions was described (Bodmeier & McGinity, 1987). This trend was not apparent for this system nor was there any obvious pattern of alterations in crystallisation events. The thermal behaviour of the microspheres formed using PVP in the primary emulsion does not appear different from those formed using PVA.

Table 3.12. Thermal behaviour of PLLA microspheres containing BSA

Description	T_g °C	T_c °C	T_m °C
Empty PLLA single emulsion spheres	56.9	88.8	145.3
Empty PLLA spheres (PVA[a])	57.1	83.2	144.8
Empty PLLA spheres (PVP [b])	56.3	78.8	144.9
Single emulsion 13.4%w/w	58.0	93.1	147.6
PVA[a]; 8.4%w/w	55.7	80.0	144.7
PVA[a]; 12.0%w/w	54.6	89.1	146.2
PVA[a]; 14.3%w/w	53.3	89.3	143.9
PVP[b]; 10.4%w/w	54.1	86.0	145.6
PVP[b]; 15.0%w/w	49.6	96.7	145.2

[a] PVA and methylcellulose used in formation of primary w/o emulsion; [b] PVP and methylcellulose used in formation of primary w/o emulsion. Results are the mean of three separate determinations

The effect of polymer degradation on the thermal events was also studied. Microspheres (20mg) were suspended in a 20mM phosphate buffer (pH 7.5) and maintained at 37°C for 30 days in a shaking water bath. The microspheres were collected, freeze-dried and

the DSC profiles obtained. The microspheres formed with PLGA co-polymer exhibited no significant glass transition event with the ordered structure of the matrix disappearing as the polymer degraded (figure 3.23). The same changes were observed with PDLA spheres, the T_g event was diffuse with a broadening of the melting transition. The appearance of multiple small crystalline melting peaks indicates the crystallisation of D- or L-lactic acid enriched oligomers, possibly produced by the differential cleavage of the two stereoisomers.

There is a gradual decrease in the T_g of low molecular weight PDLA during degradation. This may be due to the plasticising effect of the water (Park, 1994) or the formation of lower molecular weight degradation products. In higher molecular weight PDLA, this would not occur until a much later time, indicating that the polymer backbone is not initially subject to hydrolysis. The production of two T_g events for high molecular weight PDLA indicates that two different polymer domains are formed within the microsphere, supporting the mechanism for heterogeneous degradation suggested by Vert *et al.* (1991).

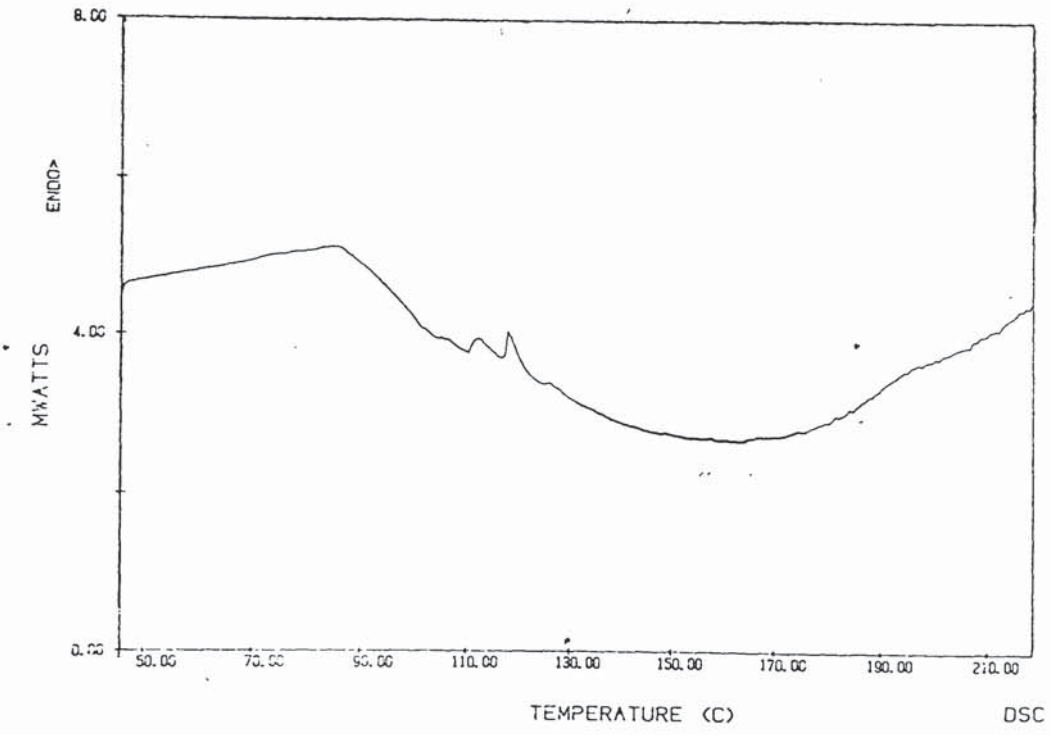


Figure 3.23. Thermal profile for PLGA microspheres after 30 days incubation in phosphate buffer

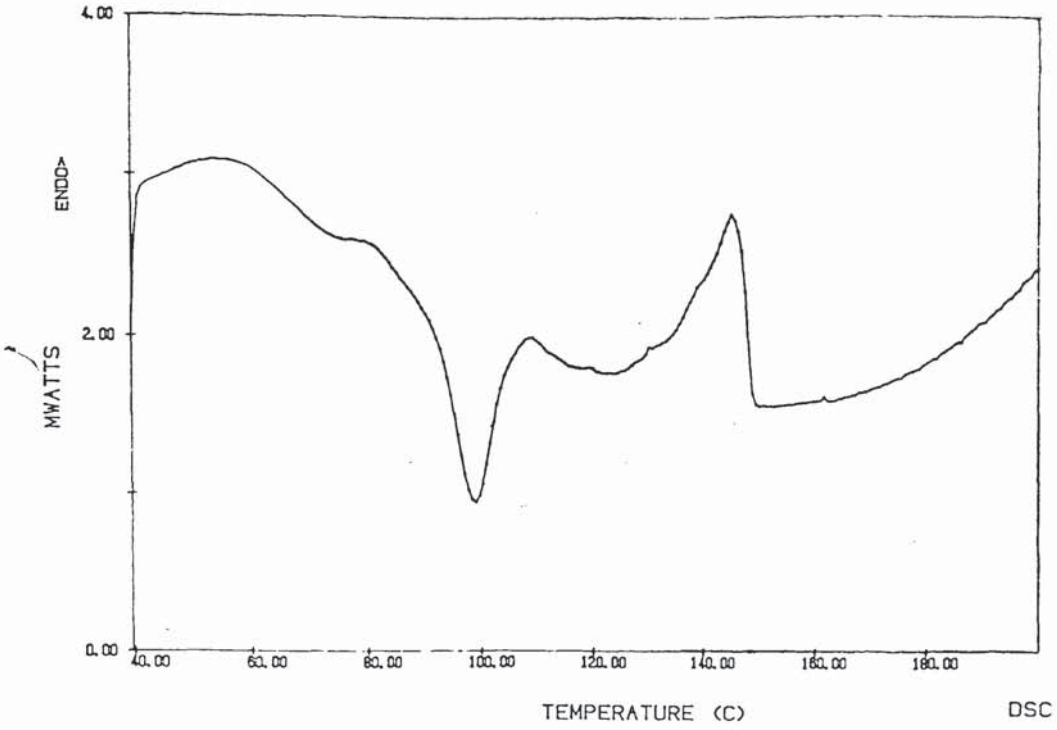


Figure 3.24. Thermal profile for PLLA microspheres after 30 days incubation in phosphate buffer

Over the time studied, for low molecular weight PLLA microspheres, the T_g became more diffuse but the other events were not altered (figure 3.24). The effects of degradation on the thermal profile of higher molecular weight PLLA may be slightly different (Pistner *et al.*, 1993). Degradation of the polymer causes a lowering of T_g, a broadening of the crystallisation and melting events and a fall in the melting point. After longer periods of incubation, the T_g for high molecular weight polymers may still be visible, but is followed immediately by a melting range with no recrystallisation.

3.3.6 pH alterations in the receiving media

The rate of polymer degradation could be responsible for some toxicity problems due to high local concentrations of degradation products leading to cell membrane damage in internalised carriers. Also, an acidic pH due to generated lactic acid accelerates polymer degradation. It is therefore important to monitor the pH in the release medium.

The generation of acid from a number of formulations was studied over a 30 day period. Acid produced from polymer breakdown lowered the pH of the buffer in some systems (table 3.13). Albumin undergoes a pH dependant N-F conformational

transformation in acidic conditions, changing from the infinitely soluble N-state to the water-insoluble F-state below pH 5. Care should be taken that the pH does not fall below these levels during *in vitro* release studies due to the production of free acids.

The largest pH decrease is seen with the fast degrading PDLA polymer (Mw 2kDa). In this most extreme case, after 40 days, the pH falls to 6.87 which is not expected to have a major effect on drug release into the surrounding medium. An extensive degradation of the polymer should result in a substantial fall in the pH of the surrounding medium (Sah *et al.*, 1994). The base reserve of the phosphate buffer ion would become depleted and no longer possess buffer capacity. There is little change in the pH of any of the suspensions, indicating that there is not extensive degradation occurring due to hydrolytic chain scission of the ester linkages and generation of lactic acid over the 40 day period. The decrease in pH of the phosphate buffer with the PDLA preparation. confirms the faster degradation of the amorphous, low molecular weight polymer. Generation of free acid is slower (<1% pH change), with the higher molecular weight. slower degrading polyhydroxybutyrates and poly(DTH carbonate) (see sections 4.1.1.1 and 4.1.2).

Table 3.13. Percent decrease in pH of phosphate buffer (pH 7.5) containing various microsphere formulations

Microsphere formulation[a]	% decrease in pH					
	Day 1	Day 4	Day 6	Day 16	Day 24	Day 40
PLGA d/e	0.15	0.32	0.42	0.57	2.01	4.23
PDLA d/e	0.40	1.36	2.42	3.49	6.08	8.39
PLLA s/e	0.15	0.82	1.03	1.87	3.02	4.58
PLLA d/e	0.22	0.63	1.11	1.32	3.13	4.80
PLLA d/e *	0.71	1.22	1.32	1.96	3.12	4.40
PHBV140	0.02	0.19	0.19	0.14	0.45	1.04
PHB540	0.00	-0.03	0.07	0.04	0.03	0.39
PHB159	0.00	-0.04	-0.03	-0.02	0.03	0.54
PHB273	-0.02	0.04	0.11	0.17	0.17	0.46
PDTHC	0.00	0.00	0.06	0.00	0.48	1.10

[a]All batches ~2μm and loaded with approximately 4% w/w BSA. Double emulsion batches include PVA in the primary emulsion except * which contains PVP (n=2), *d/e- double emulsion; s/e- single emulsion

3.3.7 Polymer Degradation Studies

Microspheres formed from all types of polymer involved in the study are generally smooth and spherical with an overall intact surface. When PLGA spheres are degraded for seven days, the particle size increases possibly due to the uptake of water and there are large pores visible on the now uneven surface (figure 3.25). After thirty days, the microsphere structure has fallen apart, with large pores visible and only the shell of the microspheres remaining intact (figure 3.26).

For PDLA particles, the pattern of results was similar with extensive erosion of the polymer after thirty days. The pattern of events was different for the semi-crystalline PLLA particles, irrespective of formulation. There was little apparent difference after seven days and although pores were visible after thirty days and the surface appeared uneven, the microsphere structure still remained intact.

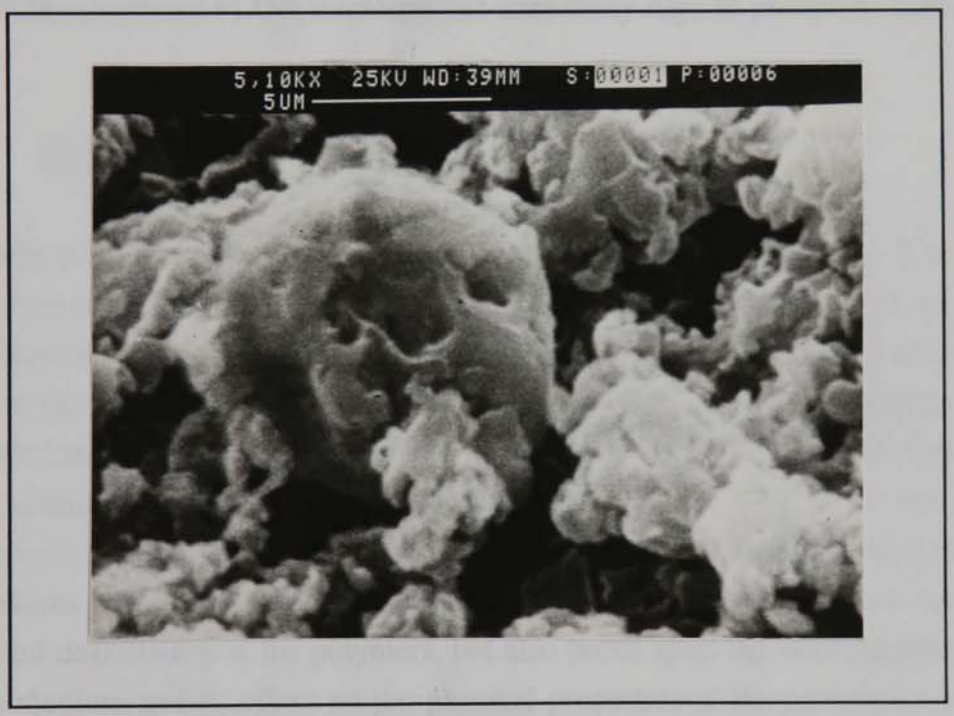


Figure 3.25. Degraded PLGA microspheres after seven days in phosphate buffer

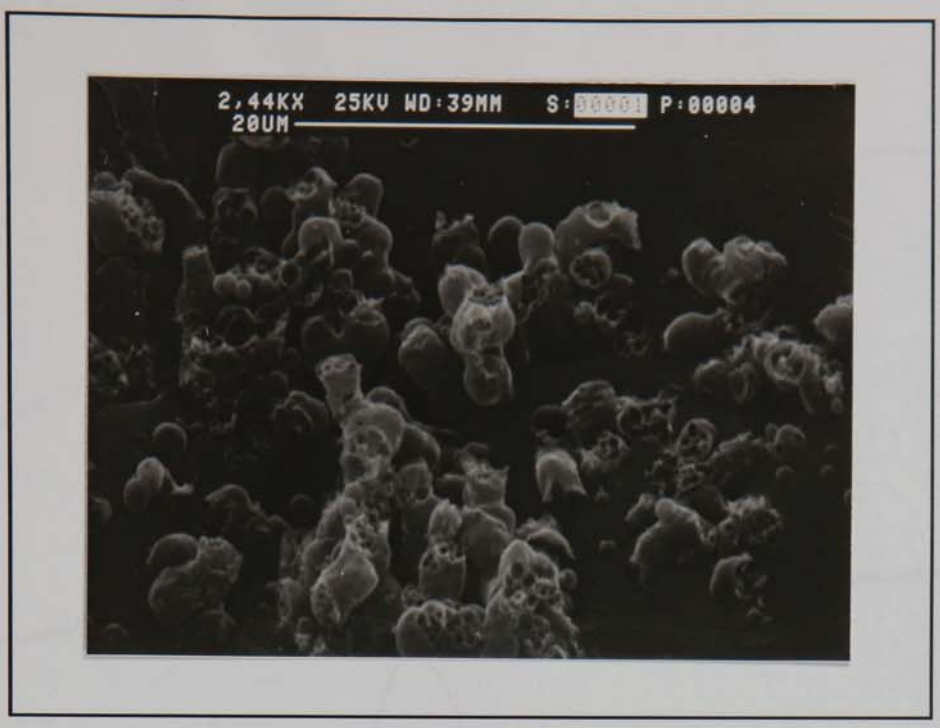


Figure 3.26. Degraded PLGA microspheres after thirty days in phosphate buffer

3.3.7.1. Gel permeation chromatography of PLA polymers

During the microsphere preparation, in this case, solvent evaporation, a hydrolysis reaction may take place with the formation of oligomers (Park, 1994). This may lead to the development of bimodal GPC traces for microspheres and for low molecular weight polymers, the formation of water-soluble oligomers during microsphere preparation will accelerate the degradation rate of the polymer. Park (1994) found acidic impurities and a sonication step to be instrumental in this degradation with substantial amounts of the oligomers (Mw 1050-1150) in samples of amorphous rather than semi-crystalline microspheres. The use of ultrasound during microsphere preparation not only accelerated degradation of the polymers, but also broke apart the microspheres in the aqueous medium and its effect on the physical properties of the particles should be taken into account. The solvent evaporation procedures used here do not require sonication at any preparative stage, an advantage when using polymers with relatively low molecular weights.

Empty or blank microspheres were employed to look at microsphere molecular weight changes in solution to prevent interference from the drug on the degradation procedure. In future, it would also be desirable to study the effects of protein loading on

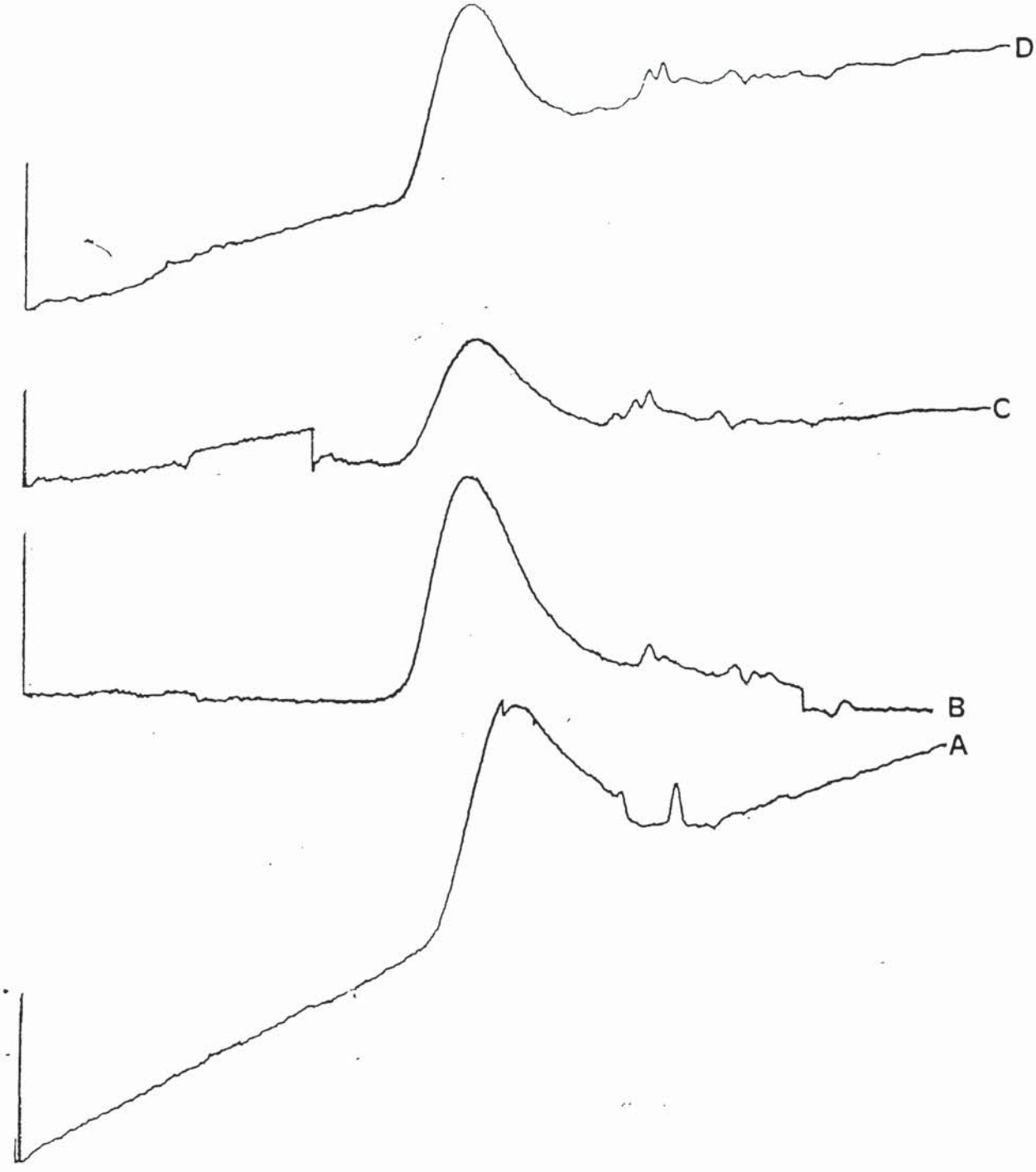


Figure 3.27. Gel permeation chromatograms for PDLA (Mw 2000) polymer and microspheres. A-polymer; B-microspheres; C-microspheres after 1 week in phosphate buffer; D-microspheres after 1 month in phosphate buffer

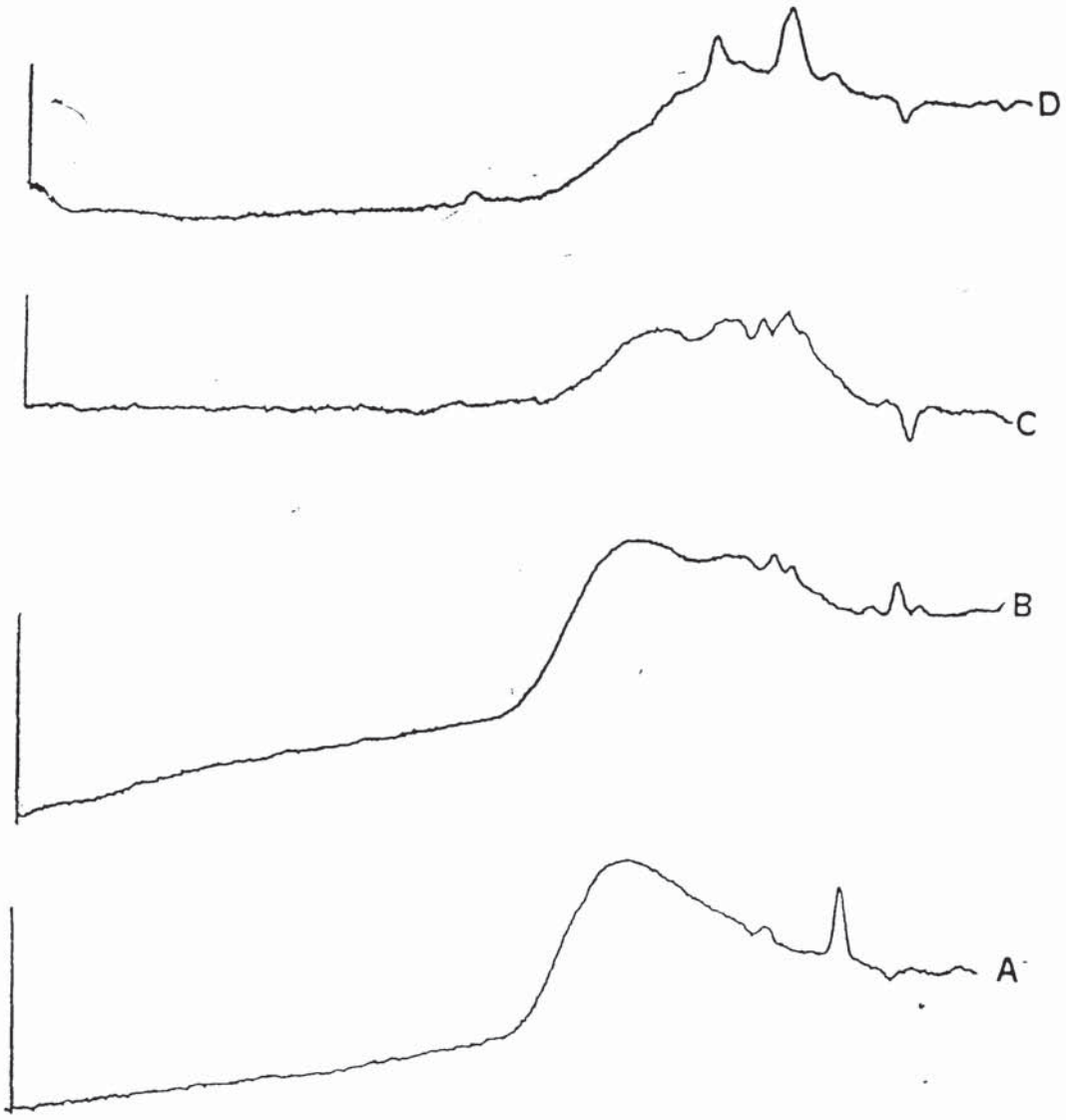


Figure 3.28. Gel permeation chromatograms for PLLA (Mw 2000) polymer and microspheres. A-polymer; B-microspheres; C-microspheres after 1 week in phosphate buffer; D-microspheres after 1 month in phosphate buffer

degradation of the polymer. The molecular weight distributions for a number of polymers, microspheres and degradation profiles are illustrated in figures 3.27-3.28. Results using the amorphous PDLA microspheres (figure 3.27) suggest that the polymer maintained a unimodal narrow distribution suggesting a homogeneous degradation throughout all the matrix. This may be due to the use of a very low molecular weight polymer which is relatively hydrophilic, permitting rapid water uptake throughout the matrix, enhancing degradation and thus protein release (Cohen *et al.*, 1991). The broadening of the molecular weight distributions (figures 3.27-3.28) suggests the co-existence of low and higher molecular weight chains (Alonso *et al.*, 1994).

The selective degradation of PLLA in the amorphous regions leads to multimodal GPC chromatograms, the peaks reflecting the molecular weights of crystalline domains (figure 3.28). Irrespective of the molecular weight of PLLA, the molecular weight should fall by relatively small amounts even after one month (Alonso *et al.*, 1993). Unfortunately, due to problems with optimisation of the columns for the low molecular weight samples involved, exact molecular weights for the polymers could not be calculated. However, it can be observed by comparison of the degradation and release profiles, that the polymer degradation rate, though contributing to the release rate determination is not the only controlling factor and release is not solely due to polymer erosion.

3.3.8 Determination of residual PVA in microspheres

Most microsphere preparation methods rely on the emulsification of aqueous and organic phases followed by the removal of the solvent. The pre-coating of PLA spheres with PVA significantly reduces their macrophage uptake (Tabata & Ikada, 1988) by reducing the particle surface hydrophobicity. Residual surfactants and chlorinated solvents are considered a drawback of these methods and quantification of these contaminants is an important consideration (Bazile *et al.*, 1992). Although PVA is generally considered safe and non-toxic, all processing may contaminate the final product and influence final product characteristics. It has been suggested that long PVA/microsphere contact times may result in incorporation of PVA into the PLA matrix, increasing the burst effect (Kwong *et al.*, 1986). Due to the extended solvent evaporation times used in this procedure, levels of residual PVA in microspheres were determined.

A calibration curve was constructed using the assay method described in section 2.10 (figure 3.29) and blank microsphere preparations were assayed for residual PVA content.

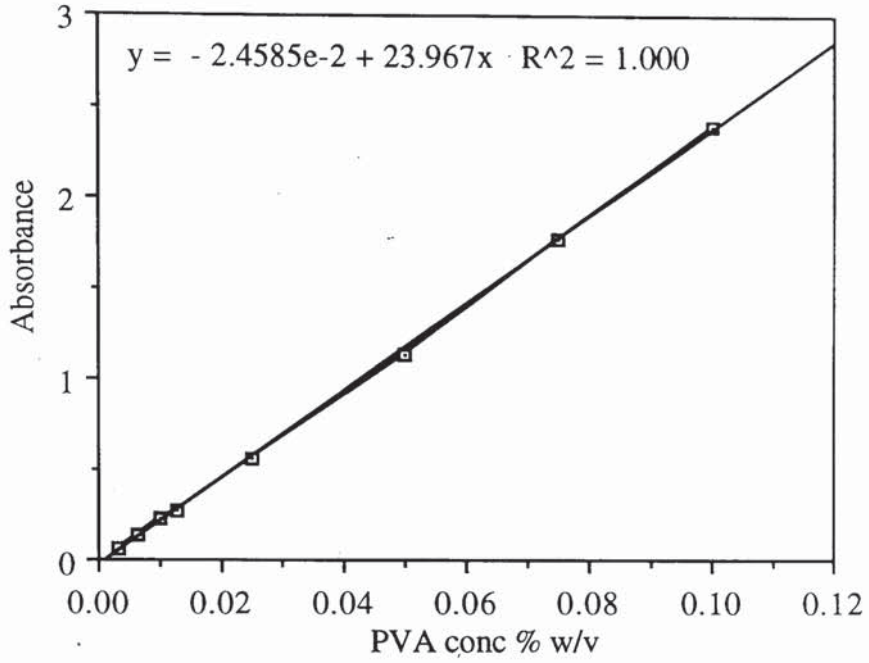


Figure 3.29. Calibration for the determination of residual PVA in microsphere preparations

The results for a number of preparations is shown in table 3.14. Values are the mean for three separate determinations on preparations of particle size 1-2μm.

Table 3.14. Residual PVA content of several microsphere formulations

Microsphere Description	Residual PVA[a]
Poly(L)lactide single emulsion	1.18±0.04
Poly(L)lactide double emulsion	1.04±0.04
Polyhydroxybutyrate-co-valerate single emulsion	1.94±0.49
Poly(DL)lactide single emulsion	1.22±0.22

[a]expressed as % w/w of microsphere

For PLLA microspheres which were freeze-dried without the washing procedure (see section 2.1), the value calculated for residual PVA was 4.02±0.05% w/w. This shows that the washing procedure is successful at removing most of the surfactant retained by the microspheres.

Where reported, residual PVA levels vary widely, with some preparations containing up to 10% of the total mass of freeze-dried product (Leroux *et al.*, 1995). As residual PVA is roughly linearly related to the specific surface area of the particles (Alléman *et al.*, 1993), the low levels of residual PVA found in these relatively small microspheres show a successful removal of the hydrocolloid. A consequence of residual PVA is the easier dispersion of small nanoparticles in water which may have an effect on the biodistribution and release of drug from microspheres.

3.3.9 Determination of residual dichloromethane content in microsphere formulations

As the production of polylactide formulations often requires the use of chlorinated organic solvents, the amount of residual solvent and its toxicity must be determined. Excess solvent is often removed by extended drying times, which may increase cost and alter the properties of the final product. The excess residual solvent is bound to the polymer and the amount bound depends on the drying process and the hydrophobicity of the polymer, i.e. increased hydrophobicity leads to increased solvent binding. Also, a more compact microsphere structure may be expected to retain more solvent. As these preparations are freeze-dried, the amount of residual solvent was expected to be low. In o/w and w/o/w emulsion solvent evaporation methods, DCM is the solvent of choice due to its low boiling point (42°C) and its low aqueous solubility (2%), leading to a suitable solvent evaporation rate.

The retention time under the conditions described (see section 2.10), with a flow rate of 24.5ml/min, for DCM was 1.15 minutes and for chloroform was 2.95 minutes. As the levels of DCM in the samples were very low, the sensitivity of the instrument had to be increased. As a result, low levels of DCM were detected in the HPLC grade chloroform used to dissolve the samples. The peak height remained consistent over a large number of injections of solvent and was treated as a constant (figure 3.30).

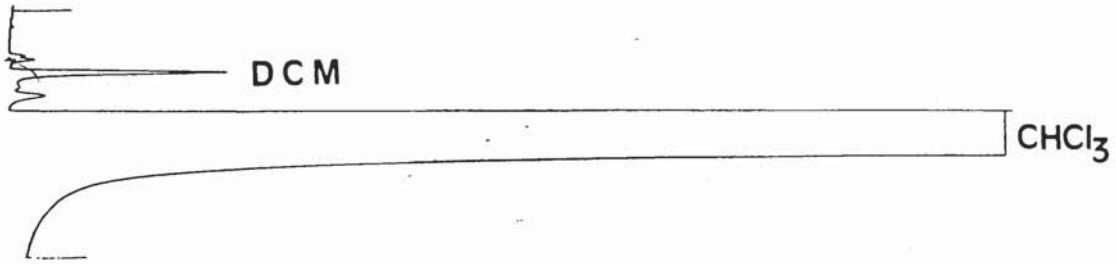


Figure 3.30. GC trace for PLLA microspheres dissolved in chloroform

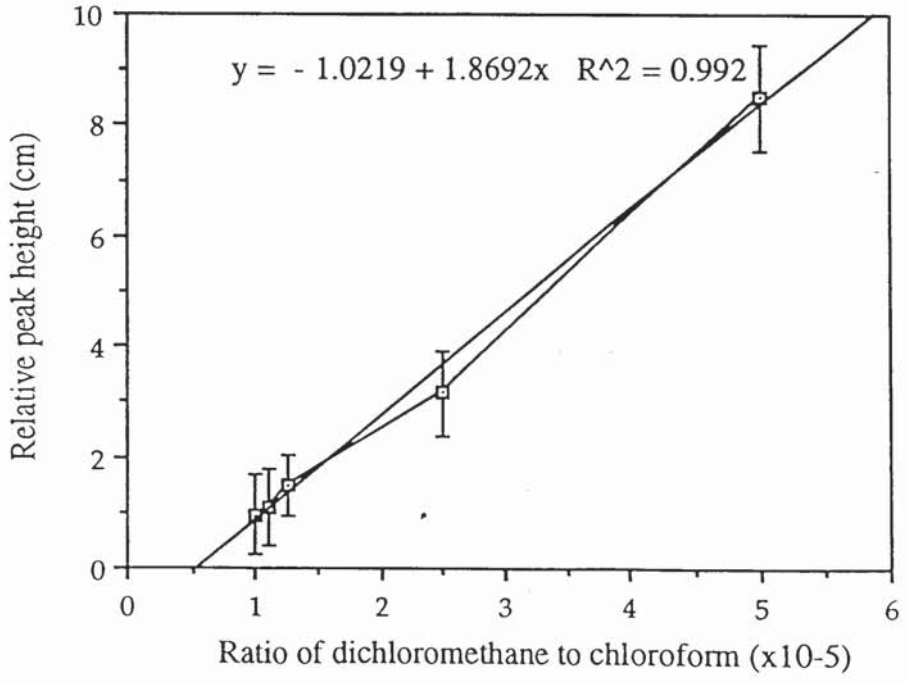


Figure 3.31. Calibration curve for the determination of residual dichloromethane, using solutions of dichloromethane in chloroform

A calibration curve was constructed for standards in the range 1:100,000 to 1:20,000, samples with higher DCM content being off scale at the sensitive settings used (figure 3.31). Each microsphere sample batch was analysed in three separate studies and each run was performed in triplicate.

Results for all microsphere formulations tested gave results below, or similar to, 10 parts DCM *per* million, the detection limit of the system. The use of different polymers, PLA, polyhydroxybutyrate or poly(DTH carbonate) for microsphere production, was shown to have no significant effect on residual solvent content, nor the solvent evaporation method used for microsphere production. Bitz & Doelker (1995) describe unacceptable results following a spray-drying procedure when DCM was replaced with chloroform. All microsphere preparations using a variety of methods and polymers involving DCM were below the acceptable limits stated by Martindale (30th edition, 1993); recommended limits for short-term exposure to DCM of 250ppm and 100ppm long-term in Great Britain. In United states, the permissible limits are 500ppm long-term with 2000ppm as the maximum short-term value.

Typical results from other studies show DCM levels below 250ppm; the lowest detection level for the instruments used (O'Hagan *et al.*, 1994) and Bodmer *et al.* (1992) found residual organic solvents below 0.5%. An elevated inner aqueous phase volume would lead to an increased removal of DCM during the solvent removal process (Crotts & Park, 1995). The low values obtained for DCM retention in the systems prepared may be due to the relatively large inner aqueous phase volume (25% of initial emulsion) providing a porous layer allowing free solvent diffusion.

3.3.10 BSA stability in microsphere formulations

Recent studies have indicated that more complex viral and bacterial antigens can be incorporated into microspheres with preservation of their antigenicity (Moldoveanu *et al.*, 1993; Reid *et al.*, 1993). From SDS-PAGE results, encapsulation of BSA in PLA microspheres shows no fragmentation of the protein during the encapsulation or irreversible aggregation after contact with DCM (figure 3.32) indicating there is no chemical interaction between the polymer and the protein being retained by the dense polymer matrix. These results are further supported by the release of >80% of the protein from the microspheres (section 3.3.2). These results show good agreement with those of Sah & Chien (1993) who showed for three different microsphere formulations of PLGA and PDLA containing BSA, that there were no adverse effects after encapsulation and after storage for three months at room temperature. They also

showed no alteration in the secondary structure of BSA after encapsulation using circular dichronism analysis.

Encapsulation of fluorescein isothiocyanate-horse radish peroxidase (FITC-HRP) into PLGA microspheres had no significant effect on enzymatic activity and allowed retention of activity for a prolonged period (Cohen *et al.*, 1991). SDS-PAGE and isoelectric focusing (IEF) rule out the possibility that the purity of the protein has been affected by the process, there has been no non-reducible dimer or covalent oligomer formation nor the production of any new charged species. This does not conclude that the protein has not been oxidised or reduced nor does it rule out the formation of covalent adducts with lactic or glycolic acid. Retention of immunogenicity after microencapsulation is also illustrated by the serum antibody responses detected after i.m. administration of antigen (section 4.3.8).

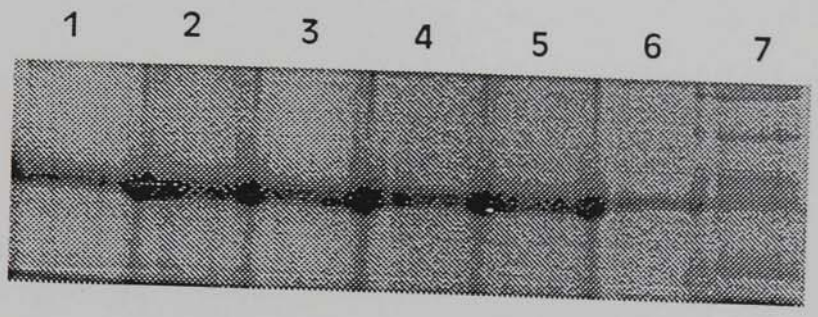


Figure 3.32. SDS-PAGE (15%) pattern of BSA microencapsulated by solvent evaporation techniques. Lanes (1) BSA prior to encapsulation (2) PLLA d/e (3) PLLA s/e (4) PDLA d/e (5) PLLA d/e* (6) adsorbed to PLLA d/e solvent extraction (7) molecular weight standards
(PLLA d/e* contain PVP in formation of the primary emulsion, all other batches contain PVA)

3.4 SUMMARY

The solvent evaporation technique employed in this study was suitable for producing microspheres of 1μm in size and with controllable protein loading and yields, depending on the application of the formulation. The influence of the surfactant used during formation of the primary emulsion was evident from the loading and the release patterns. Using PVP in the emulsification procedure, produced particles with a more hydrophobic surface demonstrating decreased burst effect and a reduced amount of protein released, resulting in a significant extension in the protein release period. Analysis of the physicochemical characteristics of the microspheres produced allows detection of differences between different formulations in terms of crystallinity, hydrophobicity, surface charge, residual solvent and surfactant levels. The effects of these parameters on the loading and release profiles obtained have also been studied and the stability of BSA within the microsphere structure has been confirmed.

4.0 PHB AND PDTHC POLYMERS FOR MICROENCAPSULATION OF A MODEL ANTIGEN

4.1 INTRODUCTION

As absorption of microparticles is partially dependant on hydrophobicity (Eldridge *et al.*, 1990), it is anticipated that the development of more hydrophobic biodegradable microspheres may lead to improved uptake and slower release, enhancing the adjuvant effect conferred by microencapsulation. Although PLA microspheres are being used experimentally with a high degree of success for the stimulation of immune responses, the administration of a preparation containing a combination of microspheres with different properties, may assist in the production of a single dose vaccine preparation. To examine the extent and duration of immune responses generated following the administration of microspheres with different properties, particles were prepared encapsulating a model antigen, BSA. The particles were fully characterised and the antibody response following i.m. administration studied.

4.1.1 Polyhydroxybutyrate polymers and polyhydroxybutyrate co-polymers

Poly-3-hydroxyalkonates are linear, semi-crystalline, hydrophobic, thermoplastic polyesters which occur naturally in a wide variety of organisms including bacteria and algae, where they function as intracellular energy and carbon storage materials. Poly-R-3-hydroxybutyrate (PHB) was the first to be discovered and is the most widespread in nature. PHB and co-polymers can be extracted in high purity from living organisms with essentially similar properties when extracted from different sources. They are commercially produced by a bacterial fermentation process using renewable foodstuffs such as glucose. PHB and poly-3-hydroxybutyrate-hydroxyvalerate co-polymers (PHBV) co-polymers have recently emerged as possible agents for drug delivery (Arshady 1991). They are structurally related to polylactides (figures 4.1 and 4.2).

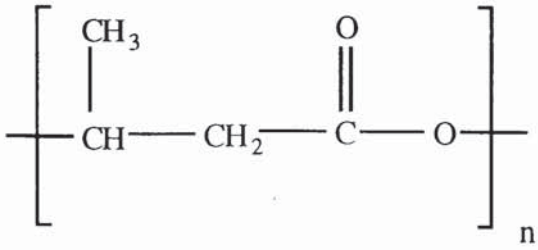


Figure 4.1. The structure of polyhydroxybutyrate polymer

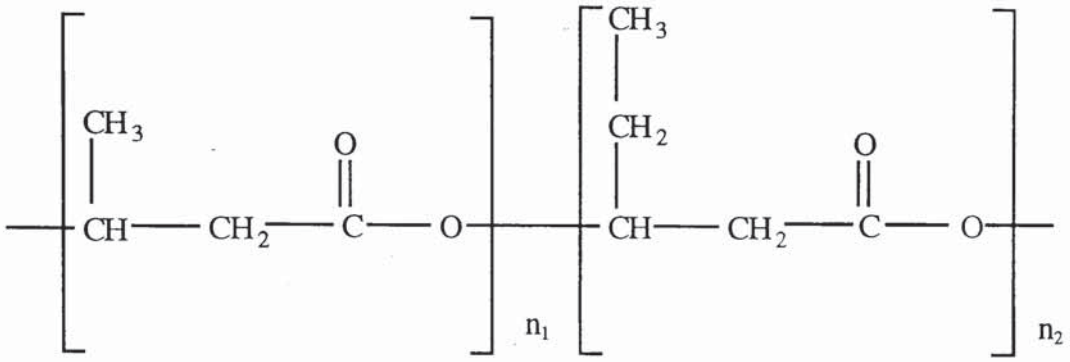


Figure 4.2. The structure of polyhydroxybutyrate-co-valerate polymer

The homopolymer, PHB, has high crystallinity (80%), leading to a stiff molecular structure, which is reduced to 30-40% following introduction of 24% w/w valerate residues, altering the bulk properties of the polymer including its degradation (Gangrade & Price, 1991). At molecular weights of less than 200 000, the mechanical properties of PHBs are altered and the material becomes quite brittle (Cox, 1992). Co-polymerisation with hydroxyvalerate (HV) allows reduction of the hardness and brittleness associated with the PHB homopolymer. Poly(β-hydroxybutyrate) is a very promising biodegradable polymer which has been used as a material for the preparation of microparticles by solvent evaporation processes and nanoparticle have been produced using high pressure emulsification (Koosha *et al.*, 1989; Juni *et al.*, 1986).

4.1.1.1 Degradation of polyhydroxybutyrate and co-polymers

As PHB is a polyester, biodegradation by hydrolysis can be expected (Juni *et al.*, 1986). The same factors influence degradation of PHB and PLA, such as molecular mass, co-polymer ratio, physical form of the sample, temperature, pH and crystallinity.

PHB crystallises readily with a melting point in the range 160-180°C, depending on extraction procedure and molecular weight. The poly-3-hydroxybutyrate-hydroxyvalerate (PHBV) co-polymers are also semi-crystalline, with lower melting points depending on valerate content.

PHB has a relatively slow rate of *in vivo* hydrolytic degradation compared with polylactides (Gogolewski *et al.*, 1993) and polymers with higher valerate degrade more extensively (Wang *et al.*, 1990). The decrease in molecular weight is accompanied by a narrowing of Mw, with only 15-43% degradation following 6 months s.c. implantation and weight losses ranging from 0-1.6%. The rate of biodegradation of polymers can possibly be responsible for some toxicity problems due to high local concentrations of degradation products leading to cell membrane damage in internalised carriers and the very slow degradation of PHB may account for its low toxicity (Engelberg & Kohn, 1991).

4.1.1.2 Biocompatibility of PHB

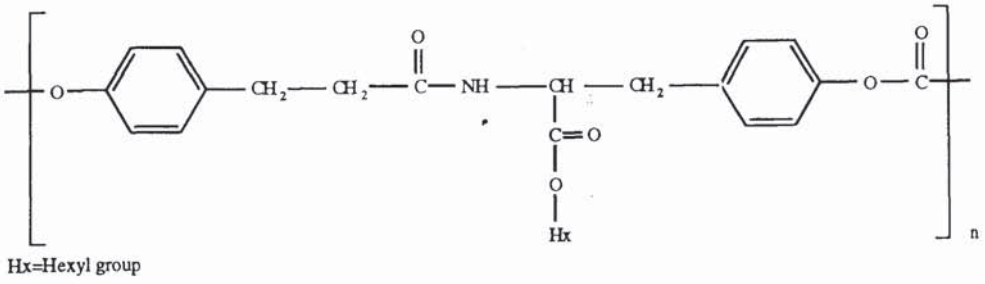
Early tissue responses developed after implantation of PLA, PHB and PHBV polymers have been studied and compared (Gogolewski *et al.*, 1993). Long term tissue responses to and *in vivo* degradation of PHB and PHBV co-polymers are less well documented than the more commonly used PLA. However, all the polymers involved in this study exhibited good tissue tolerance up to six months following s.c. administration. As the valerate ratio of the polymer increases, the number of inflammatory cells increases and overall, early tissue response to PHB and PHBV(1-3 months) is greater than PLA but after 6 months, the tissue response to both groups of polymers was similar (see section 1.1.6). The suitability of PHB polymers as biodegradable polymeric implants was assessed by Saito *et al.* (1991) and PHB films did not produce any detectable inflammation.

4.1.2 Poly(DTH carbonate) polymer

Systems based on PLA polymers are the most likely candidates to receive approval first for vaccine delivery. However, polymers with built-in adjuvanticity, such as iminocarbonates, may be ultimately better suited for the delivery of poorly immunogenic subunit vaccines. Due to the known adjuvant behaviour of L-tyrosine and its derivatives (Miller & Tees, 1974), a polymeric antigen delivery system which would degrade into a tyrosine was designed. Polyiminocarbonates are little known, biodegradable polymers that can be derived from polycarbonates by the replacement of

the carbonyl oxygen by an imino group. Iminocarbonate polymers with added tyrosine, a known adjuvant, were examined for antibody titres to BSA following immunisation with and without the adjuvant. An implant containing BSA prepared from a *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine hexyl ester-iminocarbonate (CTTH-iminocarbonate) polymer induced significant anti-BSA antibodies for over 56 weeks following a single dose administration which were significantly enhanced compared with control non-tyrosine containing iminocarbonate polymer implants (Kohn *et al.*, 1986). A biologically degraded product of the polymer, CTTH, was found to be as potent an adjuvant as FCA and MDP in enhancing the immune response and the polymer could be used to construct a device capable of stimulating the immune process, simultaneously releasing antigen over a prolonged time period (Langer *et al.*, 1990). However, despite studies carried out showing more improved controlled release properties of polyanhydride microparticles (Langer, 1990) and an enhanced adjuvant effect with iminocarbonates (Kohn *et al.*, 1986), polyesters remain the only FDA-approved biodegradable polymer in common use for preparation of microspheres.

Pseudopolyamino acids differ from conventional polyamino acids: dipeptide "monomers" are polymerised through their side chains with non-amide linkages. Preliminary safety data indicates that they have the low toxicity expected with standard polyamino acids (Kohn & Langer, 1987). The tyrosine-derived diphenols can be modified along the pendant chain attached to the polymer backbone (figure 4.3). Poly(Desamino-tyrosyl-tyrosine hexyl carbonate ester), (PDTHC) was selected due to its ability to form microspheres and its hydrophobic character and release properties (Kohn, personal communication). It has a half-life of about 26 weeks (Pulapura & Kohn, 1992a) and due to its relative hydrophobicity, the microspheres' absorption by the M cells could be enhanced. Injection of a suspension of monomer was not found to elicit any inflammatory response (Haque *et al.*, 1989).



Hx=Hexyl group

Figure 4.3. The structure of poly(DTH carbonate)

The combination of this matrix with various biodegradable polymers could provide a multi-dose pulsatile release system capable of converting vaccines requiring multiple booster immunisations into a single dose vaccine with the same or increased efficacy.

4.2 MATERIALS AND METHODS

Polyhydroxybutyrate polymers were a kind gift from Zeneca Bioproducts (Cleveland, U.K.) in a range of molecular weights as detailed in table 4.1.

Table 4.1. Molecular weights of polyhydroxybutyrate polymers and co-polymers employed for microsphere preparation

Polymer	Abbreviation	Molecular weight (Mw)
polyhydroxybutyrate	PHB159	159 000
polyhydroxybutyrate	PHB273	273 000
polyhydroxybutyrate	PHB540	540 000
polyhydroxybutyrate-co-valerate*	PHBV140	140 000

*Polyhydroxybutyrate-co-valerate contains 12% mol% HV

PDTHC polymer was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) with a molecular weight of 250 000-400 000 and was stored at 0°C until use, as detailed in the product information supplied. All other materials used in these experiments are as detailed in section 3.2.

All the methods used for microsphere production and analysis in this chapter are as detailed in chapter 2. However, to dissolve the higher molecular weight polymers of PHB, the samples required refluxing in the organic solvent, DCM, followed by evaporation of the excess solvent. The resultant solutions were very viscous, and the volume of DCM employed during microsphere preparation was doubled.

4.2.1 Immunisation studies using microencapsulated BSA

Female Balb/c mice (~25g) were used for immunisation studies and during the experiments, all animals were allowed water and food *ad libitum*. The mice were immunised with a dose equivalent to 5μg of BSA in 100μl of PBS by the i.m. route on the back hind leg. The preparations used included encapsulated BSA, free BSA and BSA incorporated into the aqueous phase of a 1:1 w/o with FIA (table 4.2). A single vaccine dose was administered on day 0 and blood samples were removed by superficial venepuncture of the tail veins on days 21, 43, 58 and 79 of the immunisation schedule. On day 118, blood samples were removed by cardiac puncture under terminal anaesthesia. The blood was allowed to clot overnight and the serum was collected by centrifugation and stored frozen at -20°C until assayed for anti-BSA IgG.

Table 4.2. Preparations and their characteristics used in i.m. immunisation schedule as described in section 4.2.1

Group	Polymer	Load %w/w (BSA)	Particle size (μm±σg)
A	PLLA[b]	3.32	1.42±0.62
B	PDLA[b]	2.18	1.68±0.90
C	PLGA[b]	2.44	1.64±0.79
D	PDTHC	5.10	0.90±0.44
E	PHB540	5.01	0.99±0.64
F	PHBV140	5.52	1.02±0.99
G	PHB273	3.39	2.50±1.21
H	PHB159	4.67	2.67±2.18
I	Free BSA	-	-
J	BSA+FIA	-	-
K	negative control	-	-

[b]preparations and release characteristics presented in sections 3.2 and 3.3.2

4.2.2 Enzyme Linked Immunosorbent Assay (ELISA)

Antibody (IgG) response in serum to the BSA in immunised animals was monitored using a microplate ELISA. The 96-well ELISA microtitre plates (Immulon 2, flat

bottom plates, Dynatech, Sussex, U.K.) were coated with 100µl *per* well of a 100µg/ml BSA in PBS solution (pH 7.3) overnight at 4°C. The plates were thoroughly washed three times in a 0.05%v/v solution of Tween-20 in PBS (PBST) and allowed to dry. The serum samples were serially diluted in PBS using a 1 in 2 dilution in round-bottom microtiter plates (Fisons, Loughborough, Leics., U.K.) and 50µl of each sample was added to each well of the coated ELISA plates. These were covered and incubated for one hour at 37°C. The plates were washed three times with the PBST solution. 50µl of the goat anti-mouse horseradish peroxidase conjugate (Sigma. A4416), diluted 1 in 1000 using PBS, was added to each well and the plates covered and incubated for one hour at 37°C. The plates were, again, washed three times in PBST solution. 50µl of the substrate (2,2' azino-bis)3-ethylbenzthia-zoline-6-sulphonic acid (ABTS), at a concentration of 3 tablets *per* 50ml of a citrate buffer plus 5µl hydrogen peroxide, was added to each well and incubated at 37°C for 10 minutes for colour development. The citrate buffer was prepared in advance using 44% 0.1M citric acid and 56% 0.2M disodium hydrogen orthophosphate. The plates were read at 405nm on an ELISA reader (Anthos Reader 2001, Anthos Labtec Instruments, Austria).

End-point titres were expressed as the reciprocal $\log_2$ of the last dilution which gave an optical density (at OD_{405}) above the OD_{405} of negative controls after the 10 minute incubation. Serum samples from individual animals were studied to determine the titre range and the mean immune responses of the different groups of animals at each time point were compared and statistical significance was assessed using Student's unpaired t-test. Values with $p<0.05$ were considered significant.

4.3 RESULTS AND DISCUSSION

4.3.1. Characterisation of microspheres prepared from PHB polymers

Though most particles are spherical, PHB does tend to form particles with more variety of size and shape than PLA (Bissery *et al.*, 1984). The rough, uneven surfaces often associated with PHB microspheres can be seen in figures 4.4 and 4.5. This has been attributed to its relatively high crystallinity (Bissery *et al.*, 1984). Changing the solvent from chloroform to DCM produces microspheres with smoother surfaces (Gangrade & Price, 1991). As the morphology of the spheres depends on the morphology and precipitation behaviour of the polymer, the smoother surfaces of the PHBV spheres may be due to the plasticising effect of the co-polymer (Gangrade & Price, 1991).

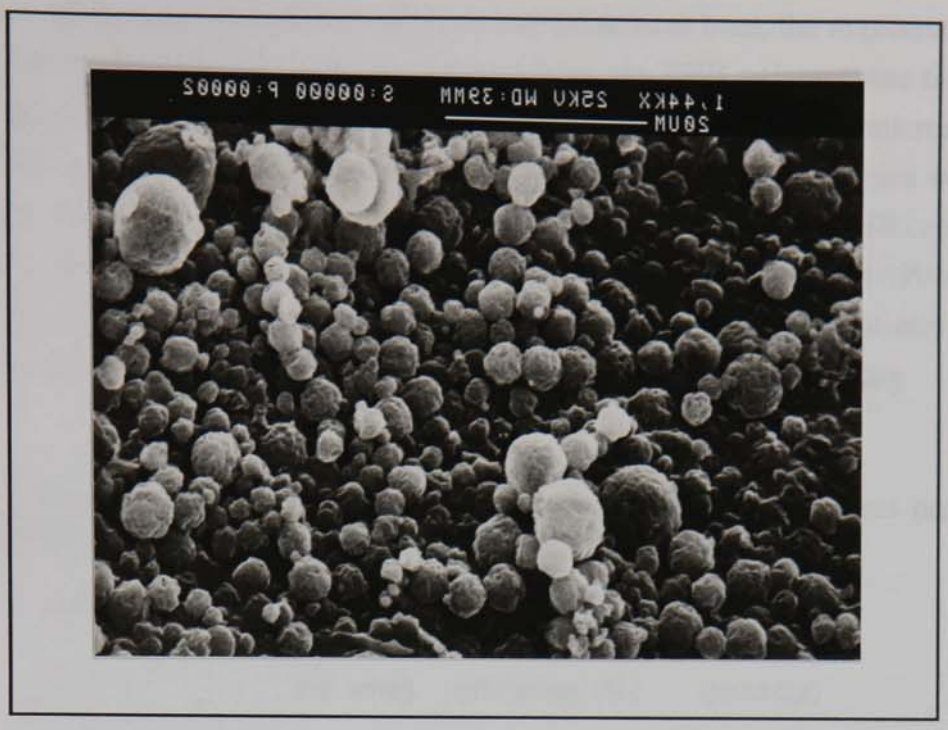


Figure 4.4. Scanning electron micrograph of PHB540 microspheres loaded with 4.26% w/w BSA

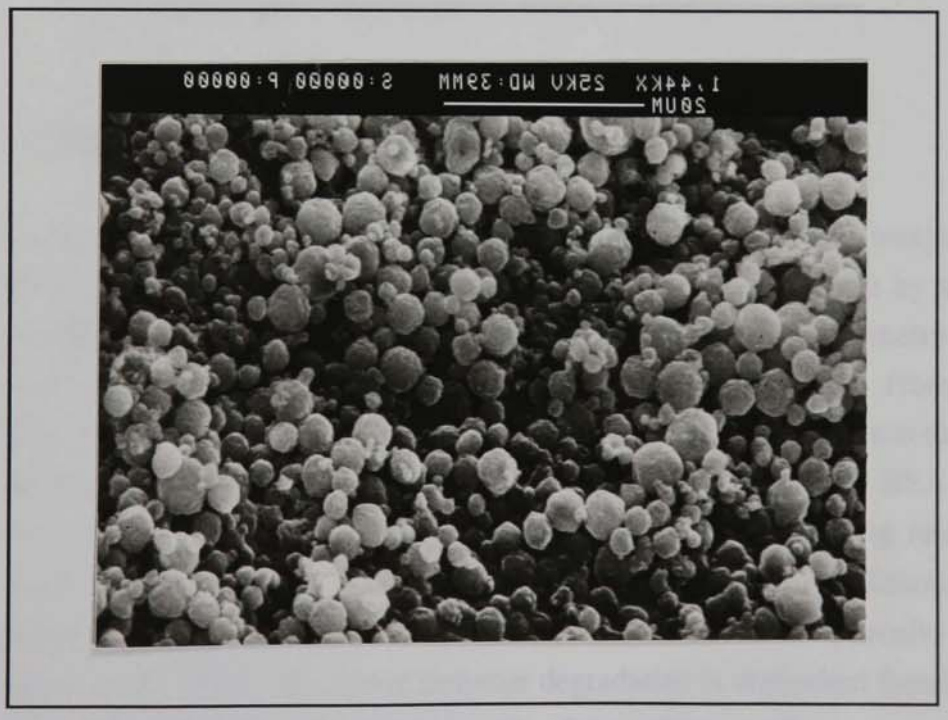


Figure 4.5. Scanning electron micrograph of PHBV140 microspheres loaded with 4.04% w/w BSA

Irrespective of the manufacturing procedure and theoretical load, the maximum BSA that could be loaded into the particles produced from any PHB polymer was 6% w/w. Even though particle size for BSA loaded was generally larger than that of microspheres produced using the PLA polymers described in chapter 3, loading efficiencies were not as high (table 4.3). The relatively high crystallinity and inflexibility of PHB compared to PLA may be responsible for its poor encapsulation efficiencies. The 12% HV content of the PHBV140 polymer has a plasticising effect, reducing the crystallinity of the polymer but not to such an extent as to significantly improve the drug loading.

Table 4.3. The loading efficiency and particle size of PHB microspheres produced using a double emulsion, solvent evaporation method

Polymer	Actual load (% w/w)	Encapsulation efficiency (%)	Particle size (μm±σg)
PHB159	3.97	39.7	1.33±1.13
PHB273	4.18	41.8	1.35±0.61
PHB540	4.06	40.6	0.91±0.54
PHBV140	4.43	44.3	1.01±0.99

4.3.3.1 Release of BSA from PHB microspheres

PHBs degrade relatively slowly *in vitro* producing the monomeric 3-hydroxybutyric acid, which is a normal blood constituent. They are reported to degrade by surface hydrolysis (Akhtar & Pouton, 1989) under the control of the surface geometry of the particle. The release of BSA, a large molecular weight molecule, from PHB microspheres, is not controlled by polymer surface erosion as predicted from the slow degradation rates of the polymer (Akhtar & Pouton, 1989) as loading of the BSA within the polymer matrix permits the formation of aqueous channels and drug release is complete before total degradation of the polymer (figures 4.6 and 4.7). Release rates from PHB microspheres are therefore affected by differences in the porosity of the matrix (Bissery *et al.*, 1984). The rate of polymer degradation is dependant therefore on the properties of the polymer surface and porosity of the polymer bulk.

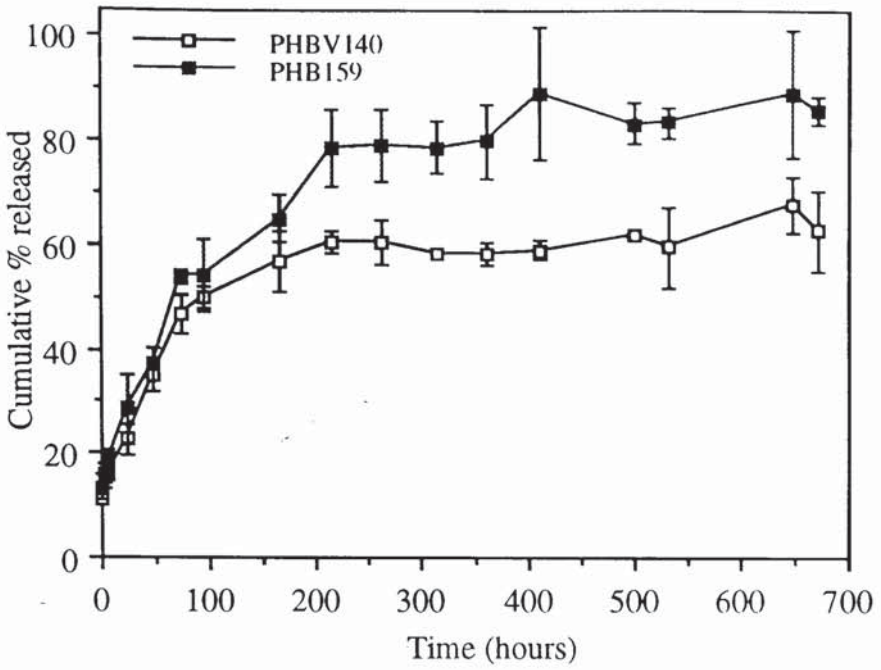


Figure 4.6. Release profiles for ~4% w/w BSA from PHB microspheres formed by a double emulsion technique (n=3; mean±s.d.)

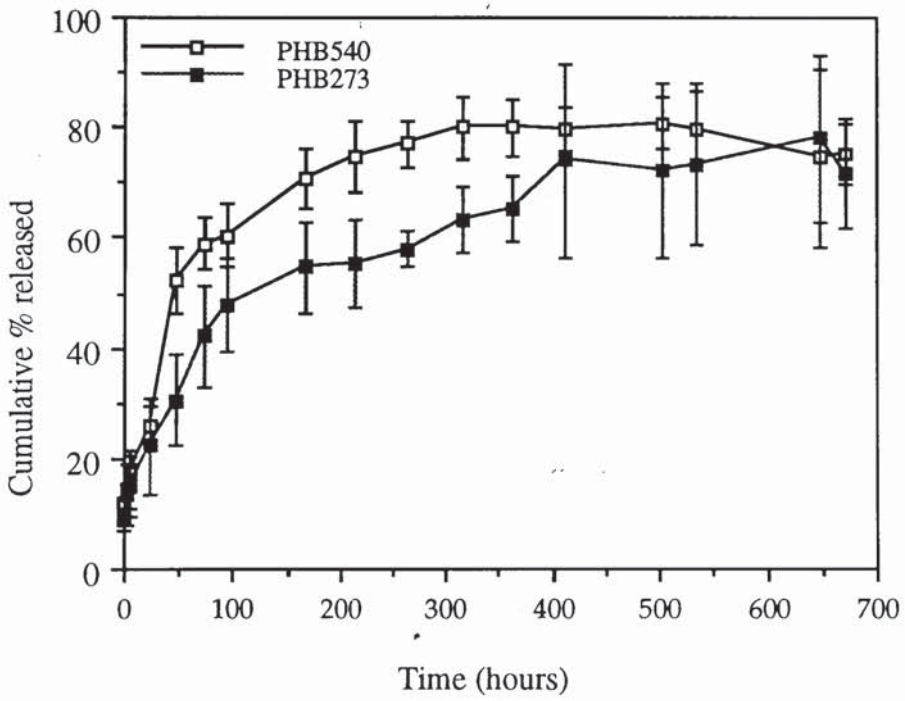


Figure 4.7. Release profiles for ~4% w/w BSA from PHB microspheres formed by a double emulsion technique (n=3; mean±s.d.)

Drug release from PHB microspheres can be considered to be due to a random chain scission of the polymer proceeding through the whole of the polymer matrix with diffusion of the drug through internal channels (Abe *et al.*, 1992). Release of low molecular weight molecules from PHB microspheres (formed using spray-drying methods) has been found to be controlled by the drug loading, occurring either by diffusion of the drug molecules through the surface matrix or through a network of water channels produced by solvent penetration. Decreased loading and increased polymer molecular weights, with corresponding increase in crystallinity, would reduce drug release for these drugs. The release of macromolecular BSA from PHB spheres formed using solvent evaporation processes does not appear to be as predictable.

The initial release is highest for the high molecular weight PHB540 polymer mainly due to a high rate of release up to 50 hours (with almost 50% of the BSA released) (figure 4.7). This may be caused by poor matrix formation by the crystalline polymer (Brophy & Deasy, 1986) leading to deposition of the protein at or near the surface domains of the microsphere. PHBV140 microspheres released less BSA (after 150 hours) than those formed using a homopolymer with a similar molecular weight (PHB159) (figure 4.6). This may be due to differences in the porosity of polymeric matrices formed as the solvent evaporates (Gangrade & Price, 1991). The HV plasticises the polymer, increasing the chain mobility and giving rise to a less porous microsphere matrix. A 9% level of valerate has an optimal plasticising effect for the system reported by Gangrade & Price (1991), with higher levels leading to excessive plasticisation of the polymer. Release rates of BSA from microspheres of PHB159 and PHB273 polymers show little difference with 80% of the drug released in 750 hours. Good correlation between *in vitro* and *in vivo* release behaviour was reported for PHB microspheres (Brophy & Deasy, 1986) and the *in vitro* profiles may provide guidelines for predicting the *in vivo* behaviour.

The release profiles for BSA from PHB microspheres was studied using kinetic models (see section 3.3.2.4). The best correlation coefficients for all systems were obtained using the square-root of time (Higuchi) model (table 4.4). These values were still low for some systems, i.e. <0.9 for microspheres made from PHBV140 and PHB540 polymers. The release rate is very slow for these systems beyond ~200 hours and the correlation coefficients for fit to the Higuchi model were higher at these earlier time periods (0.951 and 0.971 for PHB540 and PHBV140 respectively). The release of BSA from these systems is mainly diffusion controlled, especially at early time points (before 200 hours) with the BSA dissolving slowly in the permeating fluid and diffusing through pores formed in the system (Higuchi, 1963).

Table 4.4. Correlation coefficients for fit of dissolution results for PHB microsphere systems (described in table 4.3) loaded with BSA

Polymer	Zero order	First order	Cube root	Square root
	r^2	r^2	r^2	r^2
PHB159	0.754	0.863	0.834	0.922
PHB273	0.813	0.900	0.876	0.954
PHB540	0.592	0.654	0.637	0.810
PHBV140	0.661	0.674	0.704	0.855

4.3.3.2 Thermal behaviour and crystallinity of PHB microspheres

The thermal profiles for untreated PHB polymers and PHB microspheres are those characteristic of semi-crystalline solids with three separate and well-defined transitions as outlined in section 3.3.5 (figure 4.8). Reductions in the T_m of 1-2°C described by Gursel & Hasirci (1995) following conversion of PHBs into microspheres occurs in the lower molecular weight polymer and co-polymer (table 4.5). The melting point varies with polymer composition but also reflects polymer thermal history and typical of many co-polymer systems, upon addition of valerate residues into the polymer, T_m decreases as the crystallinity of the polymer is reduced with a substantial decrease in T_m of the co-polymer, PHBV140, of around 40°C. The glass transition temperatures for all homo- and co-polymers are in the range -5°C to 5°C and appear to be independent of the co-polymer composition. These results are consistent with the observations of thermal behaviour of PHB and its co-polymers reported by Engelberg & Kohn (1991). Encapsulation of BSA does not consistently reduce the T_g as for PLA polymers (section 3.3.5) or the T_m of the polymer as described by Gursel & Hasirci (1995) which was attributed to encapsulation of a water soluble drug. As the level of BSA incorporated in the PHB microspheres is relatively low (<5% w/w) any water associated with the protein would be minimal and no consistent reduction of T_m for loaded particles could be detected.

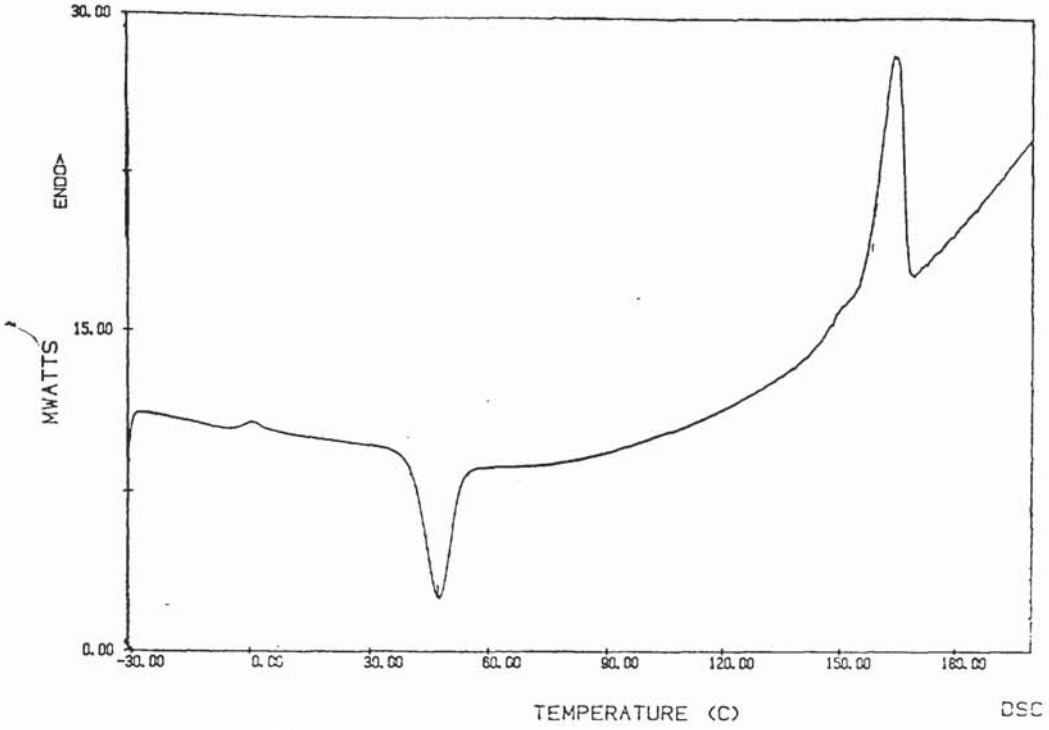


Figure 4.8. Thermal profile for PHB540 microspheres loaded with 4.4% w/w BSA

Table 4.5. DSC results (T_g and T_m) for various PHB preparations. Loaded spheres contain ~4% w/w BSA and were made by a double emulsion method

Sample	T_g (°C)	T_m (°C)
PHBV140 polymer	-4.6	129.6
PHBV140 empty spheres	-4.4	127.3
PHBV140 loaded spheres	-2.2	133.8
PHB159 polymer	4.8	172.6
PHB159 empty spheres	4.4	171.8
PHB159 loaded spheres	3.1	171.6
PHB273 polymer	2.6	162.8
PHB273 empty spheres	5.1	173.8
PHB273 loaded spheres	5.1	173.7
PHB540 polymer	-2.4	157.4
PHB540 empty spheres	1.0	172.6
PHB540 loaded spheres	0.7	164.7

4.3.3.3 Surface characteristics of PHB microspheres

The surface hydrophobicity of blank PHB microspheres was analysed using hydrophobic interaction chromatography as described in section 2.11. Due to the hydrophobic nature of the PHB particles, formation of a fine suspension by vortexing was difficult for the microspheres loaded with BSA. Therefore, the study was carried out using blank microspheres only. This limitation still allows comparison of the surface of microspheres formed from the PHB polymers.

Table 4.6. Final cumulative elution values of 1-2μm PHB particles from a series of HIC columns (n=3; mean±s.d.)

Formulation	Stationary Phase			
	Agarose	Propyl-	Pentyl-	Hexyl-
Latex 1.0μm	82.1±3.0	40.6±1.4	9.6±0.2	4.1±0.2
PHBV140	42.4±9.7	13.1±2.5	12.2±4.8	21.5±1.0
PHB159	28.0±5.8	12.9±1.6	13.0±0.7	-
PHB273	25.6±10.3	8.1±0.2	10.6±3.1	-
PHB540	52.3±5.8	16.2±4.8	24.1±7.7	34.8±0.9

Over the range of agarose to pentyl-agarose, the PHB particles are retained on the columns at similar or higher percentages than the surfactant-free latex control (table 4.6). This illustrates the hydrophobic nature of the surface of particles formed from PHB polymers. It is anticipated that these more hydrophobic particles will be strong adjuvants, increasing phagocytosis by antigen-presenting cells (Van Oss, 1978). The immune response generated following i.m. injection (i.e. the depot effect) of PHB microspheres compared with PLA (and PDTHC) has been investigated (section 4.3.3).

The surfaces of PHB microspheres, either empty or loaded with ~4% w/w BSA are not highly charged when compared to a surfactant-free latex standard (table 4.7). Like PLA spheres (section 3.3.4), there is no consistent trend for zeta potentials relative to polymer molecular weight, composition or microsphere loading.

Table 4.7. Zeta potentials of a number of formulations in two buffer systems (10mM phosphate, pH 7.0 and 10mM phosphate-citrate buffer, pH 7.0) (n=5; mean±s.d.)

Formulation	Zeta Potential (mV)	
	10mM phos. buffer	10mM phos/cit. buffer
latex spheres 1.0μm	-81.9±2.3	-97.7±2.2
PHBV140 empty spheres	-0.4±0.4	-1.3±0.9
PHBV140 loaded spheres	-3.3±1.1	-3.9±1.0
PHB159 empty spheres	-1.0±0.6	-0.3±0.3
PHB159 loaded spheres	-0.2±0.2	-2.9±0.7
PHB273 empty spheres	-1.0±0.5	-0.3±0.3
PHB273 loaded spheres	-0.2±0.5	-2.2±0.4
PHB540 empty spheres	-0.9±0.5	-1.9±0.4
PHB540 loaded spheres	-0.2±0.5	-1.0±0.1

4.3.2 Characterisation of microspheres prepared from PDTHC polymer

Due to limited availability if the PDTHC polymer, microsphere batches were generally produced on a smaller scale than usual, employing 50mg of polymer *per* microsphere batch. Scaling down the manufacturing process had no visible effect on yield (>80% for all batches) or loading of BSA into the particles which is very efficient (>90% at 10% w/w theoretical load). The PDTHC polymer formed even, spherical particles with little or no aggregation visible (figure 4.9).

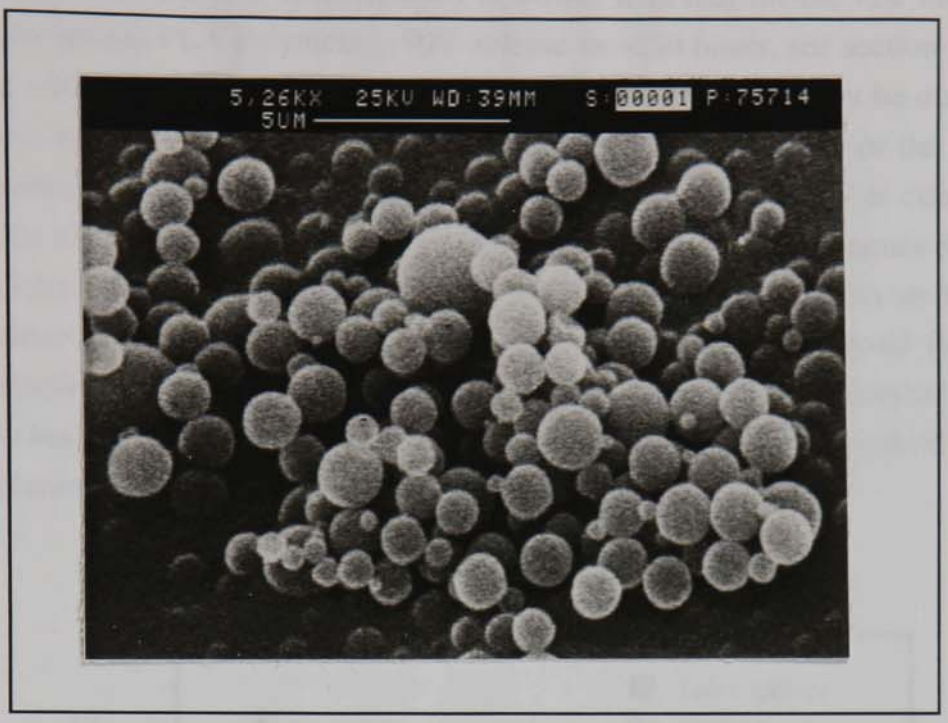


Figure 4.9. Scanning electron micrograph of PDTHC microspheres loaded with 10.2% w/w BSA

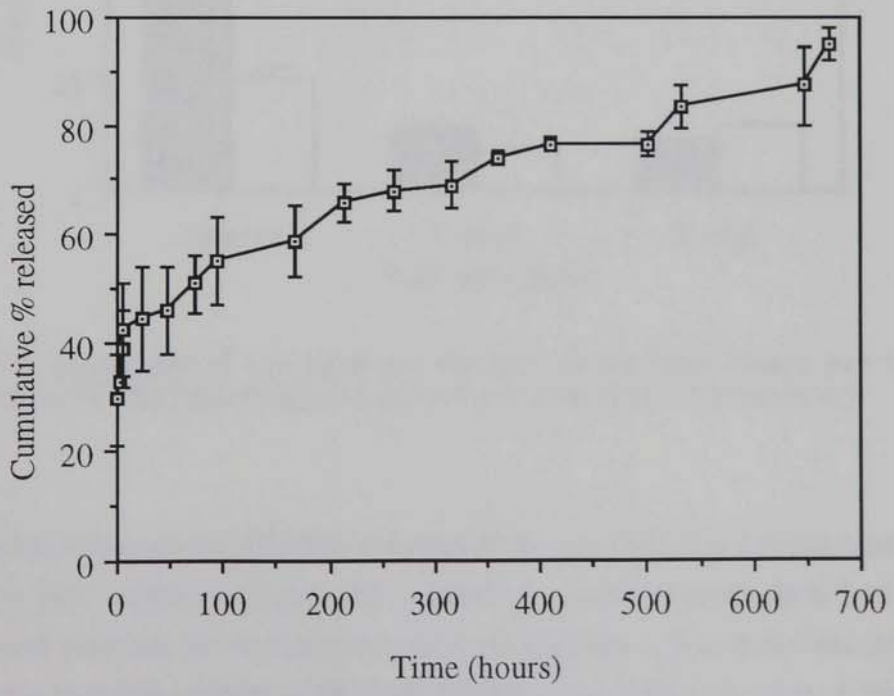


Figure 4.10. Release profile for BSA from PDTHC microspheres (size 0.70±0.56μm; load 10.2% w/w) (n=3; mean±s.d.)

BSA release from PDTHC microspheres is slower than that for the low molecular weight amorphous PLA polymers (~90% release in ~250 hours, see section 3.3.3.3) releasing ~90% of the protein after 700 hours (figure 4.10). This may be due to the amorphous nature of the polymer leading to better matrix formation or the relative hydrophobicity of the polymer, retarding water penetration. PDTHC is completely amorphous exhibiting only a Tg at 65.9°C (62°C, literature value; Pulapura & Kohn, 1992b). Microsphere formation and encapsulation of BSA had little effect on the Tg of the polymer (64.5°C). Zeta potentials were similar to those obtained for other biodegradable microsphere systems studied (-0.2±0.3mV in 10mM phosphate buffer pH 7.08) but examination of the relative hydrophobicity of the microsphere surface produced some interesting results (figure 4.11).

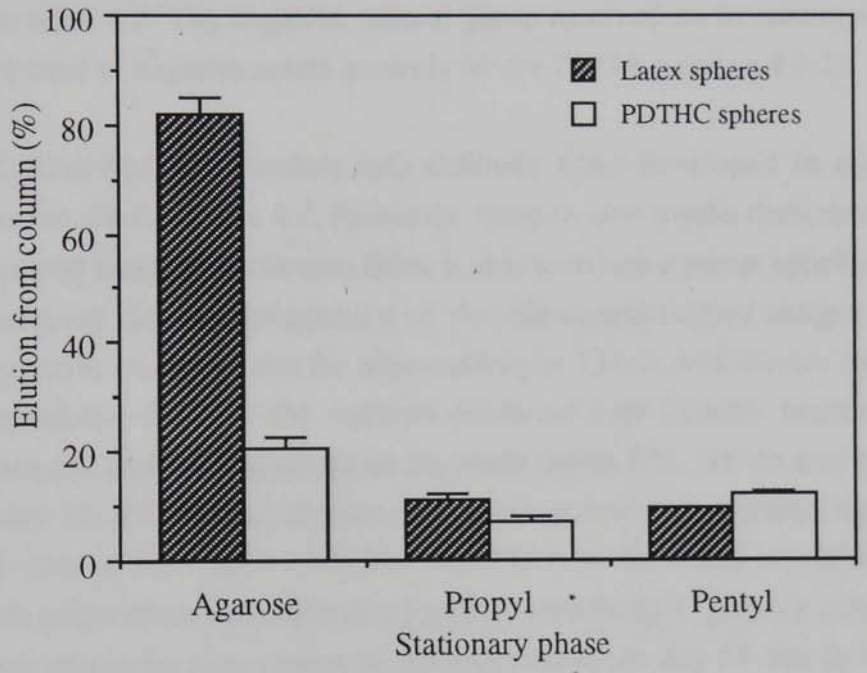


Figure 4.11. Comparison of the final percentages of particles eluted *per* type of agarose for empty PDTHC microspheres and a latex control (n=3; mean±s.d.)

The hydrophobic nature of the PDTHC polymer (Pulapura & Kohn, 1992a) causes it to be retained on HIC columns (figure 4.11) after microsphere production and it is a promising novel polymer for vaccine delivery. In addition to the improved adjuvant effect due to the tyrosine content of PDTHC (Kohn *et al.*, 1986), the slow release and higher surface hydrophobicity associated with this formulation may aid the development of single-step immunisation formulations.

4.3.3 Immune response to microencapsulated intramuscularly delivered BSA

Microspheres have been reported to possess adjuvant properties when delivered by i.m. and s.c. routes (Staas *et al.*, 1993; O'Hagan *et al.*, 1991b; Esparza & Kissel, 1992; Singh *et al.*, 1991). The sustained release from the microspheres provides a small amount of the antigen, in this case BSA, to be presented to the immune system for better stimulation. Consequently, proliferation of the immune cells results in prolonged raised antibody titres. Ten groups of mice (n=5) were immunised with 5μg of free or microencapsulated BSA, administered as a single dose on day 0, as detailed in section 4.2.1. The groups were selected to allow comparison of the ability of a number of microsphere formulations to induce and maintain an immune response to BSA and are as detailed in table 4.2. The negative control group received no treatment and serum samples were used as negative serum controls for the ELISA (section 4.2.2).

Figure 4.12 illustrates the systemic IgG antibody titres developed in mice to the formulations described in table 4.2. Primarily, these *in vivo* results demonstrate that a microencapsulated antigen, in this case BSA, is able to induce a potent specific antibody response, verifying the immunogenicity of the microencapsulated antigen. Group J served as a positive control, since the adjuvanticity of FIA is well known (see section 1.4). This emulsion form of the vaccine produced significantly higher immune responses than the free form throughout the study (table 4.6). At the earliest sample time point, day 21, the only microspheres to elicit an immune response significantly greater than that of free BSA ($p<0.005$) are those of the semi-crystalline PLLA polymer. This preparation also maintains a greater specific IgG antibody response then free BSA throughout the time course of the experiment. At day 58, the IgG antibody response peaks and is not statistically significantly different to that elicited by the FIA vaccine ($p>0.1$) suggesting that, at this time point, the formulation has an adjuvant activity at least equivalent. Interestingly, the IgG antibody titre following immunisation with PDLA particles is slower to develop than that of PLLA, but peaks at day 58, with a titre not statistically different to that of the FIA preparation. It was expected that the PDLA, though of the same Mw as PLLA, would generate a higher immune response at early time points, due to its faster release. Small differences in the loading and particle size of the microspheres used may affect the results. The PDLA microspheres were slightly larger and contained lower BSA levels than the PLLA particles used, both of which would contribute to a slower overall rate of release.

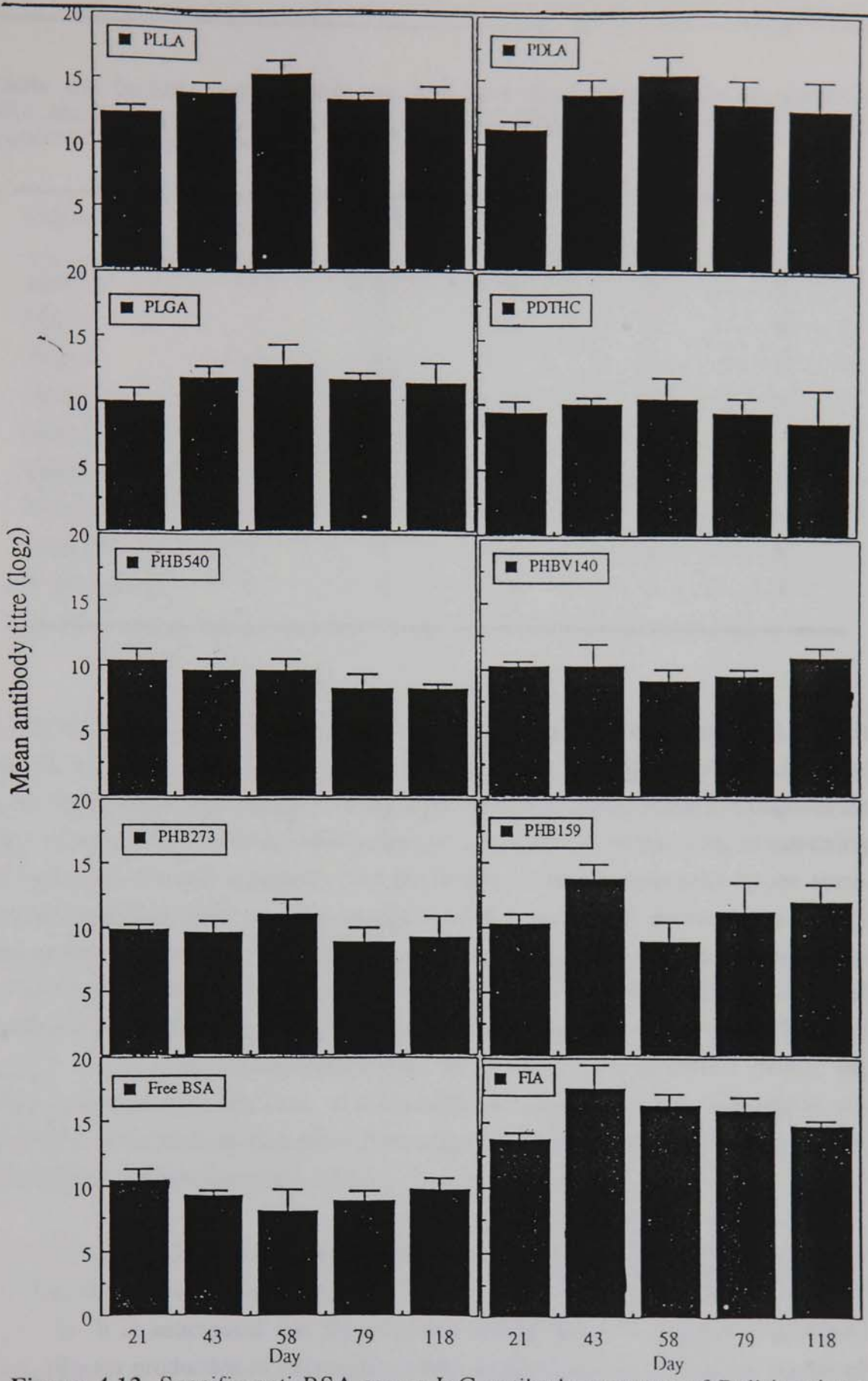


Figure 4.12. Specific anti-BSA serum IgG antibody response of Balb/c mice after immunisation by the i.m. route with 5µg of microencapsulated or free BSA (n=5; mean±s.d.)

Table 4.8. Immune response (specific IgG antibodies) produced by encapsulated BSA showing significant differences from free BSA determined using Student's unpaired t-test, significance level $p<0.05$ (n=5)

Polymer	Day 21	Day 43	Day 58	Day 79	Day 118
PLLA	√	√	√	√	√
PDLA	×	√	√	√	√
PLGA	×	√	√	√	√
PDTHC	×	×	√	×	×
PHB540	×	×	×	×	×
PHBV140	×	×	×	×	√
PHB273	×	×	√	×	×
PHB159	×	√	×	√	√
FIA (control)	√	√	√	√	√

All PLA formulations, including the co-polymer, produced antibody titres, maximal at day 58, which are maintained at a steady level up to day 118. The levels do not appear to be falling off, remaining significantly higher than that of free antigen throughout the time course of the experiment, indicating the success of the preparations in sustaining an improved systemic response. The production of higher responses by the semi-crystalline PLLA rather than the amorphous PDLA and PLGA microspheres at early time points is surprising. Enhanced potentiation of the immune response is reported to be due to the phagocytosis of particles <10μm in diameter (PLGA in this case), and transportation of these particles to the lymph nodes draining the injection site (Eldridge *et al.*, 1991a). An accelerated degree of hydrolysis may occur within the phagolysosomes (Eldridge *et al.*, 1991b) which may allow more rapid degradation of a semi-crystalline than an amorphous formulation due to better matrix formation of the amorphous particles (section 4.3.3.1).

The systemic IgG responses developed to the PDTHC polymer formulation were disappointing, being only significantly higher than those of free BSA at day 58 ($p<0.05$). It is anticipated that the relative hydrophobicity of this polymer and its capability for production of microspheres with small diameters, will lead to improved oral and nasal absorption and an enhanced mucosal response. It may be possible that the administration of slightly larger particles (4.0μm) may improve the immune response generated (Uchida & Goto, 1994).

The lowest molecular weight PHB polymer (PHB159) demonstrated the highest anti-BSA serum IgG titres over the time course studied, significantly higher than free BSA at day 43 and again at day 79. The levels were than maintained until the end of the study. The initial peak of immune response suggests that the smaller PHB microspheres are probably taken up by the antigen presenting cells and rapidly degraded producing antibody. Larger particles may remain at the site of injection, providing a depot release of free antigen (Eldridge *et al.*, 199b). The hydrophobicity of the particles makes generation of a fine suspension of the microspheres difficult and aggregation of the particles may occur. Antigen that is phagocytosed with microsphere fragments formed as the polymer slowly degrades may be responsible for the second peak in antibody titre. This pattern is similar to that produced following i.m. immunisation with the PHB273 microspheres but onset of the initial peak is delayed and detected at day 58. This could be due to the slower degradation rate of the slightly higher molecular weight polymer and it is anticipated that a second increase in antibody response may occur after day 118, the termination of this study.

The mean antibody titres following immunisation with the highest molecular weight, slowest degrading PHB polymer, PHB540, are similar to those of free BSA on day 21 and then continue to fall slowly throughout the study and do not produce titres statistically different from those of free antigen for the duration of the experiment. *In vitro*, PHB540 particles release almost 60% of the BSA within 70 hours (figure 4.7) which may contribute to the initial early immune response. Little more BSA was release from the particles over the time course involved and a study to examine immune responses over a longer time period is necessary to fully explore the adjuvanticity of this preparation. Microspheres formed using the PHBV140 polymer only start to demonstrate antibody titres higher than those of free antigen at the latest time point of the experiment (day 118) indicating the initiation of a slow IgG antibody response suggesting the possibility of a time range beyond the duration of this study. Microspheres of PHBV140 polymer were the slowest releasing of the PHB polymers studied (figure 4.6) which may lead to the stimulation of an immune response at later time periods. The possibility of using a combination of microspheres made from different polymers to achieve pulsed delivery is a distinct possibility.

4.4 SUMMARY

PHB and PDTHC polymers can be successfully used to produce microspheres encapsulating antigens by solvent evaporation methods. The particles formed demonstrate more hydrophobic surfaces than PLA polymers and protein release appears

to be dependant on similar mechanisms. The hydrophobic nature of the particle surface may lead to enhanced uptake *via* mucosal routes. Investigation of the extended time range of the immune response to i.m. delivered PHB microspheres would also be a worthwhile study as is the injection of a second boost dose to examine the extent and duration of the secondary response. The scope of the pulsed delivery for vaccine systems proposed by Eldridge *et al.* (1991b) may be extended by not only using different sized PLA microsphere but also microspheres of the slower degrading PHB polymer may be administered and the combination of two types of polymer microspheres for the generation of a better immune profile permitting the development of a single-dose vaccine delivery system.

5.0 ENCAPSULATION OF TETANUS TOXOID INTO MICROSPHERES

5.1 INTRODUCTION

According to the World Health Organisation, prevention of neo-natal tetanus by maternal immunisation requires at least two doses of tetanus toxoid in previously non-immunised women. Compliance with this dosage regime during pregnancy is often low and as a consequence, tetanus is endemic in 90 countries. Almost 565 000 neonates died of tetanus in 1991 (Aguado, 1991). The design of a vaccine delivery system requiring only one immunisation is highly desirable in pregnant women or women of child-bearing age so that immunity can be passively transferred to the foetus. Tetanus toxoid (TT) is a formalin-inactivated tetanus toxin which induces the production of neutralising antibodies. It is very immunogenic and is commonly adsorbed to alum prior to immunisation. The commercially available vaccine contains from 5-20Lf of toxoid *per* dose adsorbed onto aluminium phosphate and does not fulfil requirements for ease of administration, requiring cold storage. As with other inactivated vaccines and toxoid, it requires more than one dose to confer protection and persistent immunity. For practical purposes, tetanus toxoid, due to its characteristics and availability, is a suitable antigen for the commencement of investigations into alternative approaches to existing vaccines.

As early as 1976, Birrenbach & Spieser demonstrated enhancement of immune response against tetanus toxoid following entrapment into polymerised micelles. Two alternative systems have been proposed; pulsed delivery with three to four doses released over a year, or a continuous delivery of antigen over a similar period of time (Gander *et al.*, 1993; Alonso *et al.*, 1993 and 1994). Pulsed delivery was achieved by preparation of fast releasing, small, relatively high loaded PLGA microspheres to provide the initial high dose. They were co-administered with a slower releasing preparation, prepared using higher molecular weight, higher lactide ratio co-polymer. Higher titres were produced with this combined preparation rather than as separate doses. A summary of data so far from the WHO states that microencapsulated TT (in

PLGA microspheres) can induce similar or better antibody titres than those obtained from a single dose of alum-adsorbed TT and the antibodies produced have been found to be neutralising. Microspheres <10μm are superior to larger particles for priming and that TT microspheres can also efficiently boost the primary immune response induced by the injection of the same particles (Aguado & Lambert, 1992).

Delivery to the MALT can provide effective and sustained local and systemic immunity (section 1.2.2) and microspheres can be used to successfully deliver antigen to nasal (reviewed by Almeida & Alpar, in press) and oral (reviewed by Gilligan & Li Wan Po, 1991) immunoactive tissues. Investigations involving the delivery of TT to mucosal sites are reviewed by Jackson *et al.* (1994). Oral and nasal delivery of TT following encapsulation into liposomes and microspheres (Alpar *et al.*, 1992; Alpar *et al.*, 1994) and association with PLA microspheres (Almeida *et al.*, 1993) have previously been carried out at Aston.

5.2 MATERIALS AND METHODS

The polymers used for encapsulation of tetanus toxoid were PLLA, detailed in section 3.2 and PDTHC, detailed in section 4.2. Free tetanus toxoid used in these studies was obtained by dialysis of the commercially available Tetanus Vaccine BP in Simple Solution (The Wellcome Foundation Ltd., Beckenham, U.K.), containing not less than 14Lf *per* 0.5ml, followed by freeze-drying of the solution. All other materials used in these experiments were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) or as detailed in section 3.2.

All the methods used for microsphere production, single and double emulsion, solvent evaporation techniques, and particle analysis including SEM, surface hydrophobicity, zeta potential determination and *in vitro* release profiles in this chapter are as detailed in chapter 2.

Due to the low loading levels of tetanus toxoid encapsulated into and subsequently released from spheres, it was not always possible, therefore, to detect the antigen in the supernatant of the release media. For stability studies using SDS-PAGE analysis (see section 2.8), the effects of different types of conditions similar to those used for encapsulation on the physical characteristics of TT were examined. TT, in aqueous solution to mimic double encapsulation, and as freeze-dried solid were homogenised or sonicated in DCM. In some samples, emulsifying agents were added to the protein solution. The organic solvent was evaporated from all the samples, some with heating.

and the preparations were dried under vacuum. The samples were analysed using SDS-PAGE as described in section 2.8.

5.2.1 Immunisation studies using microencapsulated tetanus toxoid

Female Balb/c mice (~25g) were used for immunisation studies and during the experiments, all animals were allowed water and food *ad libitum*. The mice assigned to an oral, nasal or i.m. immunisation route and received free or encapsulated antigen according to the schedule detailed in table 5.1.

Table 5.1. Immunisation schedule for the study described in section 5.2.1

Group	Delivery route (F/E)*	Dose (µg)	Day of prime (boost)
A	i.m. (F)	5	0
B	i.m. (E)	5	0
C	i.n. (F)	5	0, 2, (22)
D	i.n. (E)	5	0, 2, (22)
E	i.n. (F)	50	0, 2, (22)
F	i.n. (E)	50	0, 2, (22)
G	i.g. (F)	50	0, 2, (22)
H	i.g. (E)	50	0, 2, (22)
I	i.g. (F)	100	0
J	i.g. (E)	100	0
K	i.m. (F+FIA)	5	0
L	no treatment	-	-
M	i.n. (E)	5	0
N	i.g. (F)	75	0, 2, (22)
O	i.g. (E)	75	0, 2, (22)

*(F) free/(E) encapsulated .

PLLA microspheres, prepared using a single emulsion technique were used in this study. They contained 4.4% w/w TT and the particles had a diameter of 0.79±0.50µm. Groups A and B were immunised (i.m.) on day 0 with a single dose of free or microencapsulated TT (5µg in 100µl PBS). One group of five animals (K) was

immunised with a single dose *via* the i.m. route on day 0 with 5µg of TT incorporated into the aqueous phase of a 1:1 w/o with FIA (100µl). Priming of the other groups, C-H, N and O (n=10/group) was carried out by the nasal or oral route on days 0 and 2. Groups I and J received a single oral dose of 100µg of TT, either free or encapsulated, on day 0. Half the animals within each group were sacrificed on day 21 when serum samples and gut and lung secretions were collected. Boosting of the remaining animals within each group, where appropriate, was carried out on day 22. On day 45, the animals were sacrificed and samples taken as before. A single nasal vaccine dose of microencapsulated TT (5µg) was administered on day 0 to the mice in group M, half of which were sacrificed on day 21, and the remaining animals boosted as detailed for the other groups.

Nasal delivery was carried out with a dose equivalent to 5 or 50µg TT in 10µl (5µl into each nostril) of a well mixed suspension of microspheres containing encapsulated or free antigen in 1.5% w/v $NaHCO_3$ using a Gilson pipette (P-20). It was carried out under light sedation using halothane as an inhalation anaesthetic (RMB Animal Health Ltd, Dagenham). Oral dosing (with a dose equivalent to 50, 75 or 100µg of TT) was effected through a specially adapted ball-tipped gavage needle in a volume of 400µl 1.5% w/v $NaHCO_3$. Negative serum controls from mice receiving no treatment were also collected. In these studies, the levels of antigen-specific antibody isotype responses were determined in serum and in gut and lung washes as representative of the systemic and mucosal responses, respectively.

5.2.2 Collection of biological samples

Blood was sampled from individual animals by cardiac puncture under terminal anaesthesia, allowed to clot overnight and the serum was collected using centrifugation and stored at -70°C until required for analysis. To determine the immune response in the intestinal and respiratory tracts, gut and lung wash secretions were collected using an adaptation of the procedure described by Eldridge *et al.* (1991b).

The gut and lung wash media are prepared according to table 5.2. For lung washes, 1.5ml of the wash fluid was injected slowly into the trachea using narrow plastic tubing. The solution was removed immediately, centrifuged and stored frozen at -70°C until ready for analysis.

The gastro-intestinal tract was then exposed and 2ml of fluid (table 5.2) was used to thoroughly wash the length of the tract. The liquid was centrifuged and stored frozen at -70°C until ready for analysis.

Table 5.2. Composition of fluids for gut and lung washes

Composition	Lung	Gut
phenylmethylsulfonylfluoride (PMSF)	1mM	1mM
NaCl	0.9% w/v	-
tween 20	0.5% v/v	-
sodium azide	0.1% w/v	-
iodoacetic acid	-	1mM
soybean trypsin inhibitor (1-S)*	-	0.1% w/v
ethylenediaminetetraacetic acid (EDTA)	-	10mM

*added immediately prior to use

5.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

Analysis of antibody response to TT in immunised animals was carried out using a microplate ELISA method, described by Farzad *et al.* (1986), by Dr E.D. Williamson at the Chemical and Biological Defence Establishment, Porton Down, Salisbury, U.K. The 96-well ELISA microtitre plates (Immulon 2, flat bottom plates, Dynatech, Sussex, U.K.) were coated with 50µl *per* well of Tetanus Vaccine BP in Simple Solution (The Wellcome Foundation Ltd.) diluted 1 in 10 PBS and incubated overnight at 4°C. The plates were blocked with 200µl *per* well of 0.1% w/v BSA in PBS for one hour at 37°C. The plates were thoroughly washed three times using PBST (see section 4.2.2) and allowed to dry. The serum, gut and lung wash samples were serially diluted in PBS and 100µl of each sample was added to each well of the coated ELISA plates. These were covered and incubated for two hours at 37°C. The plates were washed three times with the PBST solution and 100µl of goat anti-mouse IgG peroxidase conjugate and polyvalent Ig peroxidase conjugate, diluted 1 in 1000 using PBS, was added to each well and the plates covered and incubated for one hour at 37°C. The plates were washed

five times in PBST solution. 100µl of ABTS, at a concentration of 1 tablet *per* 50ml of a citrate buffer plus 5µl hydrogen peroxide, was added to each well and incubated at 37°C for 1 hour for colour development. The citrate buffer was prepared in advance using 56% 0.1M citric acid and 44% 0.2M disodium hydrogen orthophosphate. The plates were read at 405nm (Titertek Multiskan plate reader).

Antibody titres were expressed as the maximum dilution of serum giving an absorbance reading 0.1 units above the OD_{405} of negative controls after the one hour incubation. Samples from individual animals were studied to determine the titre range and the mean immune responses of the different groups of animals at each time point were compared and statistical significance was assessed using Student's unpaired t-test. Values with $p<0.05$ were considered significant.

The biological wash samples were freeze-dried and reconstituted in 200µl of PBS and diluted 1 in 5 in 5% w/v BSA in PBS. The samples were double diluted down the plate as before and the ELISA carried out as previously described with detection of polyvalent mouse Ig (IgA, IgM and IgG). Relative titres are expressed as (mean optical density minus background optical density) x the end-point dilution.

5.3 RESULTS AND DISCUSSION

5.3.1 Formulation of tetanus toxoid in PLA microspheres

PLLA microspheres encapsulating TT were prepared using single emulsion and double emulsion techniques. The microspheres formed were small and spherical with smooth surfaces (Figure 5.1). Typical loading amounts of TT into microspheres are in the range 0.1-10% w/w (Aguado & Lambert, 1992) depending on the method of preparation. The particles prepared using solvent evaporation techniques, as described in chapter 2, contain a maximum of ~5% w/w TT. Efforts to increase the loading over these levels resulted in decreased encapsulation efficiencies (<60%) which, due to the cost of the antigen, were not pursued.

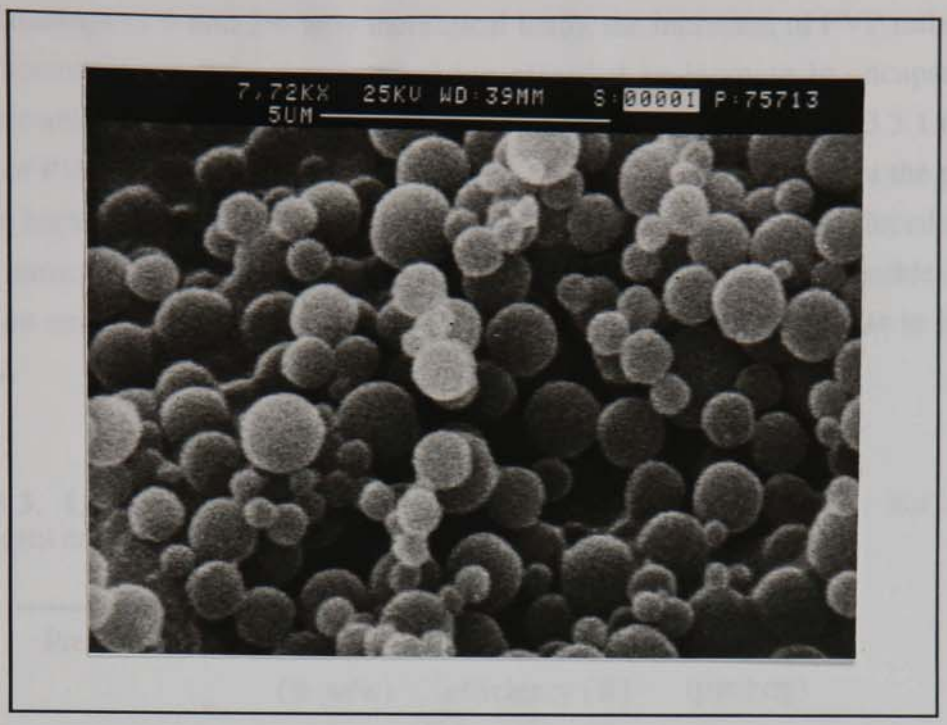


Figure 5.1. Scanning electron micrograph of PLLA microspheres loaded with 4.4% w/w TT formed using a single emulsion solvent evaporation method

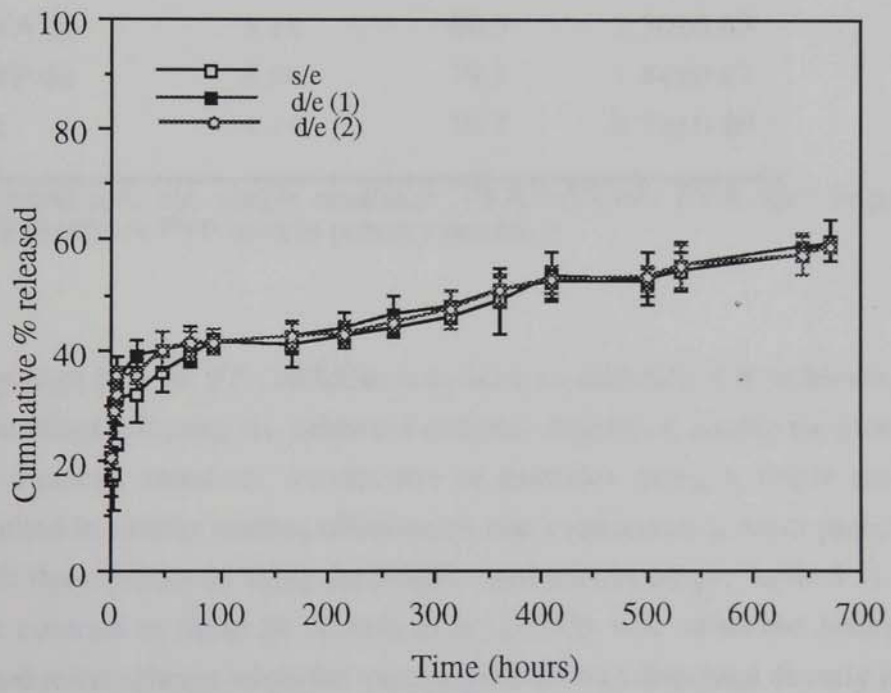


Figure 5.2. Release profiles for TT from PLLA microspheres prepared using solvent evaporation techniques (n=3; mean±s.d.). (1)-PVA in 1° emulsion; (2)-PVP in 1° emulsion

At lower loadings (1% and 2% w/w theoretical load), the inclusion of PVP rather than PVA for formation of the primary emulsion afforded an increase in encapsulation efficiency (table 5.3), similar to results obtained using BSA (see section 3.3.1.6). The inclusion of PVP had no noticeable effect on the encapsulation efficiency or the particle size at the higher loadings used (table 5.3) or on the release profiles produced (figure 5.2). The particles may already encapsulate the maximal amount of TT possible for this formulation and changes in surfactant may not permit any further increase in loading efficiency.

Table 5.3. Loading, encapsulation efficiencies and particle size for PLLA microspheres encapsulating TT

Preparation*	Actual load (% w/w)	Encapsulation efficiency (%)	Particle size (μm±σg)
PVA d/e	1.09	109.0	1.37±0.83
PVP d/e	1.29	129.0	1.44±0.73
PVA d/e	1.37	68.5	1.37±0.83
PVP d/e	2.15	86.0	1.26±0.56
PVA d/e	5.14	68.5	1.30±0.67
PVP d/e	3.96	79.2	1.44±0.67
s/e	4.24	70.7	0.79±0.50

*d/e- double emulsion; s/e- single emulsion; PVA-0.5%w/v PVA used in primary emulsion; PVP-0.5%w/v PVP used in primary emulsion

The relatively high Mw of TT (160kDa) may lead to difficulties in achieving high microsphere loadings affecting the pattern of polymer deposition around the protein (as a powder or aqueous solution). Production of particles using a single emulsion technique resulted in similar loading efficiencies and a reduction in mean particle size compared with those produced using the double emulsion technique (table 5.3). These results are in contrast to those of Alonso *et al.* (1993) who observed broken and irregular shaped microspheres when the vaccine powder was dispersed directly into the organic solution.

TT was also encapsulated into PDTHC microspheres for the purpose of investigating the surface hydrophobicity, release and eventually, mucosal antibody stimulation of

such particles. Loading was efficient (>80%) at a 6% w/w theoretical load and small particles could be produced (0.90±0.44μm) (figure 5.3).

The influence of formulation factors on the *in vitro* release profiles obtained, as outlined in section 3.3.3, was investigated for TT microspheres in 20mM phosphate buffer. The microsphere batches used had loadings of ~4% w/w and particle diameters from 0.8-1.4μm. There is little difference between the profiles obtained for the formulation methods, single emulsion and double emulsion solvent evaporation with burst effects in the range of 20% and with approximately 60% of the TT released in 600 hours.

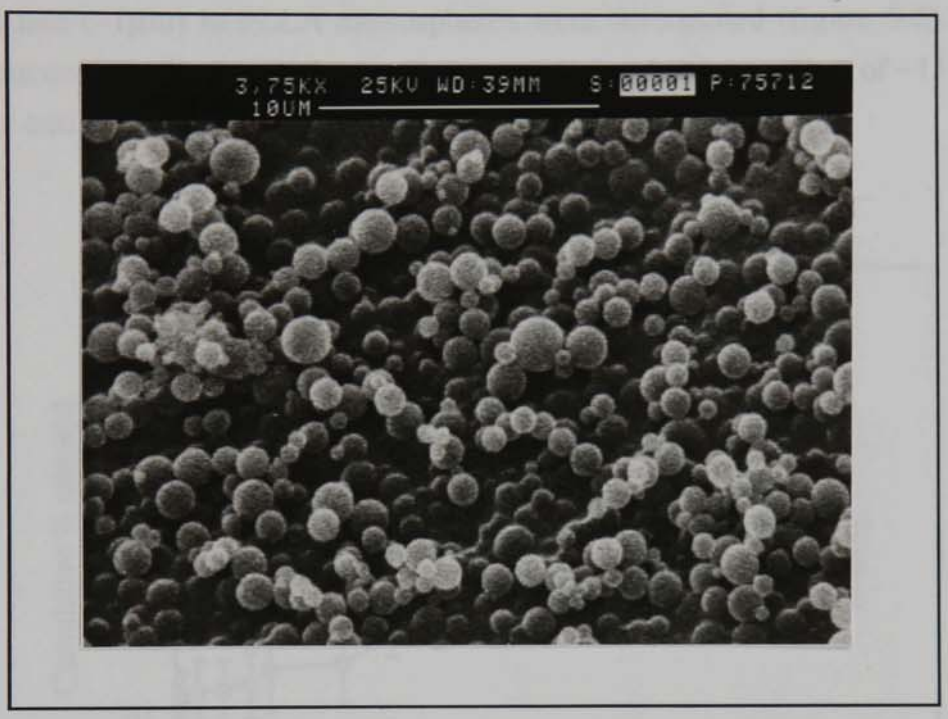


Figure 5.3. Scanning electron micrograph of PDTHC microspheres loaded with 5.1% w/w TT formed using a single emulsion solvent evaporation method

Generally, incorporation of a water-soluble drug into the internal aqueous phase of a w/o/w emulsion system gives a more controlled release than dispersion of the antigen into the organic solution and this has been reported for systems incorporating TT (Alonso *et al.*, 1993). This is reported to be due to the insolubility of proteins in the organic phase, which can give rise to a non-uniform distribution of protein islands in the polymer matrix. However, for the particles in this study, this was not the case. This disparity in results may be due to differences in the microsphere production method with Alonso *et al.* (1993) employing both high speed homogenisation and sonication to

prepare the primary emulsion. This method led to the generation of a parabolic release pattern for TT (5% and 2% w/w load) from low molecular weight (Mw 3000) PLLA microspheres, with a burst of 25%, attributed to their comparatively small size (9μm) and water uptake capacity (Alonso *et al.*, 1993). In contrast, in this study, a near constant release was obtained after the initial burst for the PLLA microspheres. Release rates are greatly affected by the purity of the batch of TT (Alonso *et al.*, 1993), emphasising the importance of extensive dialysis of the commercial form of TT used prior to encapsulation as the presence of salts was found to increase the rate of release.

The release profile for TT from PDTHC microspheres (formed by a single emulsion method) was also studied. The release profiles for microspheres of similar loading (4% w/w) and size (~1μm) to PLLA microspheres were determined (figure 5.2). Drug release from comparable PDTHC spheres was reduced with a burst effect of ~15% and 45% of the antigen release in 600 hours (figure 5.4).

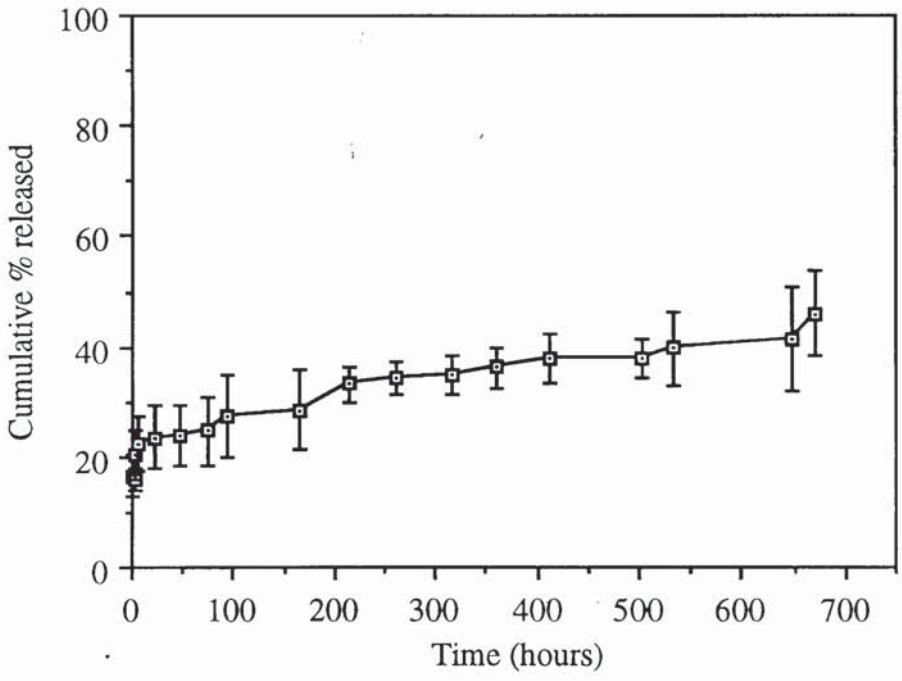


Figure 5.4. Release profile for 5% w/w loaded TT from ~1μm PDTHC microspheres (n=3; mean±s.d.)

5.3.2 Surface properties of microspheres encapsulating tetanus toxoid

The surface hydrophobicity of TT loaded PLLA and PDTHC microspheres was studied using hydrophobic interaction chromatography as described in section 2.11. The final elution percentages for control latex particles, PLLA and PDTHC spheres containing ~4% w/w TT are shown (figure 5.5). HIC results show that empty PDTHC spheres possessed markedly different surface characteristics to PLLA spheres, displaying similar hydrophobicity to the control hydrophobic latex particles (see section 4.3.2). Elution profiles from a series of gels for microsphere formulations loaded with tetanus toxoid and the latex control are illustrated in figures 5.6-5.8. These follow similar trends to the blank microspheres (see section 3.3.3) but display more hydrophobic surfaces due to the deposition of the protein within the microsphere matrix.

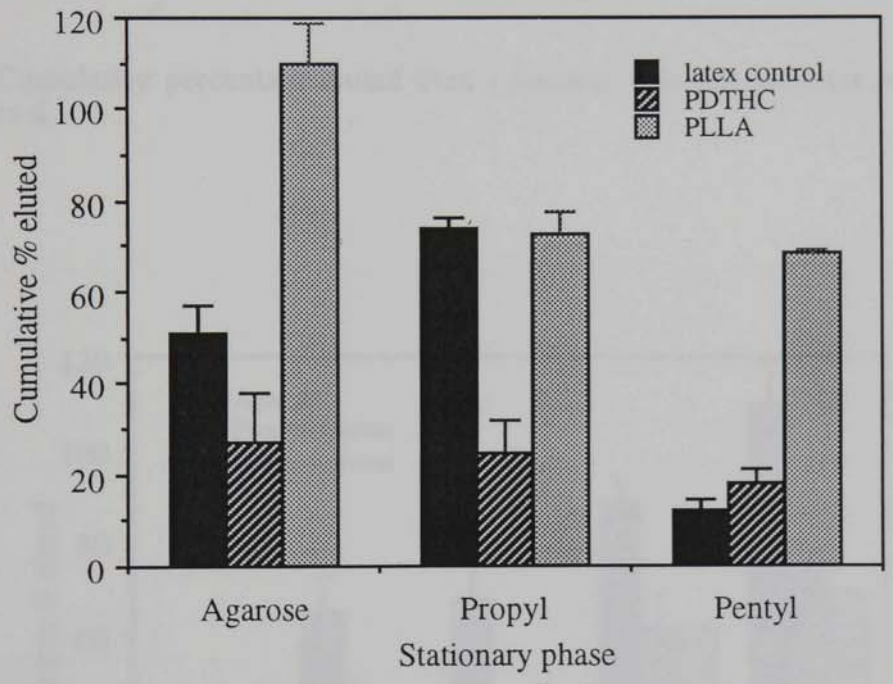


Figure 5.5. Final cumulative elution values for TT loaded microspheres formed from different polymers (n=3; mean±s.d.) from a series of stationary phases

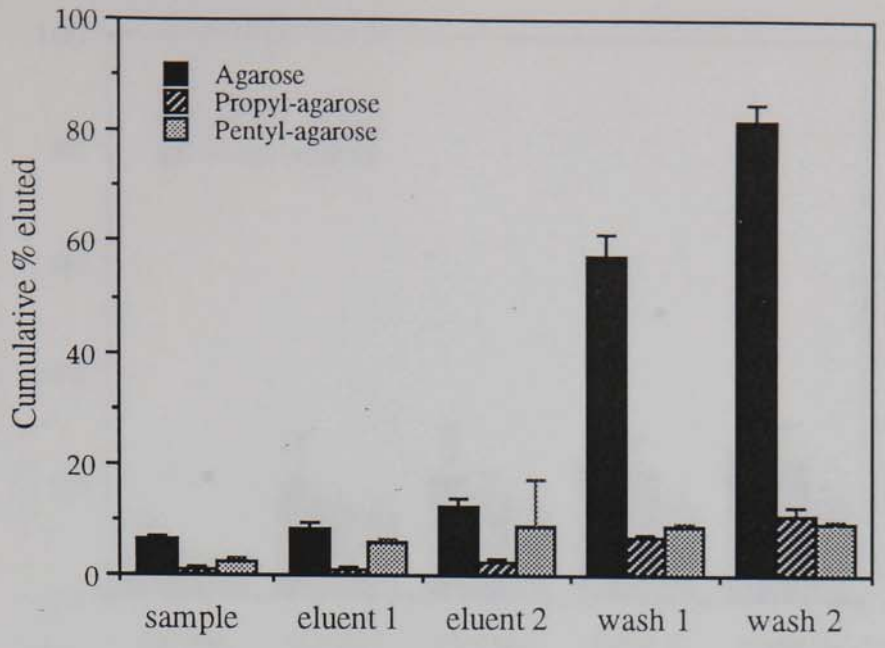


Figure 5.6. Cumulative percentage eluted from a series of columns for latex particles (n=3; mean±s.d.)

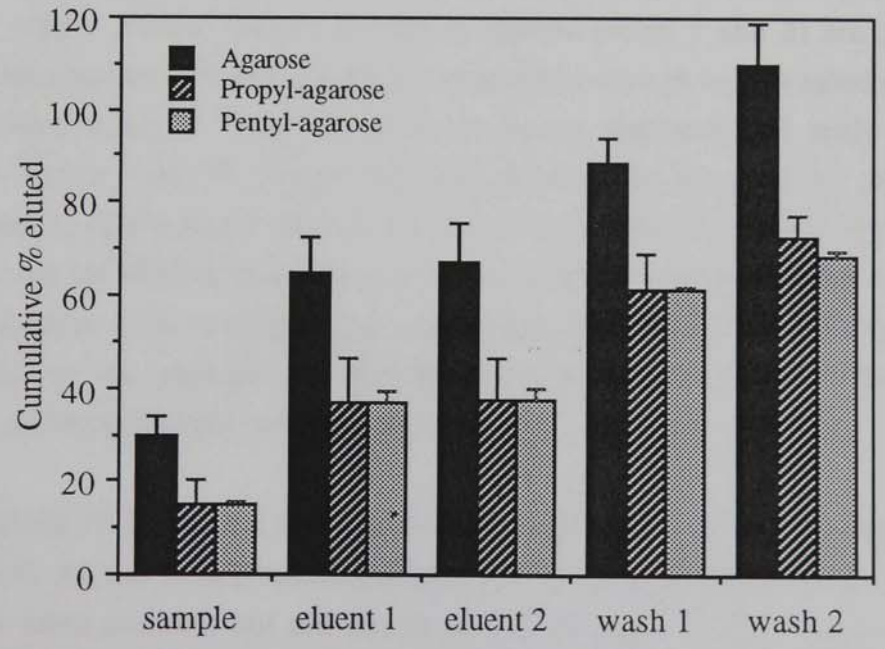


Figure 5.7. Cumulative percentage eluted from a series of columns for TT loaded PLLA particles (n=3; mean±s.d.)

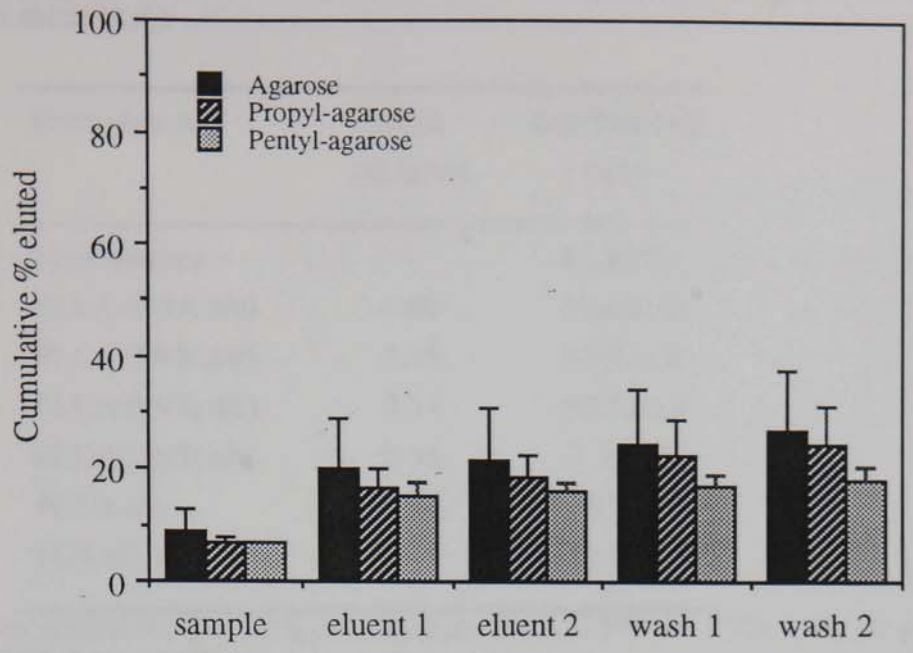


Figure 5.8. Cumulative percentage eluted from a series of columns for TT loaded PDTHC particles (n=3; mean±s.d.)

The elution profiles clearly demonstrate a heterogenicity in the systems regarding microparticle surface hydrophobicity. The more hydrophobic spheres which are washed off when the elution buffer (Triton X-100) is applied (wash 1 and 2) are a more hydrophobic sub-population than the particles hydrophilic enough to be washed of with the NaCl washes (elution 1 and 2). This is clearly demonstrated with PLLA microspheres loaded with TT (figure 5.7) and is generally the case for particles formulated from PLA polymers (section 3.3.3) and latex control particles. However, this is not the case for PDTHC microspheres which are not removed from the columns following application of the detergent. This may suggest that there is little variation in hydrophobicity of the surfaces of PDTHC particles within a single batch of microspheres, all being strongly hydrophobic.

The zeta potentials of TT loaded microspheres were determined in 10mM phosphate buffer (table 5.4). All the biodegradable particles were less negatively charged than the surfactant-free latex controls but did not show any consistent trend. The negative charges are, however, larger for TT loaded microspheres than for similar BSA loaded particles (see section 3.3.4). This may be due to chemical differences between the proteins and their distribution at the particle surface.

Table 5.4. Zeta potentials of various microsphere formulations encapsulating tetanus toxoid (n=5; mean±s.d.)

Formulation*	Load (% w/w)	Zeta Potential (mV)
latex spheres	-	-81.9±2.3
PLLA (PVA d/e)	1.09	-19.6±1.0
PLLA (PVP d/e)	2.15	-16.8±1.8
PLLA (PVA d/e)	5.14	-20.5±0.5
PLLA (PVP d/e)	3.96	-7.9±0.5
PLLA s/e	4.24	-14.3±0.4
PDTHC s/e	4.46	-28.8±0.9

*d/e- double emulsion; s/e- single emulsion; PVA-0.5%w/v PVA used in primary emulsion; PVP-0.5%w/v PVP used in primary emulsion

5.3.3 Stability of tetanus toxoid in microsphere formulations

Formulation of TT into microsphere preparations has been associated with some problems. TT loses most of its activity below pH 7 and the reduction in pH due to the formation of free acids with faster degrading preparations may affect the stability. Also, the is a possibility of interactions between relatively hydrophobic polymers with hydrophilic proteins. Aggregation of tetanus toxoid following release from microspheres could include exposure to moisture, adsorption to hydrophobic polymer surfaces, interaction with oligomeric and polymeric products of erosion and exposure to acidic pH (Manning *et al.*, 1989).

TT stability was assessed using SDS-PAGE techniques and protein samples were studied before encapsulation and after release using a 7.5% gel. Also, due to the low levels of protein released, the effects of different conditions, inherent to the microencapsulation process, on the structural integrity of TT were investigated (section 5.2). No differences were observed between the bands on SDS-PAGE gels produced by TT prior to encapsulation and TT after release from the particles (figure 5.9) indicating that the structure of the molecule is retained throughout the encapsulation process and during release from the particles. This in agreement with the observations of Gander *et al.* (1993) who found no changes in TT antigenicity after 6 weeks incubation at 37°C in aqueous solutions of neutral pH. Aggregation of TT occurring

during the freeze drying process reported by Alonso *et al.* (1994), was not observed, using SDS-PAGE techniques. The incorporation of hydrophilic stabilisers, such as Pluronic F68 and sodium glutamate, during the microsphere production process may help to stabilise the TT should any problems occur (Alonso *et al.*, 1994).

The stability of TT seems unaffected by entrapment into PLLA microspheres by single or double emulsion techniques and no breakdown or aggregation is detected following exposure to DCM (figure 5.9). The antigen does not under go any chemical interaction with the polymer, under the conditions studied, which leads to modification of the SDS-PAGE migration pattern, indicating that it is retained within the microsphere matrix by physical means.

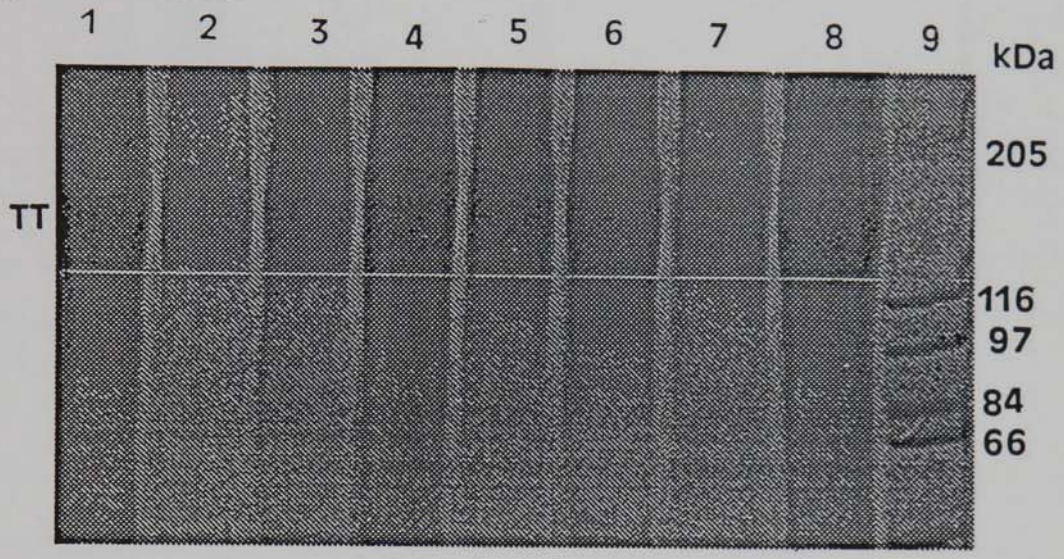


Figure 5.9. SDS-PAGE (7.5%) pattern of TT microencapsulated by solvent evaporation techniques and following exposure to DCM. Lanes (1) TT prior to encapsulation (2) PLLA d/e (3) PLLA s/e (4)-(6) TT following incubation in DCM (4) 3 minutes (5) 1 hour (6) 24 hours (7) 1 hour + PVA (8) TT prior to encapsulation (9) molecular weight standards

5.3.4 *In vivo* results

It has been shown that microencapsulated TT is able to induce a potent specific antibody response *in vivo* following parenteral (Alonso *et al.*, 1994; Esparza & Kissel, 1992) and mucosal delivery (Alpar *et al.*, 1994). An evaluation in mice of tetanus toxoid encapsulated in microspheres which were sterilised with gamma-radiation showed that a good primary immune response was produced, that gamma-irradiation modified some of the epitopes and that encapsulation of alum-absorbed toxoid did not enhance the primary response (Esparza & Kissel, 1992).

Serum IgG responses for some nasally and most orally delivered free and encapsulated preparations were low (<1000), except groups E and F for i.n. delivery and group J, which received a single i.g. dose of 100µg of microencapsulated TT, producing a mean titre of >16000. No responses were detected for free tetanus under the same conditions. The serum IgG antibody responses on day 45 of the schedule are illustrated in figure 5.10.

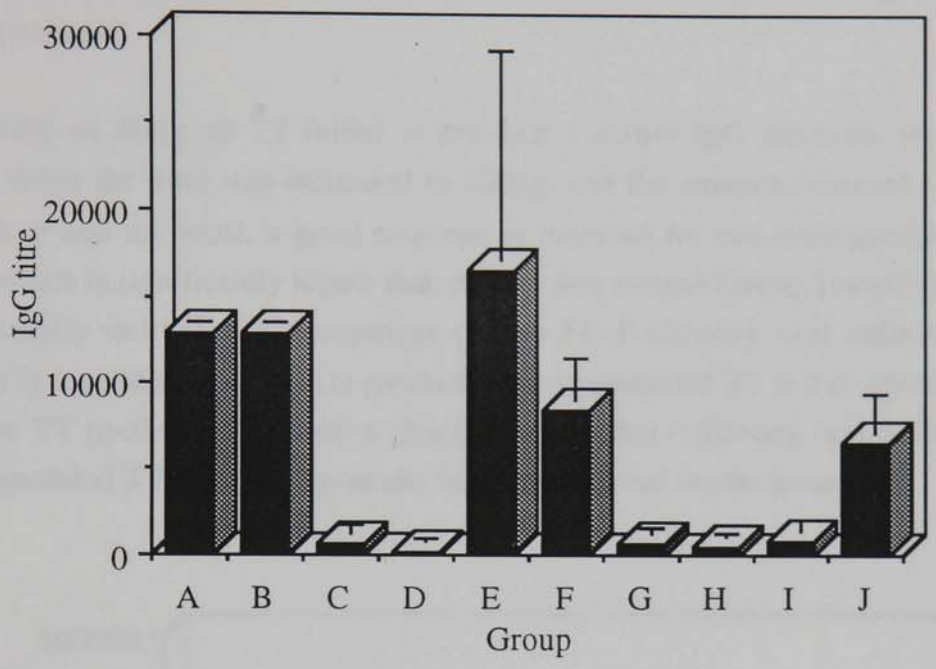


Figure 5.10. Serum IgG antibody titres induced on day 45 (following booster) (n=5; mean±s.e.)

Similar titres are produced following i.m. immunisation of free or microencapsulated TT. This may be due to the relatively slow release of the antigenically active protein from the preparation, the PLLA preparation only releasing 60% of the encapsulated drug within thirty days. Similar observations have been made by Alonso *et al.* (1994) when, despite a near constant release rate of tetanus toxoid from low molecular weight PLLA (3kDa) microspheres, the formulation in their study did not produce a higher immune response than aluminium phosphate-adsorbed control.

I.n. immunisation with 5µg of either free or microencapsulated TT failed to stimulate IgG serum immune responses. However, when the dose was increased to 50µg of TT, significant IgG responses were detected in the serum. The results with free antigen were highly variable (figure 5.10), demonstrating the need for improved reproducibility

of dosage administration of free antigen while delivery of microencapsulated antigen resulted in less interanimal variation. It must also be noted that the delivery of large doses of microspheres in the small volumes required for nasal delivery is more problematic than administration of free antigen. This may account for the higher response generated following delivery of the free form. Results from other studies using larger animals demonstrated increased IgG responses with microsphere-associated TT (Almeida *et al.*, 1993) and further studies are necessary examining the effects of animal species, immunisation protocol and formulation on the immune response generated.

Oral delivery of 50µg of TT failed to produce a serum IgG antibody response. However, when the dose was increased to 100µg, and the animals received a single priming dose and no boost, a good response is detected for microencapsulated TT (group J) which is significantly higher than that for free antigen (group I) and similar to that for nasally delivered microspheres (group F). Following oral delivery, the polyvalent Ig response (figure 5.11) generated by encapsulated TT is also greater than that of free TT ($p<0.05$). The results clearly indicate that following oral delivery of microencapsulated TT, a primary systemic immune response can be generated.

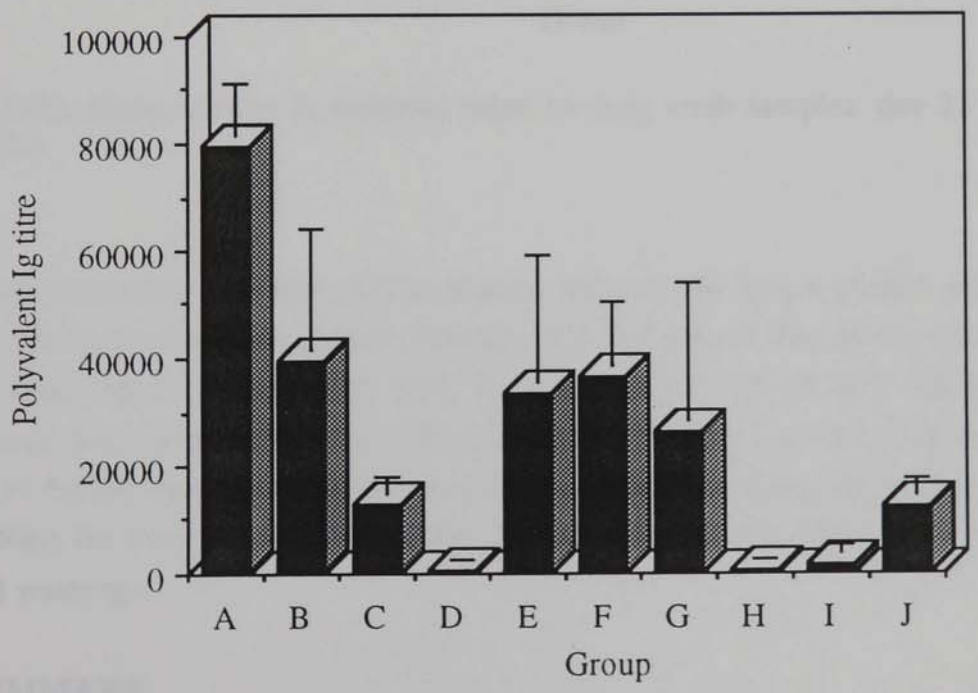


Figure 5.11. Serum polyvalent Ig titres induced on day 45 (following booster) (n=5; mean±s.e.)

The potential of single immunisation for tetanus toxoid containing microspheres has also been studied by (Alonso *et al.*, 1993) who detected an early and persistent neutralising antibody response. The higher dose used for nasal delivery (50μg) also stimulated a polyvalent Ig response for both free and encapsulated antigen and again, variations were smaller with TT delivered in microspheres.

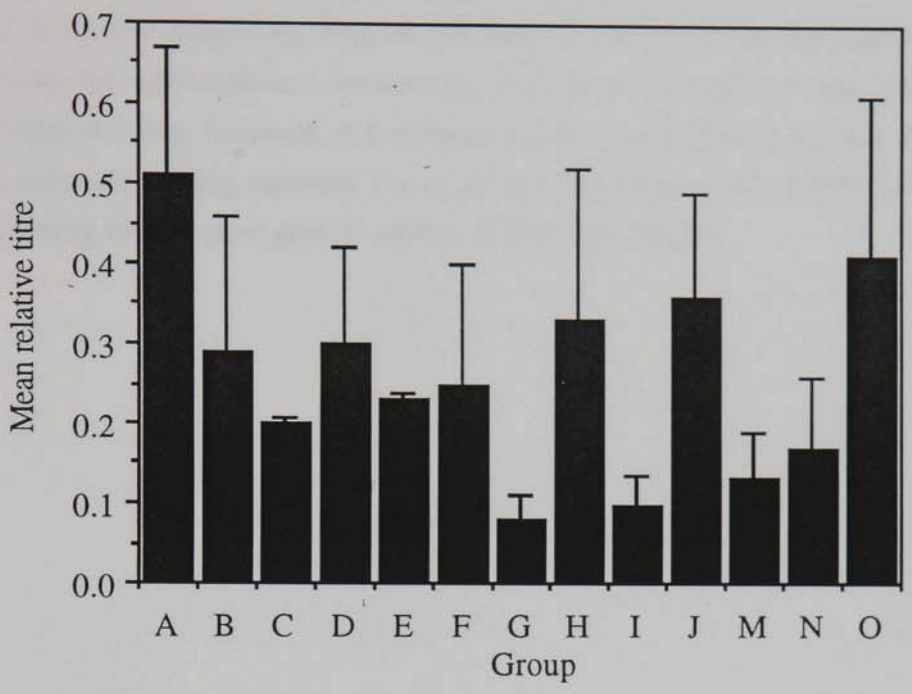


Figure 5.12. Mean relative Ig antibody titres for lung wash samples, day 21 (n=5; mean±s.e.)

In all cases, including low dose oral microsphere delivery, the lung wash IgA antibody titres for mucosally delivered, microencapsulated TT are greater than those induced by the free form (figure 5.12) after 21 days. The immune response induced following a single nasal dose (group M) is lower than that induced using a double priming dose (group D), though the difference is not statistically significant. Increasing the dose from 5μg to 50μg for nasal delivered TT did not affect the immune response generated at mucosal washings.

5.4 SUMMARY

Tetanus toxoid has been successfully encapsulated in PLA microspheres by solvent evaporation methods and the particles characterised. The production of micron sized

microspheres has enabled the investigation of these as a mucosal delivery system and further results remain to be determined in this study. Significantly higher immune responses were generated following oral delivery of a single dose of encapsulated TT compared with free antigen. IgA antibody titres were detected in the mucosal tissue in the lungs following oral and nasal delivery. The generation of much higher immune responses compared to free TT, following nasal delivery of microsphere-associated TT previously reported by our group (Almeida *et al.*, 1993; Alpar *et al.*, 1994), were not detected in this investigation. This may be due to variability in the administered dose, especially for microsphere formulations and the animal species and protocols employed. Nasal delivery, however, still remains a promising route for vaccine delivery with recent successes being reported for microspheres (Yan *et al.*, 1995) and other systems, including bioadhesive gels (Duchêne & Ponchel, 1993).

6.0 CO-ENCAPSULATION OF PROTEINS INTO PLA MICROSPHERES

6.1 INTRODUCTION

Using BSA as a model protein/antigen, the process and formulation variables affecting microsphere formation have been investigated by us (chapters 3 and 4) and others (Cohen *et al.*, 1991; Sah & Chien, 1993). Due to the tendency of BSA to form a semi-solid interfacial film with the organic polymer phase and due to differences between the physicochemical properties of proteins, results cannot be directly extrapolated to other proteins. The properties of different proteins will affect cross-interactions between the phases, thus affecting entrapment and release, leading to different optimum conditions for microsphere formation (figure 6.1). The combination of two (or more) proteins for encapsulation into microspheres will lead to different encapsulation efficiencies, surface and release properties of the microspheres formed.

The number of proteins undergoing evaluation for potential clinical is very large and many cytokines, such as gamma-interferon are being developed for treatment of chronic disorders (Talmadge, 1993). The full potential of these bioactive drugs has not yet been realised due to difficulties in selectivity or in achieving temporal control of their systemic or tissue levels in response to biological needs. However, the very high potency of cytokines renders them very suitable for incorporation into, and also release from, sustained or targeted dosage forms. The low loading levels required for such potent bioactive agents would result in a slow release profile, with a lag phase occurring after the initial burst effect. The inclusion of carrier proteins as excipients to mediate a continuous release is one approach for overcoming this problem.

The inclusion of human serum albumin (HSA) as an excipient to mediate the release of IL-2 from microspheres (Hora *et al.*, 1990b) and IFN-α from mini-pellets (Fujioka *et al.*, 1995) has been reported. In both cases, the diluent protein promoted release of the bioactive agent without a lag period. The addition of SDS into the buffer medium for *in vitro* release studies was not sufficient to overcome difficulties in resolubilisation of the encapsulated protein, IL-2 (Hora *et al.*, 1990b). HSA, a highly soluble molecule, was

co-encapsulated with the lower molecular weight protein in order to facilitate rapid resolubilisation allowing elution of HSA from the microsphere and creating a highly porous structure within the polymer matrix, permitting a more rapid diffusion of the IL-2. The same principle lies behind the inclusion of HSA into the IFN minipellet, and IFN release was reported to be sustained for extended periods (Fujioka *et al.*, 1995).

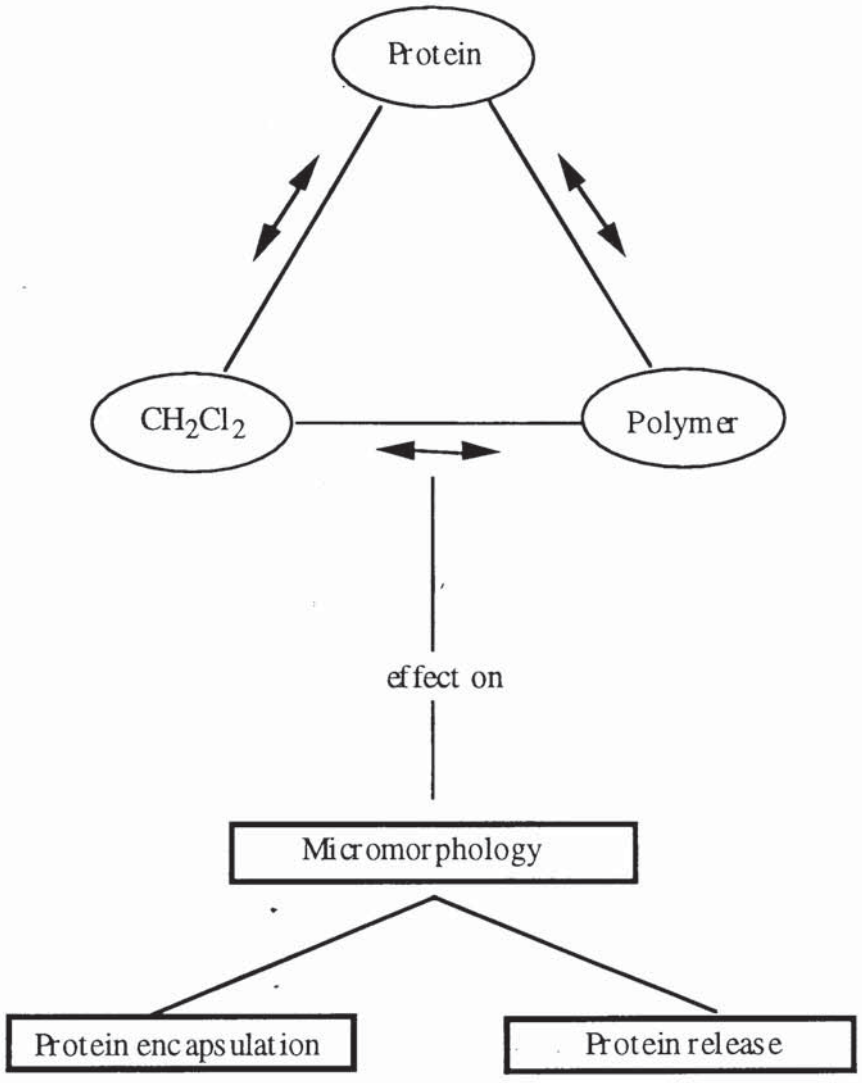


Figure 6.1. Scheme showing potential protein/polymer interactions (adapted from Reich, 1995)

The delivery of proteins is an important research area with considerable interest in the development of systems involving biodegradable polymers for both oral and parenteral administration. As proteins, they are subjected to digestive degradation and have a short half-life in the body proving a drawback to long term therapy. Although, encapsulation of bioactive polypeptides and proteins into microsphere formulations for sustained

release has been the subject of many investigations (reviewed by Hutchinson & Furr, 1990; Langer & Moses, 1991), only a few studies have been carried out on co-encapsulation of bioactive proteins. One report, using PLGA, involved the co-encapsulation of nerve growth factor (NGF) and monosialoganglioside (GM1) into biodegradable microspheres for the treatment of lesioned cholinergic neurones (Maysinger *et al.*, 1993). The proteins were loaded into the internal aqueous phase of a double emulsion preparation at a ratio of 1:200. The ratio between the two in the final lyophilised product was 1:125. The release of both drugs followed a similar biphasic, first order pattern with the release of the NGF enhanced by the co-encapsulated GM1 and some promise was derived from delivery of these co-encapsulated trophic factors directly to the brain.

The next generation of adjuvant formulations may consist of several different adjuvant formulations, where each component is added to augment a particular type of immune response, either having additive or synergistic effects. A systematic study should be performed for each antigen to determine the best preparation to evoke the desired response. Despite the potential of microencapsulated vaccines for pulsed delivery, the degree to which different antigens and adjuvants can be combined in the same microsphere or different microspheres in the same injection has not been determined. Co-encapsulation of materials will be necessary in the design of polyvalent vaccines and when immunopotentiation by co-encapsulation with immunomodulators such as muramyl dipeptide (MDP) and its derivatives and gamma-interferon (IFN-γ) is desired. A decreased loading efficiency and altered release profiles have already been reported following the encapsulation of a small amount of IFN-γ with bovine serum albumin (Conway *et al.*, 1994).

The amphipathic nature of proteins, resulting from their mixture of polar and non-polar side chains, causes them to be concentrated at interfaces. A protein's ability to compete with other proteins and situate itself at an interface has a marked effect on the deposition of protein deposits within the microsphere matrix. Size, shape charge and thermal stability will all influence the surface activity of the protein. The milk proteins, α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) were selected along with BSA to study how molecular properties and surface activity affect loading into and release from PLLA microspheres formed by single and double emulsion techniques (table 6.1). Though much is known about the chemistry of these molecules (Suttiprasit *et al.*, 1992), there is little known about the mechanisms influencing a protein's ability to compete with other proteins in a solution and situate itself at a surface. Encapsulation and release of IFN-γ, either singly or in combination with carrier proteins such as BSA and HSA, was also

studied. The use of this cytokine as an adjuvant has been discussed in section 1.4.3.1 and the immune responses generated following administration of formulations co-encapsulating IFN-γ and subunit antigens for prevention of bubonic plague have been discussed elsewhere in this report (section 7.3.4). This chapter discusses incorporation of proteins with a range of molecular weights, flexibility and charge permitting investigations into the effects of emulsifying agents, drug and polymer properties on the loading and release from such biodegradable microparticles. The potential competitive adsorptive behaviour and individual molecular influences of proteins on surfaces during particle preparation will lead to differences between loading efficiencies and release profiles of individual proteins and protein combinations from single and double emulsion microspheres prepared by solvent evaporation techniques.

Table 6.1. Some physical properties of three model proteins (adapted from Suttiprasit *et al.*, 1992)

Protein	Amino acid residues	Mw (kDa)	pI
α-Lac	123	14.2	4.2-4.5
β-Lg	162	18.3	5.13
BSA	582	66.3	4.7-4.9

6.2 MATERIALS AND METHODS

The polymer used in this section, unless otherwise stated, was PLLA (Mw 2000), detailed in section 3.2. BSA, alpha-lactalbumin and beta-lactoglobulin was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). PLLA microspheres were prepared incorporating these proteins and their binary mixtures in different ratios (1:160 to 1:1) to examine the influence of the presence of various ratios of another protein on encapsulation and release behaviour. ^{125}I-bovine serum albumin was supplied by ICN Biomedicals Inc. (High Wycombe, Bucks., U.K.) and used as described in section 3.2.

HuIFN-γ was a gift from Dr G.R. Adolf (Bender+ Co. GesmbH, Wien, Austria) and was supplied in a buffered solution containing 10mg/ml IFN and an unspecified quantity of other stabilising proteins. (3-[^{125}I]iodotyrosyl)gamma-interferon (I-IFN-γ)

was purchased from Amersham Life Science (Bucks., U.K.) and used as described in section 7.2. All other materials used in these experiments are as detailed in section 3.2. All the methods used for microsphere production and analysis in this chapter are as detailed in chapter 2.

6.3 RESULTS AND DISCUSSION

6.3.1 Encapsulation of alpha-lactalbumin, beta-lactoglobulin and BSA

α-Lac and β-Lg were encapsulated into PLLA microspheres using single and double emulsion techniques at a theoretical loading of 10% w/w. Though microspheres were produced under similar conditions, there was a wide variation in encapsulation with α-Lac loading more efficiently than β-Lg. Microsphere production using double emulsion techniques was more successful then single emulsion methods (from the % w/w loading point of view), in contrast to results with the high molecular weight tetanus toxoid (see section 5.3.1). Particle sizes were typically in the range 1-2μm. The magnitude of the surface charges (zeta potentials) is larger for than for similar BSA loaded particles (~-1 to -6mV) (see section 3.3.4). Although there were no obvious trends in the magnitudes of the surface charges of the particles formed, the increase may be due to chemical differences between the proteins and their distribution at the particle surface (table 6.2).

Higher loadings were achieved using a double emulsion preparation technique for both α-Lac and β-Lg than a single emulsion technique. This may be due to the reduction in potential protein losses using the w/o/w method. The improvement in loading is also demonstrated by mixtures of proteins as detailed in table 6.2. When small quantities of the lower molecular weight proteins (1:160) were incorporated with BSA into microspheres, the loading efficiency of the process was reduced by up to 60% (table 6.2). Generally, this occurred to a larger extent with α-Lac and β-Lg.

Molecular charge and hydrophobicity play a role in protein adsorption and deposition at interfaces. The adsorption of BSA and β-Lg is largely governed by non-electrostatic interactions (Luey *et al.*, 1991). Proteins are dynamic structures and those with low stability and/or high flexibility will be preferentially adsorbed. General characteristics of protein adsorption are similar at air-water and oil-water interfaces (Graham & Phillips, 1979) and reports describing adsorption at air-water interfaces can be extended to afford

some explanation of the processes involved during encapsulation of a mixture of proteins and their subsequent release. Proteins cause a decrease in water/DCM interfacial tension, the extent of which depends on their hydrophobicity (the ratio and distribution of the polar/apolar amino acids) and/or their structural flexibility. As the cross interactions between the aqueous phase and the organic polymer phase are dependant on the chemical structure of the protein, these have an effect on protein entrapment and release characteristics of the resultant microspheres, leading to different optimums for different protein/polymer combinations.

Table 6.2. Total protein loads and zeta potentials (in 10mM phosphate buffer) of PLLA microspheres loaded with single proteins and protein combinations (1:160)

Formulation	Load (% w/w)*	Zeta potential (mV) (n=5)
α-Lac d/e	12.69	-8.6±0.4
α-Lac+BSA d/e	7.90	-8.4±0.3
β-Lg d/e	11.61	-14.0±0.5
β-Lg+BSA d/e	10.32	-6.8±0.5
α-Lac s/e	6.90	-6.7±0.6
α-Lac+BSA s/e	3.66	-9.7±0.6
β-Lg s/e	3.59	-7.1±0.3
β-Lg+BSA s/e	4.18	-12.1±0.9
BSA d/e	8.12	-1.3±0.7

* total protein load, determined using BCA assay

The affinity between a protein and adsorbent increases with increasing hydrophobicity of the surface and proteins desorb more easily from hydrophilic than hydrophobic surfaces but there are many variables affecting protein adsorption, e.g. there is an increase in adsorption near the isoelectric point of the protein. At the isoelectric point, the protein is more globular but becomes more extended for larger or smaller values of pH. A more extended molecule requires a larger number of sites and adsorbs with an increased difficulty compared to a globular molecule, therefore adsorption is maximal at or near the isoelectric point. The isoelectric point of β-Lg is slightly higher than those of BSA and α-Lac which may account for its better loading at the pHs involved in microsphere formation. It is probable that the contribution of electrostatic interactions in

influencing the deposition of proteins is only minor and hydrophobic interactions are more significant (Suttiprasit *et al.*, 1992).

Although α-Lac, β-Lg and BSA are all globular proteins, at neutral pH, BSA and α-Lac exist as monomers whereas β-Lg is a dimer. The relatively compact α-Lac structure may allow a more easy accommodation into an interfacial film, which may lead to more efficient loading. Even in a crowded interface, α-Lac can renature after denaturation, aiding its ability to adopt an energetically stable state. Although BSA is relatively large and consists of a high number of disulphide bridges, its multidomain and high helix content, render it somewhat flexible (Suttiprasit *et al.*, 1992). β-Lg, though nearly half the size of BSA, is less flexible due to its dimer structure and thiol-disulphide interchange reactions, which may decrease the stability of the emulsion formed. BSA can compete more effectively with α-Lac for surface sites than the less flexible β-Lg, resulting in a reduced overall encapsulation efficiency for α-Lac/BSA combinations, not observed with β-Lg/BSA combinations (table 6.2).

6.3.2 Release from microspheres containing alpha-lactalbumin, beta-lactoglobulin and BSA

Release profiles for single proteins, α-Lac and β-Lg, from PLLA double emulsion microspheres are shown in figure 6.2. Profiles were similar for spheres formed using single emulsion techniques but demonstrated a faster overall release (e.g. figure 6.3). Generally α-Lac and BSA exhibit similar release profiles though those of β-Lg were faster. The accumulation of a protein at an interface during solvent evaporation process has been described by Bazile *et al.* (1992). The less flexible structure of β-Lg may affect its loading into and distribution throughout the microsphere matrix in such a manner as to cause faster release.

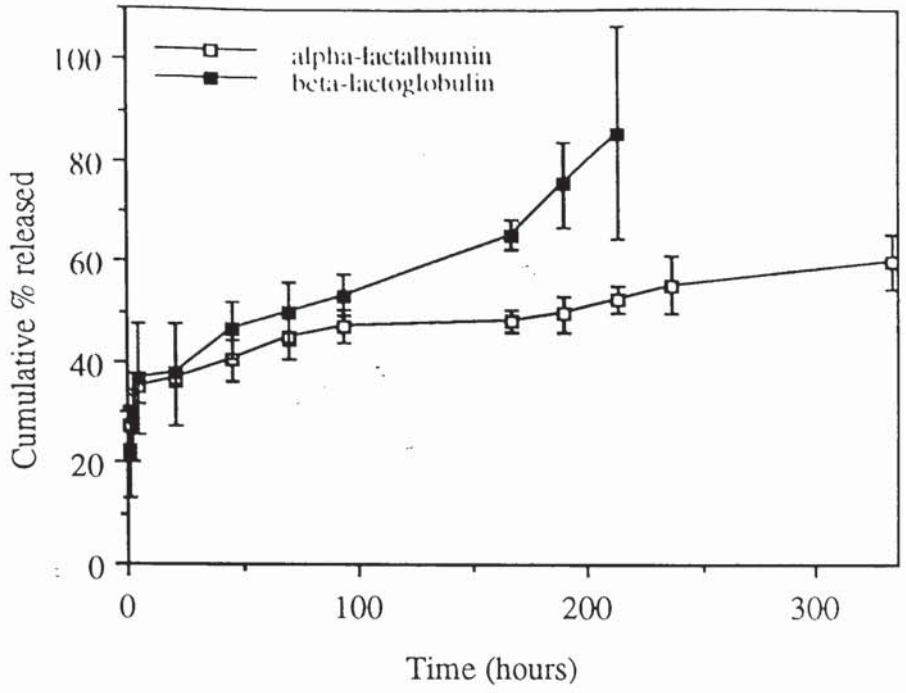


Figure 6.2. Release profiles for β-Lg and α-Lac from PLLA microspheres prepared using a double emulsion method (n=3; mean±s.d.) Protein loading is ~12% w/w and particle size is ~1μm

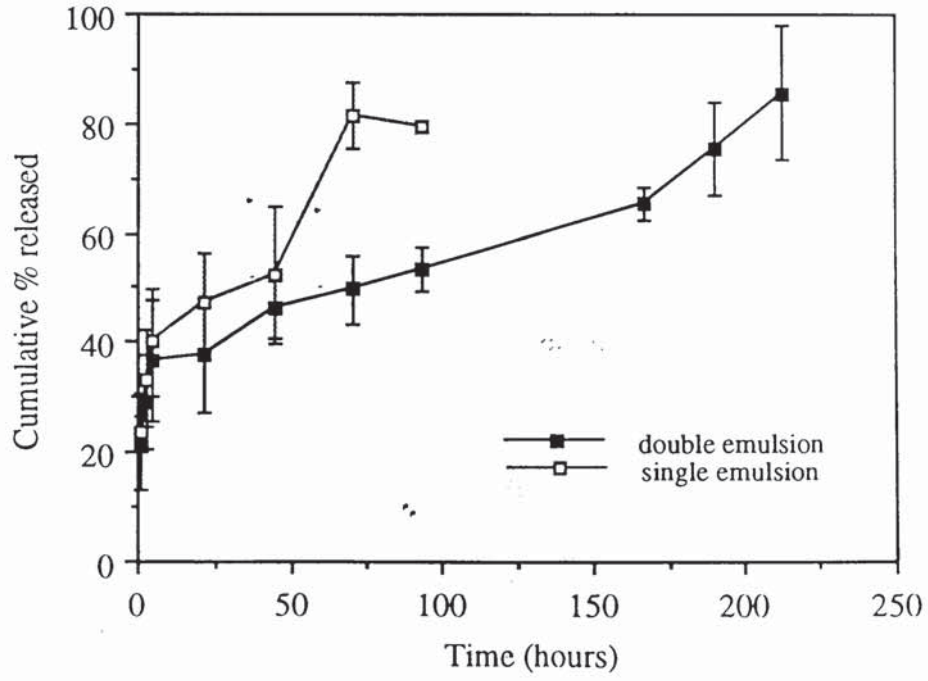


Figure 6.3. Release profiles for β-Lg from PLLA microspheres prepared using single or double emulsion, solvent evaporation techniques (n=3; mean±s.d.). β-Lg loading is ~16% w/w for both preparations and particle size is ~1μm

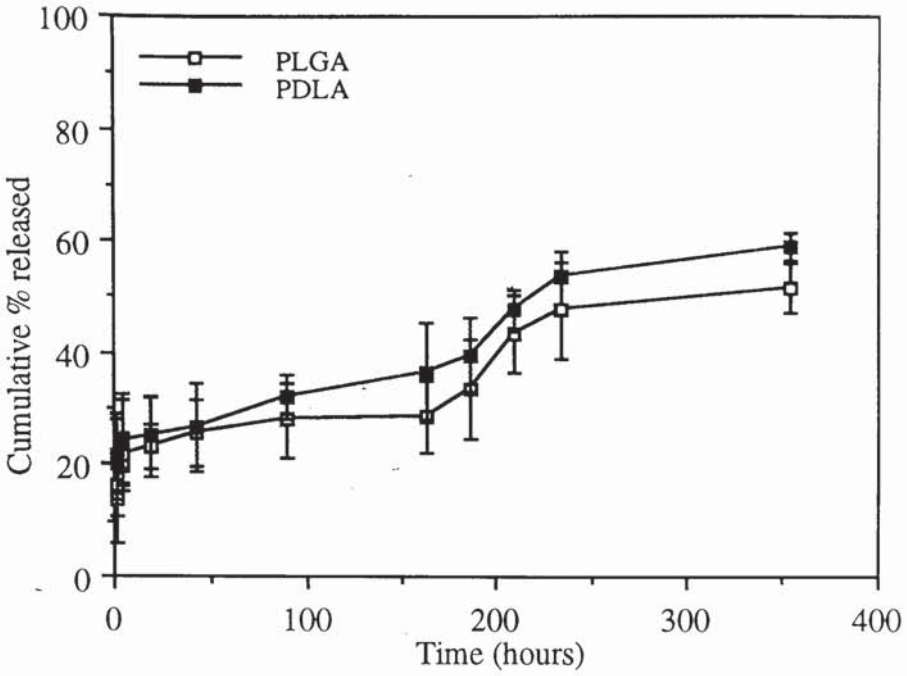


Figure 6.4. Release profiles for β-Lg from PDLA and PLGA microspheres prepared using a double emulsion technique (n=3; mean±s.d.). β-Lg loading is ~10% w/w for both preparations and particle size is ~1μm

The loading and release of β-Lg into the amorphous PLA polymers, PDLA and PLGA was also studied (figure 6.4). Loading was more efficient than for similar BSA loaded particles (table 6.3), the burst effect was reduced and overall release rate was slower, with 50-60% of the β-Lg released in 350 hours (see section 3.3.3.2). The poor stability of β-Lg for prolonged periods above pH 6.5 lead to difficulties in sustaining the release studies for prolonged periods (Suttiprasit & McGuire, 1992).

Release from spheres containing binary mixtures was quicker than from those with a similar BSA load (figure 6.5 and section 3.3.2.1). Burst effects were also reduced and in contrast to results from spheres containing single proteins those with α-Lac and BSA binary mixtures demonstrated a slightly faster overall release than β-Lg and BSA combination. Radiolabelled BSA was incorporated into PLLA microspheres, along with the lower molecular weight proteins, to allow differentiation between the proteins. Release profiles obtained are shown in figures 6.6 and 6.7 and it can be seen that for mixtures of both α-Lac and β-Lg with BSA, the overall rate of protein release is different to that of the radio-labelled BSA. The presence of small amounts of the low molecular weight proteins, therefore, causes the radio-labelled BSA to be released more rapidly than the total protein (determined by BCA assay).

Table 6.3. Loading, encapsulation efficiencies and particle sizes for BSA and β-Lg loaded PLGA and PDLA microspheres

Polymer	Protein	Actual load (% w/w)	Encapsulation efficiency (%)	Particle size (μm±σg)
PLGA	BSA	4.88	48.8	1.21±0.49
PDLA	BSA	5.04	50.4	1.15±0.52
PLGA	β-Lg	10.74	107.4	0.83±0.36
PDLA	β-Lg	11.18	111.8	1.02±0.41

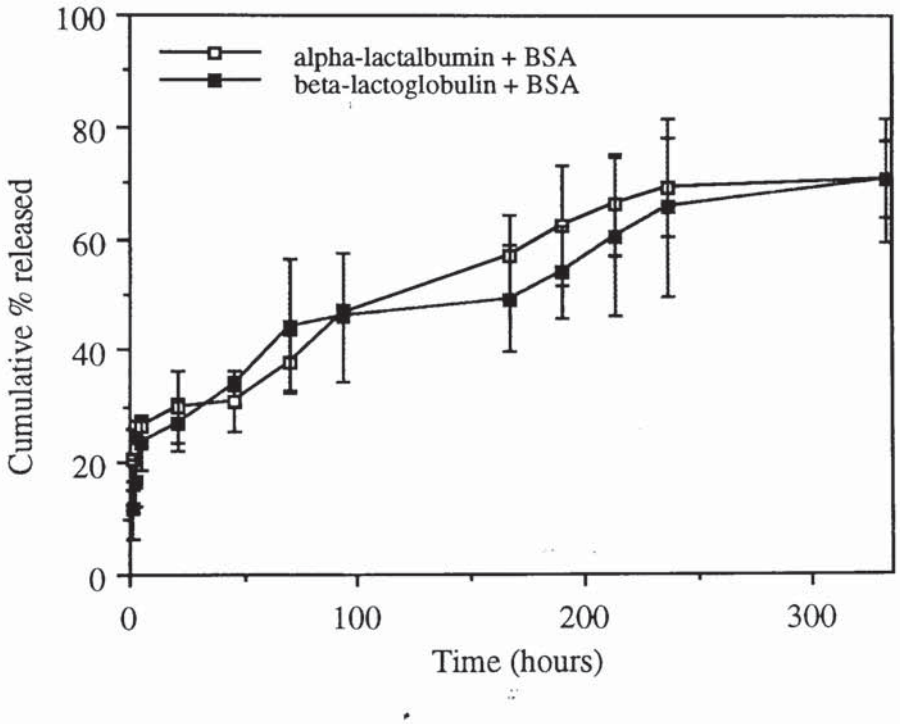


Figure 6.5. Release profiles for total protein from PLLA microspheres prepared using a double emulsion technique (n=3; mean±s.d.). Protein loading is ~10% w/w for both preparations (ratio 1:160) and particle size is ~1μm

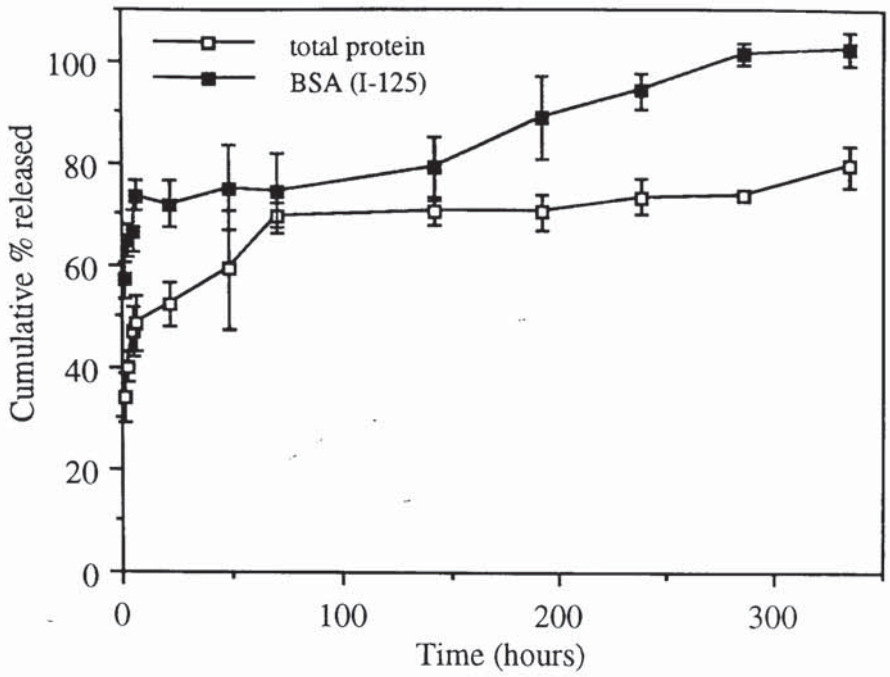


Figure 6.6. Release profiles for ^{125}I-BSA and total protein (determined by BCA) from PLLA microspheres prepared using a double emulsion technique (n=3; mean±s.d.) using 160:1 BSA:α-Lac

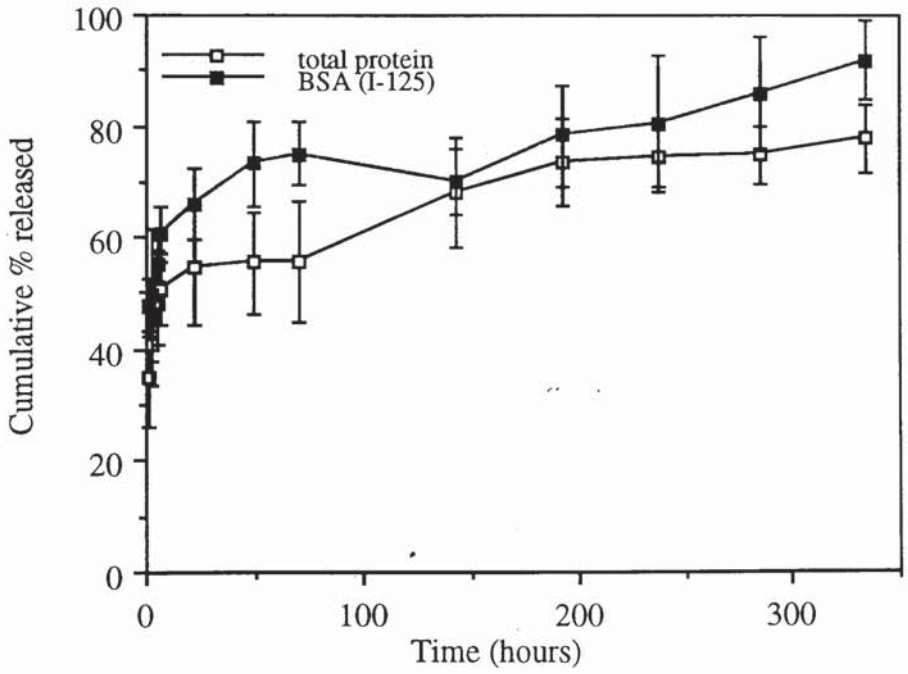


Figure 6.7. Release profiles for ^{125}I-BSA and total protein (determined by BCA) from PLLA microspheres prepared using a double emulsion technique (n=3; mean±s.d.) using 160:1 BSA:β-Lg

As β-Lg demonstrates loading and release properties different to BSA, increased ratios of the lower molecular weight protein were co-encapsulated with BSA and the release profiles studied (figure 6.8). The heightened release of BSA observed for lower ratios of β-Lg/BSA combinations, is not detected when the amount of β-Lg is increased to a 50:50 ratio, i.e. there is little difference between the overall rate of release and that of BSA.

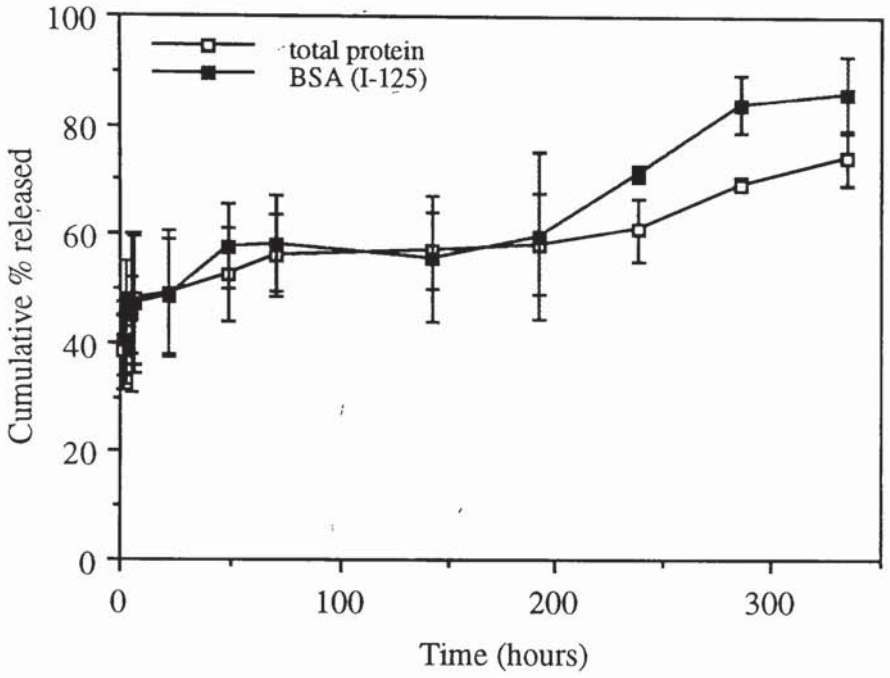


Figure 6.8. Release profiles for ^{125}I-BSA and total protein (determined by BCA) from PLLA microspheres prepared using a double emulsion technique (n=3: mean±s.d.) using 50:50 BSA:β-Lg

Proteins arrive at an oil/water interface during homogenisation at a rate proportional to their concentration. At low concentrations, there is little difference in emulsifying properties between α-Lac and BSA but both are higher than β-Lg. Less stable molecules are adsorbed at the interface more rapidly than stable molecules, in this case β-Lg is the most thermally stable and α-Lac the least stable (Suttiprasit *et al.*, 1993). α-Lac and the domain of BSA responsible for adsorption at the oil/water interface are quite flexible, allowing more protein unfolding at the surface. Thus, it may be that more α-Lac and BSA are located at the interfaces between the aqueous and organic phases within the internal microsphere structure than β-Lg, and the release of these proteins is slower than β-Lg. At higher concentrations the behaviour is more difficult to predict. β-

Lg is only slightly more hydrophobic then the other molecules. At low concentrations it is expected that the large, flexible BSA molecule may locate at the interface more efficiently due to its α-helices. α-Lac is a small molecule and it is resistant to unfolding. In more concentrated solutions, α-Lac may make more contacts with the interface. However, β-Lg is nearly half the size of BSA and its faster arrival at the interface may compensate for its more rigid structure. This is more important at high concentrations where BSA is more inhibited and the short protein-surface contact times associated with this method before particle hardening occurs make prediction of loading efficiencies and release characteristics more difficult.

Generally, the adsorption of proteins to polar surfaces does not result in major disruption of protein secondary or tertiary structure. As the surfaces become more hydrophobic, the possibility of conformational changes increases, with intrinsically less stable proteins being more susceptible. There is no simple relationship between the potential for structural change and particular structural aspects of the proteins. If these changes do not alter the conformation of the protein antigen or disrupt the neutralising epitope, they will not adversely affect the immunogenicity of the vaccine. The addition of stabilising agents such as sugars or surfactants, may afford some protection against conformational change. For sustained delivery, the drug must be stabilised at 37°C within the microspheres *in vivo* as well as during the encapsulation process. Due to conflicting results reported by Baneyx & Georgiou (1992), it is likely that charge and hydrophobicity do not correlate with protein degradation in general.

6.3.2 Encapsulation and release characteristics of interferon-gamma in microspheres

IFN-γ is a basic hydrophobic protein, which leads to a significant challenge for microencapsulation, due to the potential for protein/polymer interactions. IFN-γ is a glycoprotein consisting of 166 amino acids and carbohydrates, resulting in a molecular weight of 17 kDa. It is basic, with an isoelectric point at about pH 10, acid-labile and is denatured at 56°C.

Low and relatively high levels of IFN-γ, 0.06% w/w and 1% w/w respectively, were encapsulated into PLLA microspheres using single and double emulsion solvent-evaporation techniques. It may be advantageous to administer the IFN-γ at the same time as the antigen, but in a different preparation to give more flexibility and control over release processes. To minimise loss of the potent constituent and to permit release of the cytokine within a desirable time period, IFN-γ was diluted with albumins before

incorporation into the particles and loading and release were studied using the cytokine itself and in the presence of diluent proteins, HSA and BSA.

At 1% w/w loading levels, the encapsulation process was very efficient, with >90% of the protein contained within the particles formed (table 6.4). HSA was loaded into PLLA microspheres at a 10% w/w level and the effect of the inclusion of a small amount of IFN-γ (0.06% w/w) on encapsulation efficiency was studied.

Table 6.4. Loading of IFN-γ into PLLA microspheres; effect of method of preparation and stabilising protein on encapsulation efficiency and particle size

Formulation	Theoretical load (% w/w)	Actual load (% w/w)	Encapsulation efficiency (%)	Particle size (μm±σg)
IFN-γ d/e	trace (0.06)	-	-	0.79±0.48
IFN-γ s/e	1.0	0.91	91.0	1.12±0.67
IFN-γ d/e	1.0	0.98	98.0	1.27±0.52
HSA s/e	10.0	8.46	84.6	0.86±0.64
HSA + IFN-γ s/e	10.0*	4.75	46.5	1.02±0.98
HSA d/e	10.0	12.49	124.9	0.96±0.92
HSA + IFN-γ d/e	10.0*	8.16	81.6	1.02±0.90

* total protein load

The reduction in loading efficiency of protein, when only a trace amount of cytokine (1:160) is added to the HSA, is ~30-40%. This decrease may be due to interaction between the cytokine and the polymer or, as is more likely from investigations using model proteins (section 6.3.1), competition for loading between the carrier protein and the cytokine. Similar reductions were produced when BSA was used as the carrier protein (table 6.5). At the lower loading (10% w/w), the overall efficiency of protein loading was increased in the presence of the cytokine although the efficiency of IFN-γ loading was low (35.1%). As the protein loading was increased to 20% w/w, but IFN-γ levels maintained at 0.06% w/w, there was a decrease in the overall protein loading efficiency and loading of the IFN-γ.

Table 6.5. The effect of mixtures of proteins on the loading efficiency of PLLA microspheres prepared using a double emulsion technique

Drug loading	IFN-γ efficiency*	Total protein efficiency	BSA efficiency[(a)]
150μg IFN+ 24.85mg BSA (10% w/w)	35.1%	98.2%	80.2%
150μg IFN+ 49.85mg BSA (20% w/w)	18.6%	46.1%	69.9%

(a) BSA loading in a similar microsphere system * determined using I-IFN-γ

The loading of different ratios of proteins within the one microsphere system must be fully characterised before such a system can be reproducibly manufactured and the release profiles reliably predicted. The competition for loading sites must be studied at a number of loading levels and individual molecular influences of even structurally similar proteins, such as HSA and BSA, must be considered.

The deposition and loading pattern of the individual proteins will also has an effect on the release profiles obtained. Release profiles were carried out in 20mM phosphate buffer and for 1% w/w loaded PLLA microspheres without the inclusion of any diluent protein are faster than the profiles obtained for higher molecular weight molecules. Also, release from spheres formed using a double emulsion method is greater than from single emulsion particles at similar loadings. This is due to the reduction in burst effect exhibited by single emulsion particles (~30% vs. 50%) (figure 6.9). The increase in burst effect may be due to imperfectly incorporated drug at the polymer surface (Shah *et al.*, 1992) for the double emulsion method, indicating that a certain level of drug is necessary for the rigid structure of the microspheres (Heya *et al.*, 1991b). Also, the presence of a basic drug in the matrix may increase the release rate as the hydrolytic rate of PLA polymer increases in alkaline pH.

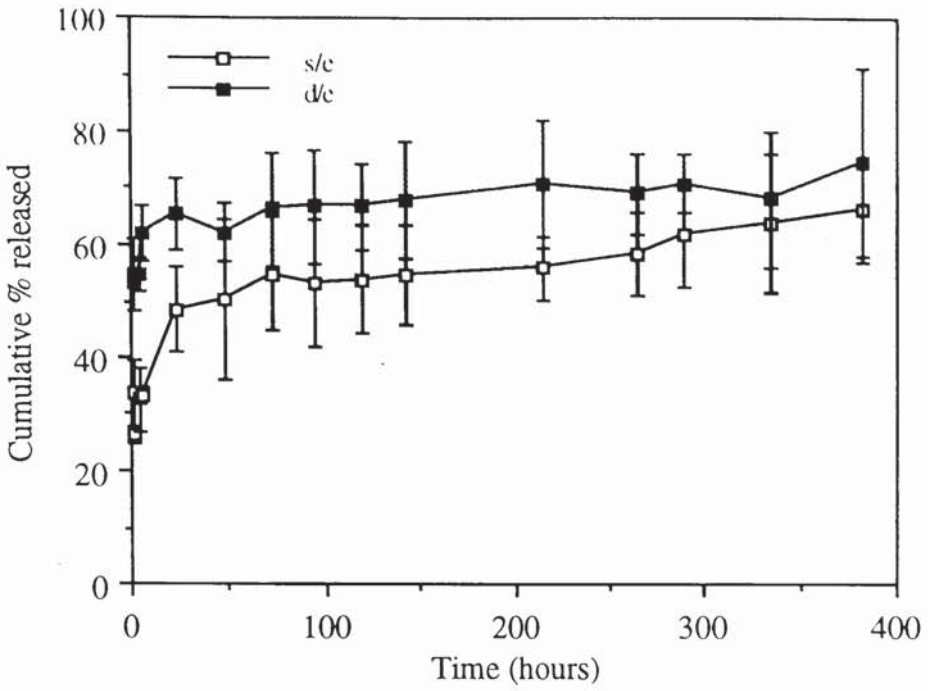


Figure 6.9. Release profiles of 1% w/w IFN-γ from PLLA spheres made using single or double emulsion techniques (n=3; mean±s.d.)

Cleland & Yang (1995) found that IFN-γ could be released intact and bioactive from PLGA microspheres, but degradation in the media maintained at 37°C does occur over time due to inherent instability of the protein. rHu-IFN-γ was most stable in release media at pH 5 (Cleland & Yang, 1995) and burst effects varied from 1-4% by mass but there was no binding to the polymer observed. Due to the expense and limited availability of the cytokine, analysis of stability was not possible. However, further studies on the stability of the protein within the system are to be carried out by Dr G. R. Adolf (Bender+ Co. GesmbH, Wien, Austria).

The overall release process is complicated by the presence of stabilising proteins in the cytokine supplied and salts in the radiolabelled preparation. Release of radiolabel into gastric simulated media (see section 2.3.2) and phosphate buffer was carried out using 1% w/w loaded particles and trace loaded particles at 37°C. Due to lower agitation rates used for this investigation, the burst effect from PLLA 1% w/w double emulsion particles was reduced. However, in the gastric simulated media, ~50 % of the drug was released in 3 hours. This was reduced to ~30% for 0.06% w/w loaded microspheres (table 6.6).

Table 6.6. Release of IFN-γ into gastric simulated media or phosphate buffer from PLLA microspheres

Time (hours)	~1% w/w d/e		~0.06% w/w d/e	
	pH 7	pH 1.2	pH 7	pH 1.2
0.25	12.5±3.5	36.1±5.6	3.7±1.0	16.0±3.8
0.75	11.6±5.0	38.0±5.2	4.1±0.6	23.3±6.0
1.00	18.6±7.7	49.8±3.4	5.0±0.5	28.3±8.0
2.00	21.0±8.9	52.0±4.7	5.4±0.5	29.1±8.2
3.00	20.7±7.9	46.1±6.1	6.4±1.1	28.0±6.5

Overall, the release rate of the hydrophobic IFN-γ was higher in the simulated gastric medium (table 6.6). Degradation of PLA, though maximal in alkaline conditions (Makino *et al.*, 1986), is enhanced in strongly acidic environments. In strongly acidic conditions, the zeta potential of microspheres becomes more positive, due to proton transfer from the bulk solution to the microsphere surface and the concentration of H^+ in the membrane surrounding the microsphere has a predominant effect on degradation (Makino *et al.*, 1985). Protection of encapsulated antigens has been demonstrated by Moldoveanu *et al.* (1993). Following incubation of PLGA microspheres in gastric simulated medium (USP XXI) for 2 hours prior to s.c. immunisation, the immunogenicity of the encapsulated influenza virus was fully retained. Suspension of microspheres in $NaHCO_3$ prior to oral administration is anticipated to reduce the acidity in the stomach, so reducing the percentage of drug lost in the harsh environment.

The effect of co-encapsulation of a carrier protein (in this case BSA) on the release profiles (in 20mM phosphate buffer) of individual proteins and protein combinations from single and double emulsion microspheres prepared by solvent evaporation techniques was studied. When release profiles were performed on particles with a comparable total protein load of BSA or BSA+IFN-γ, both systems exhibited a similar burst effect (figure 6.10).

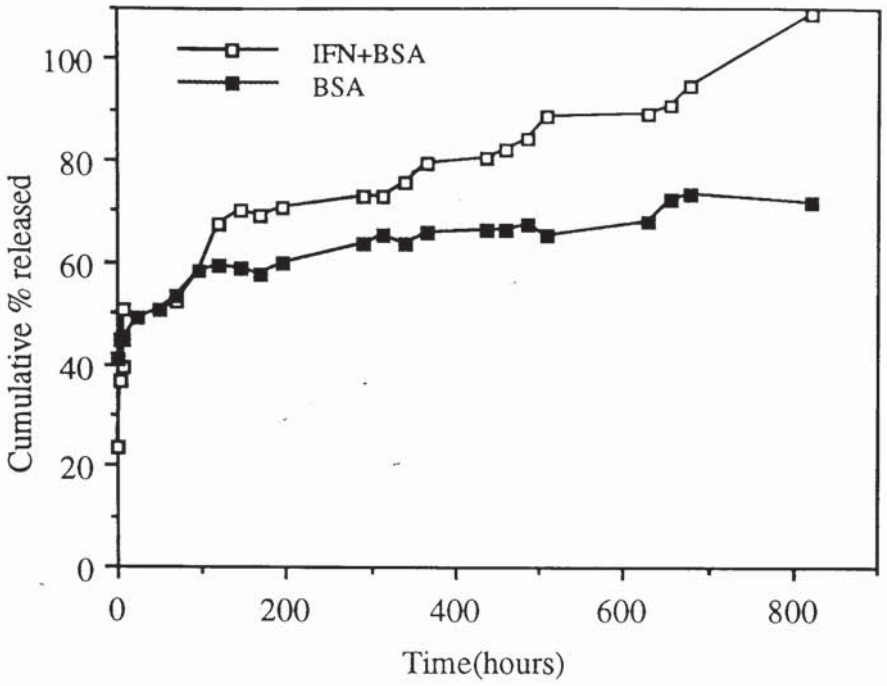


Figure 6.10. Release profiles for BSA and IFN-γ+BSA (determined using a BCA assay) from microspheres formed using a double emulsion method. Particle size ~1μm, protein loading ~9.3% w/w (results are the mean of three separate determinations and s.d.s are less than 12% of the mean)

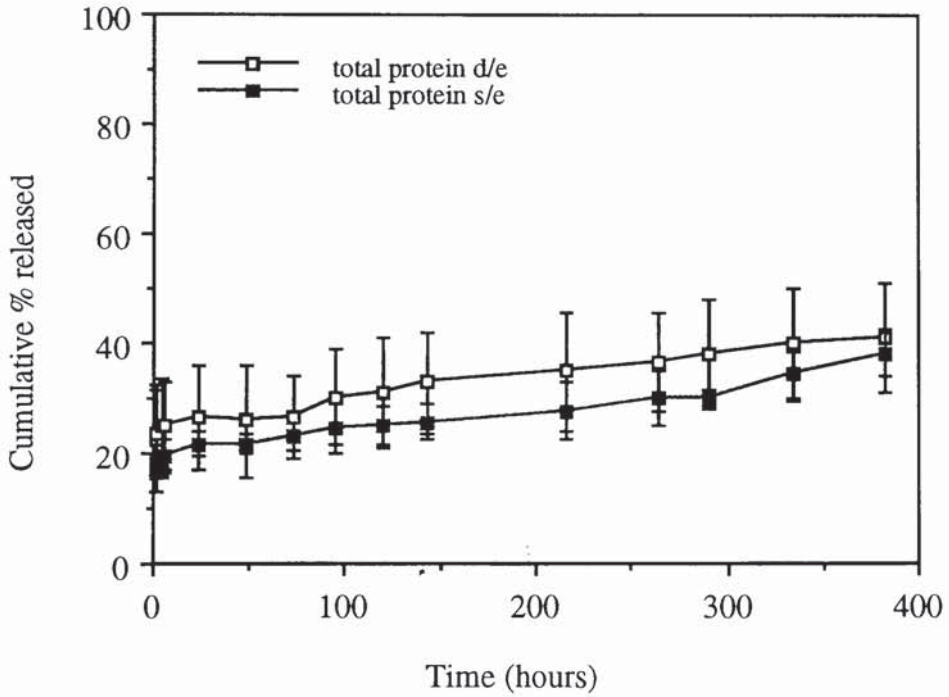


Figure 6.11. Release profiles for total protein (determined by BCA method) from PLLA microspheres encapsulating IFN-γ and HSA prepared using single or double emulsion techniques (n=3; mean±s.d.)

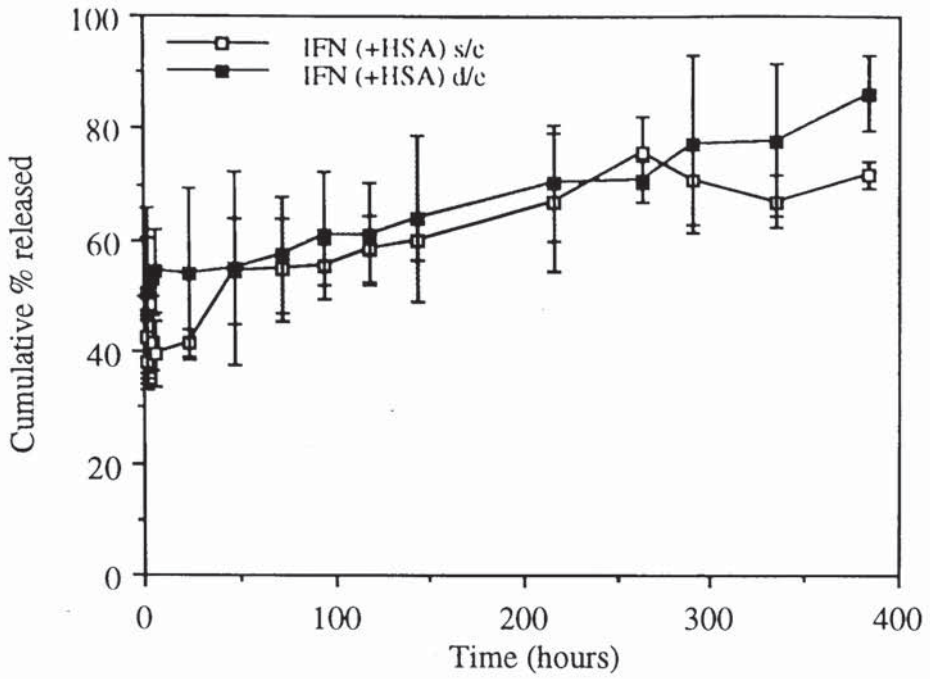


Figure 6.12. Release profiles for I-IFN-γ from PLLA microspheres containing IFN-γ and HSA prepared using single or double emulsion techniques (n=3; mean±s.d.)

After approximately 100 hours incubation time, the release profiles started to differ, with the co-encapsulated mixture being released more rapidly than the BSA. Co-encapsulation of the mixture of cytokine and BSA facilitates the enhanced release of IFN-γ and BSA, probably by the mechanisms proposed (section 6.1) by Hora *et al.* (1992b). However, when the diluent used was HSA, the overall rate of protein released was decreased (figure 6.11), with only 40% of the total protein release in 400 hours. This was the case for batches prepared using both single and double emulsions, which exhibited similar profiles, with the burst effect from double emulsion preparations only slightly higher. The release of I-IFN-γ was however, faster than the overall rate of protein released, with little difference between the preparation methods after 50 hours (figure 6.12). The presence of the soluble HSA in the microsphere formulation facilitated a faster release of the low loaded, low molecular weight protein but was not itself released from the preparation at the same rate. Thus, water penetration into the system may be enhanced but the highly porous structure proposed by Hora *et al.* (1992b) does not produce large enough pores to allow a rapid diffusion of the higher molecular weight diluent protein.

6.4 SUMMARY

During emulsification, three main processes occur, droplet reduction, adsorption of protein molecules on newly created surfaces and coalescence. The physical properties (size, shape and flexibility) of all proteins in a mixture are important in determining whether the proteins co-adsorb in a simple competitive manner (Hunter *et al.*, 1991). A more flexible structure may adsorb to small areas of exposed interface between other adsorbed molecules while a more rigid structure will preclude its inclusion into a layer of adsorbed protein. Molecular size effects on surface activity of proteins are thought to be incidental (Suttiprasit *et al.*, 1993) and proteins with low structural stability and/or high flexibility are more surface-active.

The addition of proteins to impart porosity has been successful, altering the structure of the polymer matrix, facilitating the movement of water into and the release of protein out of the microsphere. However, the addition of a hydrophobic material, such as IFN-γ, may retard the release of hydrophilic proteins, such as HSA further complicating the loading into and the release from the particles of all constituents. The co-encapsulation of protein mixtures, and other excipients such as salts or surfactants, requires investigation of the effect of each individual ingredient at different ratios on the subsequent deposition and release behaviour.

7.0 MICROENCAPSULATION OF F1 AND V ANTIGENS IN PLA MICROSPHERES

7.1 INTRODUCTION

Due to the complexities of developing safe whole-killed or attenuated vaccines, and due to advances in biotechnology, it is likely that many future vaccines will be peptide or protein subunits. Being chemically well-defined, immunisation with these entities results in safe and reproducible vaccination. However, subunit vaccines are often poorly immunogenic and adjuvants are required to reduce dosage frequency. The depot adjuvant action of controlled release formulations started being investigated more than a decade ago (Pries & Langer, 1979) but has accelerated recently. For these subunit vaccines, it may be difficult to elicit a vigorous humoral response with a controlled release system alone and the inclusion of an immunostimulant may be vital to the success of the vaccine.

Yersiniae are a member of a group of facultative intracellular parasites which commonly cause granulomatous disease, are capable of growth within phagocytes and are frequently vulnerable to processes of cell-mediated immunity (Nakajama & Brubaker, 1993). The vaccines currently available for the prevention of plague are either killed or attenuated whole-cell preparations, which are highly heterogeneous with variable endotoxin content causing a wide range of side-effects. The identification of protective subunits of *Y. pestis* which could be delivered directly to mucosal surfaces to produce protective responses may facilitate the development of an improved vaccine against plague. Two such candidate subunits are F1 and V antigens of *Y. pestis*. Recent successes in this field include the protection against virulent strains using F1 antigen expressed in *Salmonella typhimurium* (Oyston *et al.*, 1995) and co-immunisation with F1 and V subunits affording equivalent protection to the conventional vaccine (Williamson *et al.*, 1996).

Microencapsulation of these subunit vaccines may not only confer adjuvanticity, but may also provide protection from the harsh environments encountered during delivery to the immunoresponsive areas of the MALT.

7.1.1 The V antigen of *Y. pestis*

The V antigen is historically important as the first antigen discovered to be associated with virulence in *Y. pestis*, (Burrows, 1956), being a diffusible protein present in cultures of the virulent strain produced at 37°C. It is protective in both active and passive immunisation (Une & Brubaker, 1984) and the importance of V antigen, though itself, a poor antigen, renders investigation into methods to improve antigenicity relevant.

The human-pathogenic yersiniae have a multi-component virulence property called the low-Ca^{2+} response (Lcr phenotype) which contains a set of regulatory genes and 12 co-ordinately regulated virulence genes. These include a set of 11 surface proteins called Yops (*Yersinia* outer membrane proteins) and a secreted protein called V antigen (Price *et al.*, 1991). The regulatory genes modulate the expression of the virulence genes in response to environmental inputs of temperature, Ca^{2+} and nucleotides. The association of the V antigen with virulence is based on the ability of partially purified V to protect mice against fully virulent *Y. pestis* and on the passive protection provided by anti-V sera against plague (Price *et al.*, 1989). The molecular weight of ~38kDa calculated from amino-acid sequencing corresponds with that obtained by SDS-PAGE analysis and the isoelectric point of 5.42 correlates well with isoelectric focusing (IEF) results (Lawton *et al.*, 1963). V antigen stability can be reduced after prolonged storage in solution at 5°C and after lyophilisation.

V antigen may be a bifunctional protein, having a role as a virulence protein and important regulatory functions in the low-Ca^{2+} response in both causing restriction and promoting virulence gene expression. The effectiveness of V antigen, being directed against certain virulence factors, in protecting against experimental plague, demonstrates that both cellular and humoral processes of immunity can operate against wild-type strains of *Y. pestis*. There is a theory that V antigen serves as a component of the process that blocks activation of macrophages (Une & Brubaker, 1984) but the biological role of V antigen has not yet been fully resolved. Lcr+ yersiniae (capable of producing both V antigen and Yops) provide protection against subsequent challenge. This has been attributed to anti-V as indicated by the ability of polyclonal anti-V to provide mice with passive protection. However, there is concern that possible contaminating antibody to Yops, established virulence factors, may account for the passive immunity rather than anti-V itself (Brubaker, 1991). Results, however, clearly demonstrate that V antigen contains at least one epitope which prevents the occurrence of plague.

7.1.2 The F1 antigen of *Y. pestis*

At 37°C, encapsulated strains of the plague bacillus of *Y. pestis* form massive gel-like capsules which are believed to increase resistance to phagocytosis. The main constituent material of this envelope is protein fraction 1 (F1) antigen which diffuses into the surrounding medium and is one of the main immunogens of the plague microbe. This envelope material, though non toxic itself, has an important role with respect to immunity against plague infection. During cases of human bubonic plague, antigenaemia levels in the µg/ml range occur, with the liver and the spleen containing larger amounts (Williams, 1990).

As the titre of F1 antibodies correlates with the protection gained through vaccination, it has been assumed to be the primary immunogen in the current whole cell vaccines with protective inducing properties. Side effects with traditional vaccines, such as severe malaise and fever, due to the heterogeneous antigenic composition render them unsuitable for widespread use. When inoculated as a parenteral immunogen, F1 has been shown to be protective both as native antigen and a cloned product (Baker *et al.*, 1952; Simpson *et al.*, 1990). It is a glycoprotein and as such, can be relatively resistant to proteolytic degradation. The recombinant product may actually have carbohydrate components as the molecular weight, determined by SDS-PAGE analysis, appears the same. Thus, the cloned fragment containing the F1 gene may also encode enzymes involved in the modification of the F1 protein.

Secreted F1 has 149 amino acid residues with a deduced Mw of 15.5kDa and isoelectric point of 4.3 and consists of two antigenically identical subfractions, a protein and a glycoprotein (Bennett & Tornabene, 1974). Both the protein and the glycoprotein comprising F1 show unusual antigenic stability even after harsh dissociation treatments. F1 protein has mainly β-sheet structure (at least 50%) and may have 3-4 short strips of α-helix. The region between residues 105-120 may be located on the surface of the protein, since it contains β-turns (Galyov *et al.*, 1990) and may constitute antigenic determinants. The C-terminal end is supposed to be the area of great importance for immunogenicity having an amphipathic structure with opposing hydrophilic and hydrophobic surfaces which correlates with T cell antigenicity (Galyov *et al.*, 1990).

A vital influence on the state of the quaternary structure and homogeneity of F1 is exerted by its method of preparation or isolation. Differences between recombinant and purified F1 may account for some antigenic differences due to different glycosylation states. In aqueous salt solutions (e.g. 0.2M NaCl in 0.1M potassium phosphate buffer,

pH 4.4-7.5), native F1 exists as associated species of protein subunits of molecular mass 12-16kDa (Vorontsov *et al.*, 1990). The F1 subunit monomer contains about 45 mol% hydrophobic amino acid residues, (20% acid and 8% basic). The mass of the F1 associated species can reach several megadaltons and the degree of polymerisation and purity depends on the method of isolation. A hypothetical model has been postulated by Vorontsov *et al.* (1990) for the macromolecular organisation of protein F1 in which associated intact protein is made up of tens (possibly hundreds) of dimers joined in a single plane *via* numerous lateral interdimer hydrogen bonds. Native F1 is a complex dynamic system consisting of a multiplicity of subunits, the quaternary structure of which is stabilised by hydrogen bonds and hydrophobic interactions. The electrostatic interactions between the subunits destabilise the F1 associated species at physiological pH. As the isoelectric point is approached, there is an increased shielding of the charges on the subunits and the rate of self-assembly of the protein increases.

I.m. immunisation of Balb/c mice with recombinant F1 purified from *E.coli.* induced high titres of F1-specific antibody and conferred protection against parenteral challenge with 10^5 virulent plague bacteria (Simpson *et al.*, 1990). F1 is immunogenic in humans when delivered by the s.c. route (Meyer *et al.*, 1974), with protection indices comparative to those obtained in mice. Single dose delivery of purified intra-gastric (i.g.) delivery of free F1 antigen by Thomas *et al.* (1992) failed to provide protection against subsequent challenge, nor were any detectable titres against F1 produced. The lack of response following oral immunisation may be caused by several reasons: the antigen did not survive proteolysis or the quantity absorbed through the oral mucosa was too low to stimulate antibody production. This was in contrast to i.p. delivered antigen which displayed significant protection. I.g. administration of multiple doses of F1 resulted in low geometric mean positive titres but gave no significant increase in resistance to *Y. pestis* and suggested an inverse relationship to the quantity of antigen delivered possibly due to oral tolerance. However i.g. dosing of a *Salmonella typhimurium* construct producing F1 antigen induced IgG serum titres similar to those resulting from i.v. dosing (Oyston *et al.*, 1995). Anti-F1 serum IgA titres, though approximately 16 fold lower than IgG titres, were detected following i.g. administration. The highest anti-F1 titres (both IgA and IgG) were induced following i.p. administration of F1 alone but protection against parenteral challenge was >90% for the *Salmonella* strain and only 75% for the purified F1. The presentation of F1 in a live-vaccine vector also stimulated a larger cell mediated immune response, determined by splenic cell proliferation assays, than older vaccine formulations and the free antigen.

A cell mediated response is required for any successful plague vaccine as the bacilli are able to survive in intracellular locations. Microencapsulation allows targeting of macrophages, prolongation of release and protection from harsh *in vivo* environments. The encapsulation of F1 and V antigens into microspheres suitable for delivery by mucosal routes is a very attractive goal and this chapter details the successful preparation and characterisation of such particles. Formulations were prepared co-encapsulating the cytokine IFN-γ along with the subunit vaccines. Initial formulations were prepared using HuIFN-γ, being available in relatively large quantities, while preparations intended for *in vivo* studies encapsulated a murine strain.

7.2 MATERIALS AND METHODS

Microspheres were prepared using double and single emulsion solvent evaporation methods outlined in section 2.1. The polymer used for all preparations was PLLA (Mw 2000) (section 3.2) and surfactant-free polystyrene particles for surface characterisation studies were obtained from Brookhaven Instruments Ltd. (Worcestershire, U.K.). All chemicals not stated in the text were from Sigma Chemical Co. (Poole, Dorset, U.K.) and Fisons (Loughborough, Leics., U.K.) and were of Analar grade or equivalent. All the preparations were analysed (size, loading, surface characteristics, release and antigen stability) using methods detailed in chapters 2 and 3.

The *Y. pestis* sub-unit antigens were prepared by Dr E.D. Williamson at the Chemical and Biological Defence Establishment, Porton Down, Salisbury, U.K. and were supplied in 10mM phosphate buffer and stored at -20°C. The antigens were freeze-dried in the buffer and then stored below 5°C in a desiccator. HuIFN-γ was a gift from Dr G.R. Adolf (Bender+ Co. GesmbH, Wien, Austria) and was supplied in a buffered solution containing 10mg/ml IFN-γ and an unspecified quantity of other stabilising proteins. MuIFN-γ was purchased from Sigma (as above) and was diluted in PBS prior to use as detailed in the product information. (3-[^{125}I]iodotyrosyl)gamma-interferon (I-IFN-γ) (specific activity ~25TBq/mmol at reference date) was purchased from Amersham Life Science (Bucks., U.K.) and was prepared by iodination of recombinant HuIFN-γ using sodium[^{125}I]iodide and chloramine T. As stability of the product is concentration dependant, it was reconstituted in the minimum volume of water, dispensed into aliquots and stored at -20°C as detailed in the product specifications. The product also contained BSA and other stabilising agents.

The microspheres, containing antigen and IFN-γ, were prepared using solvent evaporation techniques as described in sections 2.1.1 and 2.1.2. Prior to encapsulation

of the proteins, they were combined in the desired ratios, from trace amounts of IFN (1:160) to 1:1 ratios and the water removed by solvent evaporation (DNA SpeedVac) if necessary. A predetermined quantity of I-IFN-γ was added to the mixture when desired for quantification and ^{125}I analysis was determined using a LKB WALLAC (Stockholm) 1282 Compugamma universal gamma counter. For determination of total protein, a BCA assay method was used (see section 2.7) using a mixture of the proteins for calibration.

Release studies were determined in 20mM phosphate buffer (pH 7.5) (see section 2.3.1) and the samples analysed for percentage of total protein released using a BCA method calibrated with a mixture of the proteins used. For ^{125}I quantification, a 100-200µl aliquot of the supernatant was removed and radioactivity counted as above. Fresh buffer was used to replaced the total sample volume removed.

All *in vivo* studies using F1 and V antigens were carried out by Dr E.D. Williamson (as above). Adult female Balb/c mice were used for all studies at 6-8 weeks of age and all animals received 25µg of F1 (or an equivalent microsphere dose) as a single dose on day 1 and 6 animals were sacrificed at each time point. The immune response to microencapsulated F1 antigen (in single and double emulsion formulations), F1 with the adjuvant IFN-γ and also with alhydrogel was examined. Blood samples, removed by cardiac puncture, at each time point (days 21, 35, 56 and 87) were pooled. Immune responses to F1 antigen were determined using an ELISA technique. Briefly, microtitre plates were coated with F1 antigen in PBS and the test sera were serially diluted in duplicate on the plate. Bound antibody was detected with peroxidase conjugates against mouse polyvalent Ig (Sigma), mouse IgG_1 (Sera-Lab, Sussex, U.K.) and mouse IgG_{2a} (Sera-Lab, Sussex, U.K.), each used at a dilution of 1:2000 (Titertek Multiskan plate reader). Titre was estimated as the maximum dilution of serum giving an absorbance 0.1 units above background and is expressed as (optical density at the end-point dilution minus background optical density) x the end-point dilution. The effect of ~80U of co-encapsulated murine IFN-γ on the response pattern was also examined.

On day 60 of the schedule, 5 animals were randomly selected from each group and challenged with s.c. injection with a virulent strain of *Y. pestis*. GB strain (mouse Ld_{50}<1cfu) was given to the animals at three challenge levels, 10^7, 10^5 and 10^3 cfu. Challenged mice were observed over 14 days and results are expressed as percentage survival 14 days after challenge.

Table 7.1. Details of the immunisation groups used for i.p. immunisation (carried out at CBDE)

Group	Preparation
1	F1 s/c
2	F1 d/c
3	F1 s/c (+IFN-γ)
4	F1 d/c (+IFN-γ)
5	F1 + alhydrogel
6	control

A modified ELISA method was also used to examine secretion of specific antibodies from spleen cells. The spleens were removed from six animals within each group, amalgamated and placed in ice-cold DMEM (Dulbecco's Modification of Eagle's Medium) to form crude cell suspensions. Microtitre plates were coated overnight at 4°C with either soluble F1 (5µg/ml) or a Salmonella lysate expressing F1 antigen in PBS. The plates were blocked with DMEM plus 10% v/v fetal calf serum for one hour at 37°C. 200µl of the cell suspension was double diluted on the plate and incubated for 20 hours (37°C, 5% CO_2). The plates were washed three times in PBS, followed by 2 further three washes in PBST (see section 4.2.2). The plates were soaked in PBST for 5 minutes prior to addition of the peroxidase-labelled secondary conjugate. The plates were incubated at 4°C for 20 hours, washed four times in PBST and ABTS added for colour development. Absorbance was read using a Titretek Multiskan plate reader.

7.3 RESULTS AND DISCUSSION

7.3.1 Characterisation of microspheres containing *Y. pestis* antigens

7.3.1.1 Characterisation of microspheres containing V Antigen

Microspheres loaded with V antigen were produced with high (H) and low (L) protein loadings (table 7.2). At low loadings (2% w/w theoretical loading), the loading efficiencies for single and double emulsion techniques are different, with the single emulsion method being more efficient. This situation is reversed when the theoretical load is increased to 10% w/w, with the double emulsion technique being more efficient. However, the encapsulation of this antigen at these levels leads to the production of

relatively large microspheres (mean diameter >2μm) possibly due to the pattern of protein distribution within the microsphere matrix.

Table 7.2. Loading efficiencies and particle sizes for PLLA microspheres encapsulating V antigen

Formulation	Loading (% w/w)	Encapsulation efficiency (%)	Particle size (μm±σg)
V s/e	2.04 (L)	102.0	0.82±0.65
V d/e	1.31 (L)	65.5	0.82±0.67
V s/e	6.31 (H)	63.1	2.84±1.94
V d/e	8.20 (H)	82.0	2.67±1.34

At low loadings (2% w/w) or lower, the microsphere production methods examined had little effect on the release profiles obtained, with ~20% of the protein released in 400 hours. This may be due to the almost identical particle size distributions obtained (table 7.2). As the loading is increased to 8.2% w/w for particles formed using a double emulsion technique, the percentage released is increased with almost 60% of the antigen released in 400 hours (figure 7.1). For particles produced using a single emulsion technique, the increase in release is not as dramatic, with ~40% of the antigen released in the same time period (figure 7.2).

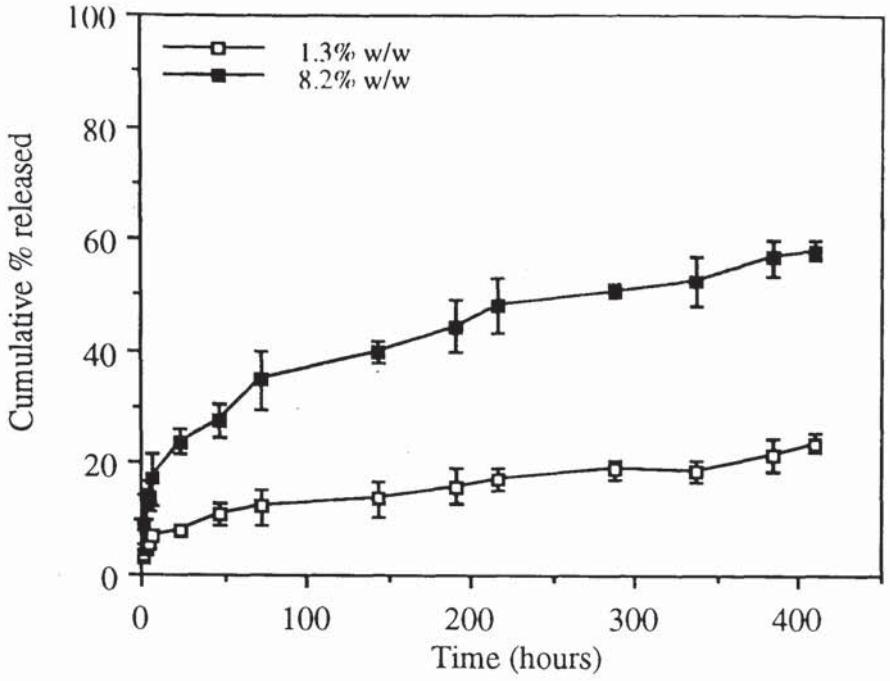


Figure 7.1. Release profiles of V antigen from high and low loaded PLLA microspheres formed by a double emulsion method

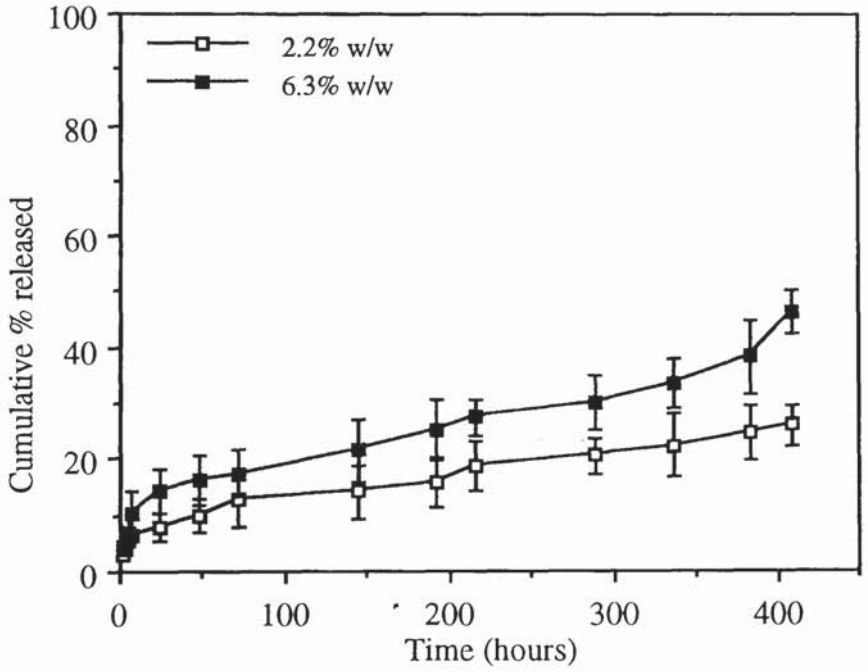


Figure 7.2. Release profiles of V antigen from high and low loaded PLLA microspheres formed by a single emulsion method

7.3.1.2 Characterisation of microspheres containing F1 antigen

PLLA microspheres containing only F1 antigen were more difficult to prepare with high loadings. At 10% w/w theoretical loads, loading efficiency was in the region of 40% (resulting in ~4% w/w loads) whether single or double emulsion techniques were employed (table 7.3). When radiolabelled HuIFN-γ was included in the formulation (0.06% w/w), the loading efficiency of the F1 antigen was increased to 60% and the cytokine was loaded at 34% efficiency (as determined by radio-labelling) . These results were used to predict the loading efficiencies of muIFN-γ combinations with F1 and V antigens and the results are shown in table 7.3.

Table 7.3. The effect of co-encapsulation of IFN-γ on the loading of PLLA microspheres containing *Y. pestis* subunit vaccines

Formulation	Loading (%w/w)	Particle size (μm±σg)
F1 s/e	4.43	1.67±0.47
F1 d/e	3.48	1.67±0.51
F1/muIFN s/e	8.82	2.84±1.86
F1/muIFN d/e	7.73	1.29±1.14
V s/e	6.31	2.84±1.94
V d/e	8.20	2.67±1.34
V/muIFN s/e	9.08	0.71±0.33
V/muIFN d/e	10.43	1.59±1.12

Release profiles of F1 antigen from PLLA spheres formed using single or double emulsion techniques show little difference, with 70% of the protein being released in 250 hours (figure 7.3). The overall rate of release is faster than that demonstrated with V loaded PLLA particles. The inclusion of IFN-γ in the formulations, both at trace levels (0.03% w/w) or at higher levels (2% w/w), has a notable effect on the release profiles obtained (figure 7.4). The presence of the cytokine appears to reduce the burst effect and the overall amount of protein released from the system. As the amount of IFN-γ is increased, the release of total protein from the microspheres is reduced. The release of IFN-γ itself, measured using radiolabelled drug, was greater than the total

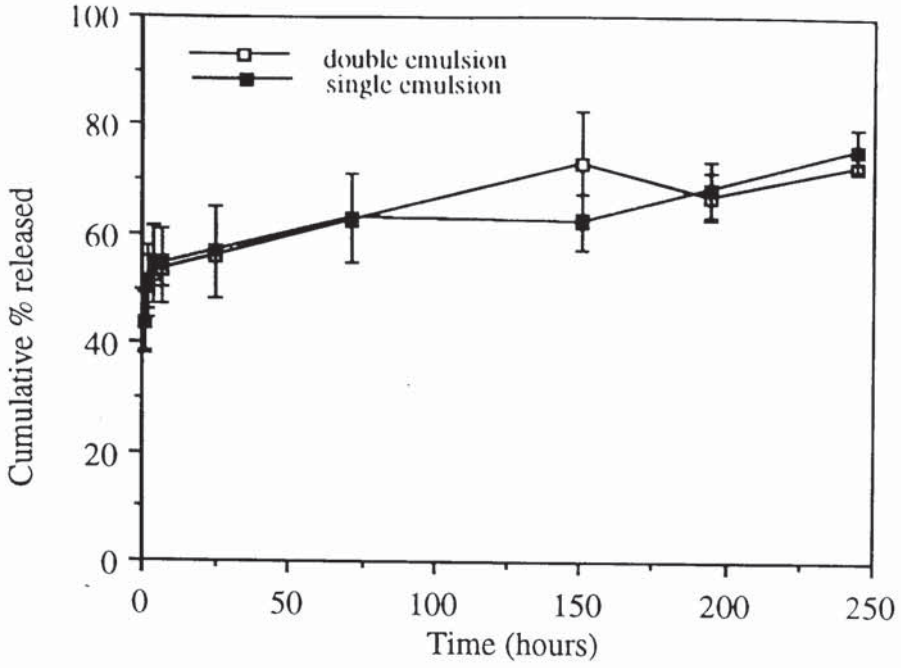


Figure 7.3. Release profiles of F1 antigen from PLLA microspheres formed by single and double emulsion methods (n=3; mean±s.d.)

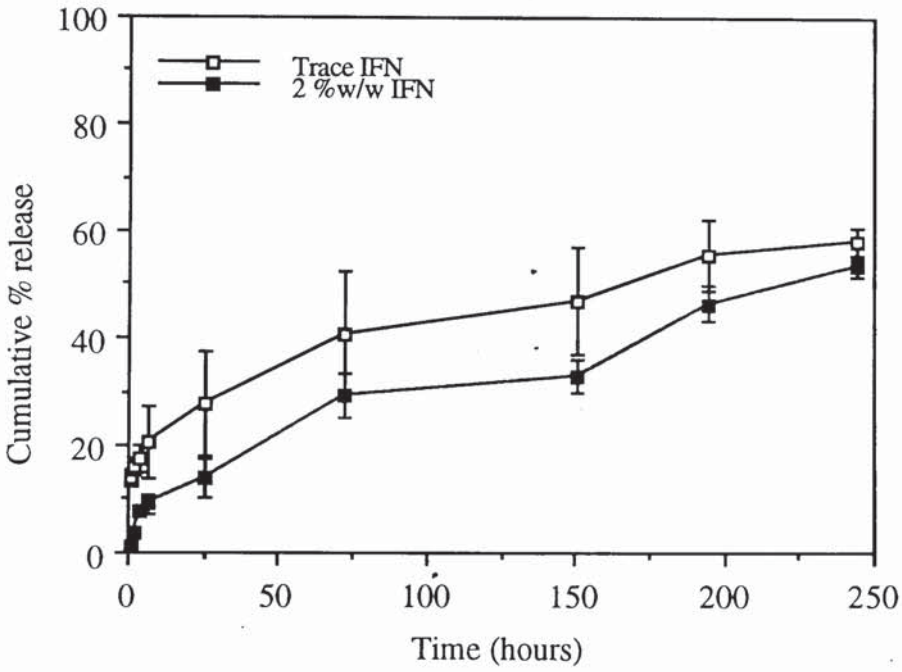


Figure 7.4. Effect of the amount of IFN-γ co-encapsulated on the release of total protein (determined by BCA assay) from PLLA microspheres formed by a double emulsion method and containing F1 antigen and IFN-γ (n=3; mean±s.d.)

protein in combined preparations (no figure shown). For a trace loaded formulation, the burst effect was low, less than 2%, but up to 80% of the IFN-γ was released in 250 hours. When the loading of IFN-γ was increased, the burst effect remained similar but the overall amount of cytokine released from the formulation was decreased (~60% in 250 hours). This illustrates the importance of examining the presence of even a trace amount of a second protein on the overall loading and release for each formulation.

7.3.2 Surface characteristics of microspheres containing *Y. pestis* antigens

The effect of encapsulation of different proteins on microsphere surface hydrophobicity has previously been reported (Alpar & Almeida, 1994) but for certain proteins, i.e. TT, was not found to alter the surface characteristics. The hydrophobic nature of the surface of PLLA microspheres containing F1 antigen is illustrated in table 7.4 which shows the percentage of the microsphere suspension eluted from a series of stationary phases. Through all columns, almost 80% of the particles remain irreversibly bound to the hydrophobic packings. The encapsulation of F1 antigen into PLLA microspheres formed by solvent evaporation techniques, produces particles with very different surface hydrophobicities to empty or BSA loaded particles (see section 3.3.3). The percentages retained are comparable with those of the hydrophobic latex control (best illustrated on pentyl- and hexyl-agarose columns) and it is anticipated that these relatively hydrophobic particles will result in an enhanced absorption after oral delivery (Eldridge *et al.*, 1990) and, due to more efficient interactions with antigen presenting cells, improved mucosal immune responses (Van Oss, 1978; Kreuter *et al.*, 1988).

The inclusion of IFN-γ, even in trace amounts, reduces the hydrophobicity of the PLLA particle surfaces (table 7.4). During the microencapsulation procedure, protein molecules may be deposited at or near the surface of the particles influencing the physicochemical properties of the carriers. The pattern of deposition within the particles is altered depending on the composition of the protein or protein mixture to be encapsulated. This pattern of deposition affects not only the release of the microsphere contents but also its surface characteristics and it is important to fully characterise the microspheres formed so subsequent immune responses can be fully understood. The relative hydrophobicity of F1 may lead to the deposition of the antigen at the surface of the particles in such a way that the hydrophobic portions (about 45 mol%, Vorontsov *et al.*, 1990) are oriented towards the surface of the microsphere. The presence of another protein, in this case IFN-γ, may alter this pattern of deposition leading to a reduction in the particle surface hydrophobicity.

Table 7.4. Final percentage for particles loaded with F1 and V antigens (1μm) eluted *per* type of agarose (n=3; mean % eluted ± s.d.)

Formulation	Stationary Phase			
	Agarose	Propyl-	Pentyl-	Hexyl-
1.3% w/w V antigen	71.7±7.3	84.3±6.9	71.4±4.6	75.7±5.9
8.2% w/w V antigen	72.6±6.9	48.5±4.2	58.2±2.9	48.1±2.3
10.4% w/w V antigen +IFN-γ	97.3±3.6	86.9±1.6	77.0±5.8	87.2±6.5
4.4% w/w F1 antigen	20.5±11.3	25.3±4.1	20.8±1.4	20.7±2.2
8.8% w/w F1 antigen + +IFN-γ*	48.2±3.6	47.3±2.4	46.2±4.6	39.4±9.8
7.7% w/w F1 antigen + +IFN-γ	52.4±5.7	43.8±9.3	44.8±3.4	40.3±4.9
Latex control	73.4±2.4	51.0±5.8	12.3±2.1	11.0±0.9

All particles formed by double emulsion techniques except * formed by single emulsion method

Encapsulation of V antigen under similar conditions does not produce particles with such hydrophobic surfaces, producing particles that demonstrate an elution pattern more similar to those of empty or BSA loaded PLLA particles being gradually more retained as the hydrophobicity of the stationary phases is increased (table 7.4). The increase in loading (8.2 vs. 1.3 %w/w) of V antigen results in significantly more hydrophobic particles probably due to more protein being present at the microsphere surface. The addition of a trace amount of IFN-γ in the formulation, again, greatly reduces the hydrophobicity of the microspheres, similar to results with F1 antigen. Thus the inclusion of even trace amounts of the cytokine may apparently greatly alter the pattern of protein deposition within the microspheres.

7.3.3 Stability of F1 and V antigens

The stability of the F1 and V antigens was investigated using SDS-PAGE. F1 antigen exists in the form of associated species of protein subunits (Bennet & Tornabene, 1974), the gel pattern shows a number of subunits between 12-16kDa. The pattern of migration for both subunit antigens appeared unaltered following encapsulation into and release from microspheres (figures 7.5 and 7.6). Figure 7.5 shows the large number of bands produced due to association of F1 monomers but all the bands occur at the same positions prior to and following encapsulation of the protein. Due to the loading levels obtained using these subunit antigens, detection of the released protein in the

supernatant was not always possible so studies on protein stability were also carried out as described in section 5.2, following homogenisation on DCM. The complexity of the pattern produced for gels of F1 antigen made any interpretation of these studies difficult, however the effect of exposure to DCM could be studied for V antigen, which produces only two bands. After 3 minutes, one hour and even after 24 hours incubation with the organic solvent, the migration pattern for V antigen remained unchanged (figure 7.7), with only two bands visible. Also, sonication of the protein in the solvent did not produce any detectable changes and it was concluded that even under much harsher conditions than those used during microsphere preparation, the structure of the protein remained unchanged.

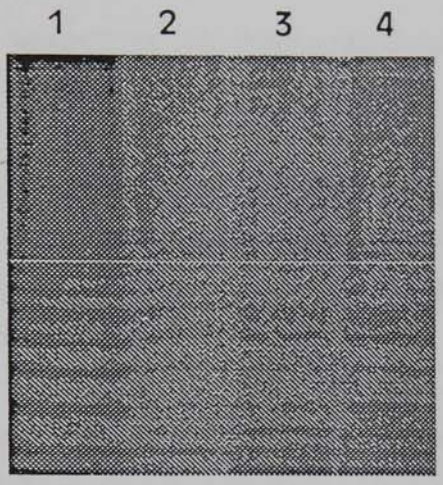


Figure 7.5. SDS-PAGE (7.5% gel) of F1 antigen before and after release from PLLA microspheres. Lanes (1) F1 before encapsulation into microspheres; (2) F1 following release from PLLA s/e microspheres; (3) and (4) F1 following release from PLLA d/e microspheres

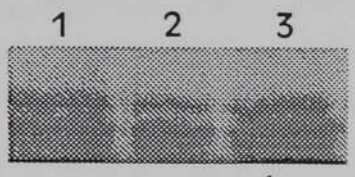


Figure 7.6. SDS-PAGE (7.5% gel) of V antigen before and after release from PLLA microspheres. Lanes (1) V before encapsulation into microspheres; (2) V following release from PLLA s/e microspheres; (3) V following release from PLLA d/e microspheres

Figure 7.7. SDS-PAGE (7.5% gel) of V antigen following homogenisation with DCM. Lanes (1) V before DCM treatment; (2) V following 3 minutes homogenisation in DCM; V in aqueous solution following 3 minutes homogenisation with DCM; (3) V following 3 minutes homogenisation with DCM and 24 hours incubation in DCM; (4) V following 3 minutes sonication in DCM

7.3.4 Immune responses to microencapsulated F1 antigen with or without interferon-gamma

The measurement of isotype and titre of the antibody response was carried out using an ELISA method using peroxidase conjugates against mouse polyvalent Ig, mouse IgG_1, IgG_{2a} and IgA (see section 7.2). All results in this section are expressed as relative titres calculated as in section 7.2.

All groups showed a maximal polyvalent Ig expression at the first time point (day 21) (figure 7.8). While there was little difference between the initial responses to single emulsion spheres compared with double emulsion spheres, co-encapsulation of IFN-γ with F1 enhanced the initial response to the single emulsion formulation relative to that of double emulsion microspheres. An enhanced response to co-encapsulated IFN-γ was particularly noticeable at the latest time-point (day 87) where the presence of IFN-γ prolonged the immune response for double emulsion microspheres (figure 7.8). The relative IgG_1 and IgG_{2a} titres were then studied at each of the time points. The vaccine formulation and delivery route used influence the activation of Th subsets (Brett *et al.*, 1990). A predominant IgG_1 response, indicating a Th2 response was produced for all the groups (figure 7.9) (see section 1.4.3), with the double emulsion (with or without the presence of IFN-γ) showing no IgG_{2a} response at day 87 (figure 7.10). Thus, the presence of IFN-γ did not switch the response to predominately Th1 (Heath, 1995). It may require co-dosing with other cytokines or anti-cytokines, such as anti-IL4 and IL12, to achieve this. Further studies are required in order to determine the effects of formulation on the immune response generated.

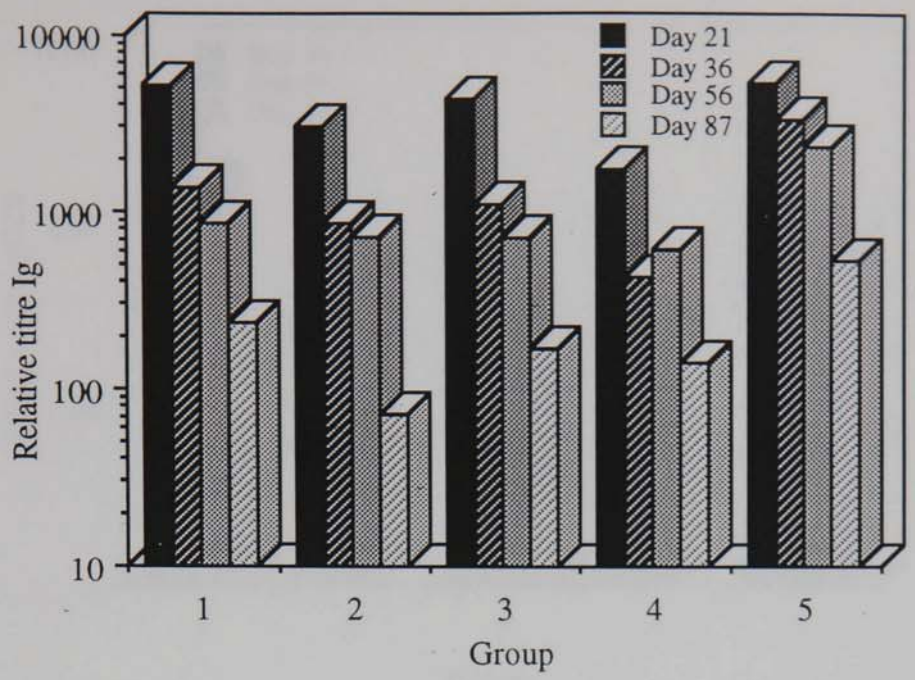


Figure 7.8. Total F1-specific Ig in serum following single dose i.p. immunisation with 25µg F1 in various formulations (n=6 *per* group, samples pooled)

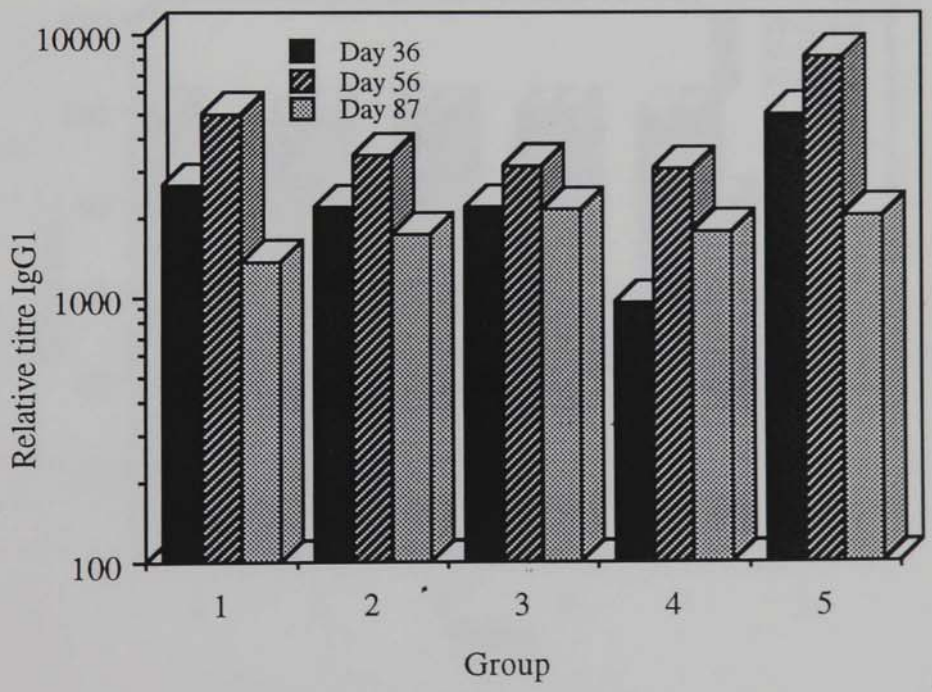


Figure 7.9. F1-specific serum IgG_1 subclass response following single dose i.p. immunisation with 25µg F1 in various formulations (n=6 *per* group, samples pooled)

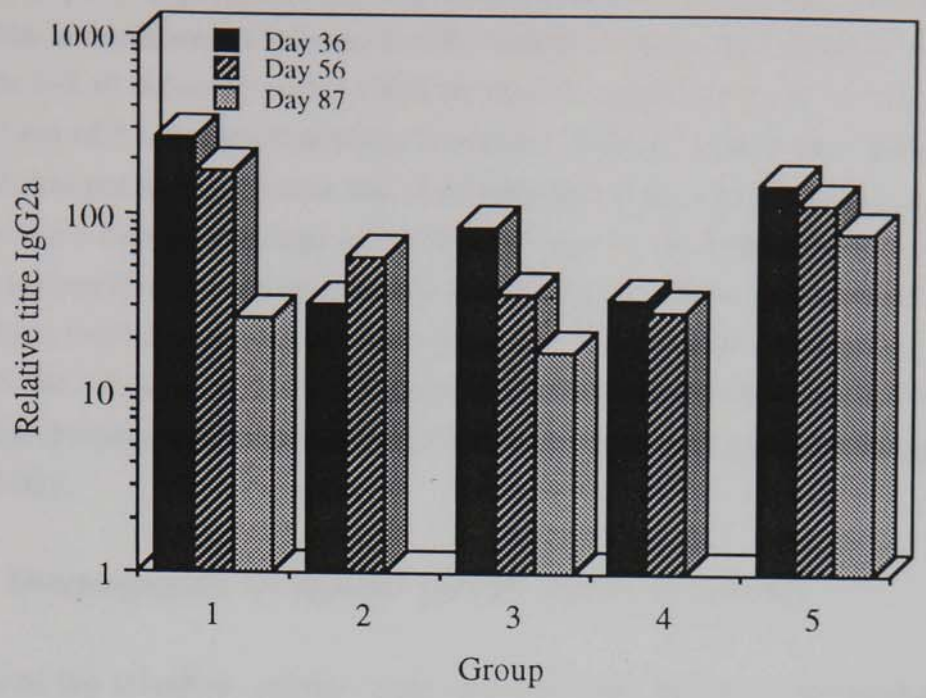


Figure 7.10. F1-specific serum IgG_{2a} subclass response following single dose i.p. immunisation with 25µg F1 in various formulations (n=6 *per* group, samples pooled)

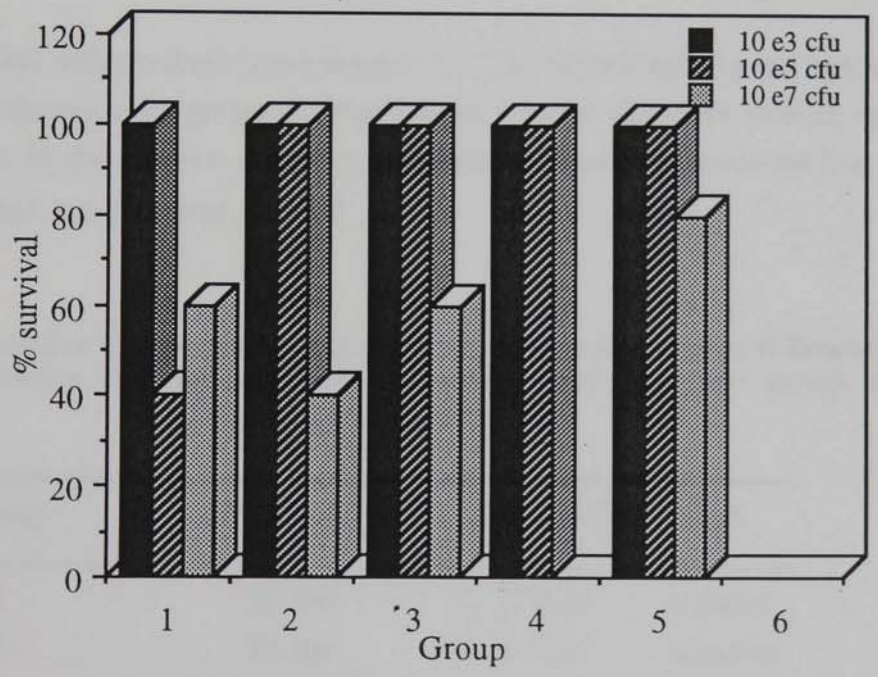


Figure 7.11. Percentage survival following i.p. immunisation with various F1 formulations and subsequent challenge with *Y. pestis* (n=5)

In order to compare the protective efficacy of immunisation with the microencapsulated formulations, animals were challenged with virulent *Y. pestis* as described in section 7.2. Groups 2-5 all demonstrated a 100% protection against up to $1x10^5$ cfu (figure 7.11). Two out of 5 immunised animals in group 1, who received F1 encapsulated in single emulsion particles, survived this challenge dose, but 3 out of 5 animals in this group survived a higher challenge of $1x10^7$ cfu. None of the groups displayed 100% protection against this high challenge level and the highest survival percentage was in group 5, those receiving F1 and alhydrogel. The protective effect of group 4, F1 plus IFN-γ in double emulsion particles was completely defeated at this challenge level and, again, further investigations are required to fully explore the effect of formulation on the survival ability.

7.3.5 Determination of specific splenic antibody activity

On day 56 of the schedule, spleens were removed from groups of six animals and assayed from anti-mouse polyvalent immunoglobulin (Sigma Chemical Co., Dorset, U.K.) and anti-mouse IgA (Sera-Lab, Sussex, U.K.) as detailed in section 7.2. The results are expressed as relative titres, i.e. (optical density at end minus point-background)x end-point dilution.

The spleen titres measured are given in table 7.5. The highest total Ig and IgA antibody titres were expressed by group 4, dosed with F1 and IFN-γ in double emulsion microspheres. In the absence of IFN-γ, microencapsulated F1 produced low total Ig titres and no IgA antibody was detected.

Table 7.5. Relative F1-specific spleen antibody titres after 56 days following single i.p. administration of 25μg of various F1 formulations (n=6 *per* group, samples pooled)

Group	Preparation	Total Ig	IgA
1	F1 s/e	0.18	negative
2	F1 d/e	0.22	negative
3	F1 s/e (+IFN-γ)	0.21	0.10
4	F1 d/e (+IFN-γ)	0.63	0.14
5	F1 + alhydrogel	0.45	0.07

Protection did not directly correlate with these results (figure 7.11) but it has been shown that direct administration of IFN-γ promotes the clearance of *Y. pestis* from the spleens of infected mice (Nakajima & Brubaker, 1993). The presence of IFN-γ in the co-encapsulated preparation may contibute to this clearance. Further studies are necessary to examine the behaviour of splenic T cells on re-exposure to F1 antigen and the effect of formulation on splenic antibody activity.

7.4 SUMMARY

F1 and V subunit antigens have been successfully encapsulated into PLA microspheres and the particles have been characterised by *in vitro* methods. The development of a microencapsulated product of the desired specifications may facilitate stimulation of a mucosal immune response following delivery to the mucosal-associated lymphoid tissue. The production of micron-sized, hydrophobic particles using PLLA polymer may enhance the immune responsiveness of the subunit vaccines and all preparations used during this study provided protection against subsequent challenges. As *Y. pestis* has been shown to suppress the production of IFN-γ in infected mice (Nakajima & Brubaker, 1993) it was prepared, with F1 antigen, in microencapsulated formulations which were delivered i.p. to mice. The adjuvant effect of microencapsulated F1 was similar to that of F1 and alhydrogel but no consistent differences between formulations were determined *in vivo*. Also, no differential effects in enhancing Th1 type immunity due to the presence of IFN-γ were detected. This may be affected by the timing and size of the dose administered or by the delivery route selected.

8.0 MICROENCAPSULATION USING SPRAY DRYING TECHNIQUES

8.1 INTRODUCTION

The application of spray drying, a technique widely used in the pharmaceutical industry, has recently been used for the preparation of microparticulate delivery systems (Conte *et al.*, 1994; Park *et al.*, 1994; Wang *et al.*, 1990). It is a single step procedure used to convert a liquid into a powder. Spray drying microencapsulation techniques generally aerosolise a solution of the drug and the polymer but for the encapsulation of proteins, a suspension of the polymer and antigen may be used to create microdroplets that solidify in a brief blast of heated air. Knowledge of the suspension rheology and nozzle design is used to control the microparticle size and shape. The drug in aqueous solution, or as solid particles, is dispersed in a solution of polymer in solvent. The mixture is pumped through the atomiser of the spray drier into the drying chamber where a heated gas dries the particles as they are carried to the jet separator for collection (figure 8.1). Typical process variables are polymer concentration, drug concentration, solution volumes, atomiser characteristics, fluid pumping rate, inlet and outlet gas temperatures and gas flow rate. Spray drying has the advantage of producing microspheres with lower levels of residual solvents, but they tend to be limited in size and are often more porous, due to the immediate evaporation of solvent, possibly reducing the capability for delayed delivery (Aguado & Lambert, 1992). Also, yields are often low using this method due to adherence of the powder to the cyclone walls (Pavanetto *et al.*, 1991).However, the production process is comparatively rapid and the ease of scale-up for the production of large batches make it an attractive alternative method of microsphere manufacture.

The applicability of the procedure to microsphere production was demonstrated by Bodmeier & Chen (1988) who prepared PDLA microspheres using spray drying procedures. A suspension of theophylline and a solution of progesterone in DCM and polymer were successfully spray dried proving the suitability of the method for both

water soluble and insoluble drugs. However, the encapsulation of proteins using spray drying techniques has only been investigated in more recent years. BSA was encapsulated into poly(ε-caprolactone) with high efficiency (~94%) and without significantly altering its structural integrity (Benoit *et al.*, 1995). Gander *et al.* (1995), in a series of studies using different solvents in the production of microspheres of PDLA, found that dichloromethane, ethyl acetate (EthAc) and nitromethane produced the highest quality microspheres.

Using the spray drying technique, microspheres loaded with BSA and β-Lg were produced using a number of methods, solvents and polymers as outlined in section 8.2

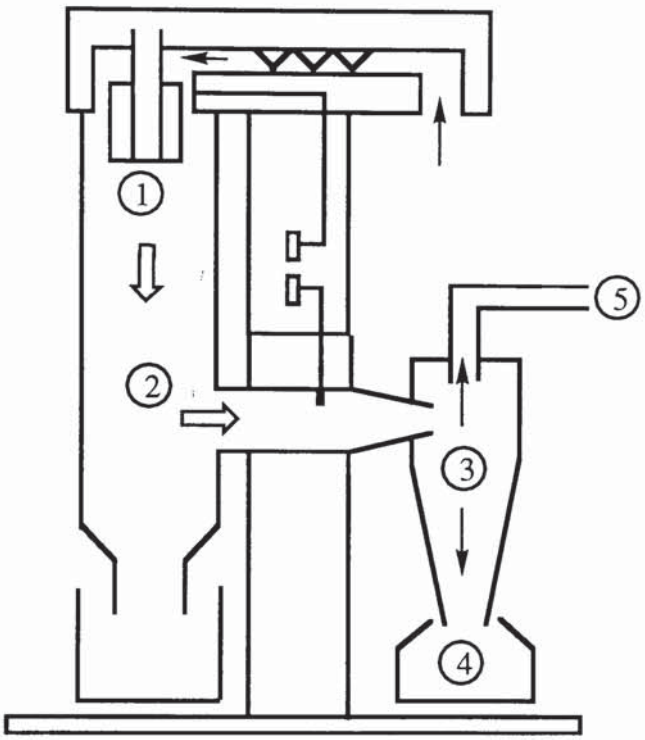


Figure 8.1. Schematic representation of Mini Buchi spray dryer apparatus (adapted from Conte *et al.*, 1994). (1) nozzle; (2) spray chamber; (3) cyclone; (4) collector; (5) aspirator

8.2 MATERIALS AND METHODS

The polymers used for formulation of microspheres using spray drying were PLLA (Mwt 2000), PDLA (Mw 2000) and PLGA 50:50 (Mw 3000) as detailed in section 3.2. Bovine serum albumin (BSA) and beta-lactoglobulin (β-Lg) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). HPLC grade dichloromethane and ethyl

acetate were supplied by Carlo Erba (Farmitalia-Carlo Erba srl, Milan, Italy). Lutrol F68 (Poloxamer 188, BASF, Cumun Nuovo, Italy) for preparation of emulsions. All other materials used in these experiments are as detailed in section 3.2.

Two methods were used for production of protein loaded, spray dried microspheres:
(a) polymer coating of pre-prepared BSA microspheres
(b) spray drying of an emulsion of drug and polymer

(a) polymer coating of pre-prepared BSA microspheres;
BSA microspheres were prepared using 200ml of a 1% w/v aqueous solution of BSA. The spray-drying was carried out using a Buchi 190-Mini Spray Drier (Buchi Laboratoriums-Technik AG, Ch9230, Flawil, Switzerland) and the operating conditions were as follows; nozzle diameter, 0.70mm; inlet temperature, 164°C; outlet temperature, 84°C; air flow rate, 700/800 L/hour; heating temperature, 10°C/min.; aspiration, -20mbar; pressure, 4atm.; pump speed set, 3ml/min. Spray flow rate was determined to be 3.6ml/min. Free flowing powder was collected and was stored under vacuum.

PLA microspheres were prepared by dissolving 1g of the polymer in 100ml of dichloromethane (or 200ml of ethyl acetate). 100mg of the BSA microspheres were suspended in this organic solution and the polymer microspheres were spray dried under the following conditions; nozzle diameter, 0.70mm; inlet temperature, 48°C; outlet temperature, 32°C; air flow rate, 700/800 L/hour; heating temperature, 1°C/min.; aspiration, -5mbar; pressure, 3atm.; pump speed set, 3ml/min. Spray flow rate was determined to be 2.2ml/min and the suspension was stirred throughout the spray drying process. The solid microspheres precipitated into the bottom collector were harvested and stored under vacuum until analysed. A higher inlet temperature was used for ethyl acetate, 86-89°C (outlet temperature 56-60°C).

(b) spray drying of an emulsion of drug and polymer;
A w/o emulsion was prepared using 200ml of solvent DCM (or EthAc) containing 2g polymer (1g for ethyl acetate) as the continuous phase. The dispersed phase was 20ml of a 0.1% w/v aqueous solution of Lutrol F68. This aqueous phase also contained 200mg of protein. The w/o emulsion was formed by homogenisation, in an ice bath, using Ultra-turrax at ~7,000rpm for 10 minutes. The emulsion was stirred continually throughout the spray-drying procedure. Approximate conditions were as follows: nozzle diameter, 0.70mm; inlet temperature, 87-88°C; outlet temperature, 50°C; air flow

rate, 550 L/hour; heating temperature, 4°C/min.; aspiration, -15mbar; pressure, 3atm.: pump speed set, 4. The rate of preparation was determined to be 3.3ml/min. A higher inlet temperature was used for ethyl acetate, 86-89°C (outlet temperature 56-60°C). The solid microspheres precipitated into the bottom collector were harvested and stored under vacuum until analysed.

All the methods used for analysis of microspheres in this chapter are as detailed in chapter 2.

8.3 RESULTS AND DISCUSSION

8.3.1 Characterisation of microspheres formed by spray drying methods

Microsphere preparation using spray drying was carried out in the Facolta' di Farmacia, Universita' Degli Studi di Pavia, Italy using equipment supplied by Prof. U. Conte. Due to limited time and technical difficulties, only a small number of microsphere samples were prepared (table 8.1). Methods (a) and (b) for microsphere production are as detailed in section 8.2.

Table 8.1. Particle size, encapsulation efficiency and yields for 10% w/w theoretical loaded microspheres produced using spray drying methods (a) and (b)

Batch	Polymer	Protein	Solvent	Size (μm±σg)	Encapsulation efficiency (%)	Yield efficiency (%)
1	PLLA	BSA	DCM (a)	2.31±0.98	388.3	8.0
2	PLLA	BSA	DCM (b)	2.36±0.86	205.0	13.6
3	PLLA	BSA	EthAc (a)	2.54±1.17	427.4	14.2
4	PLGA	BSA	DCM (a)	2.22±0.94	353.4	4.3
5	PDLA	BSA	DCM (a)	4.55±2.00	316.5	9.0
6	PLLA	β-Lg	DCM (b)	2.64±1.22	110.7	15.2
7	PLGA	BSA	DCM (b)	2.87±1.49	143.9	12.0
8	PLLA	BSA	EthAc (b)	2.42±1.07	14.6	2.5

SEM of the protein loaded microspheres showed that spray drying of BSA produced particles of 1-5μm with irregular surfaces (figure 8.2). Due to the water solubility of the

Figure 8.2. Scanning electron micrograph of BSA microspheres prepared by spray drying

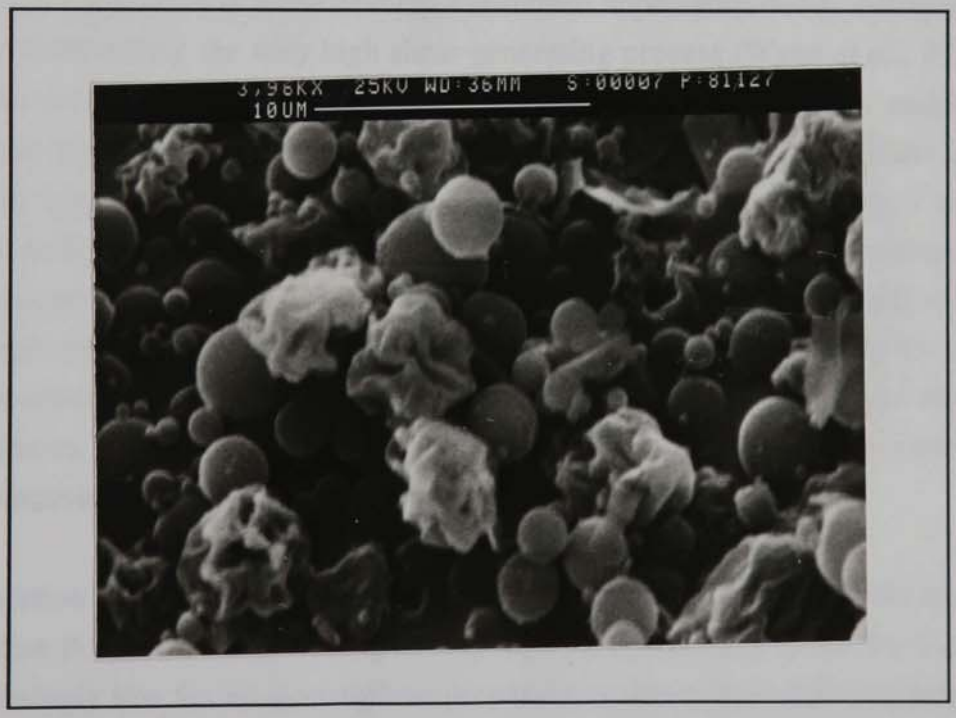


Figure 8.3. Scanning electron micrograph of PLLA microspheres prepared by spray drying method (a) (batch 1)

BSA microspheres, particle sizing using the Malvern MasterSizer/E was not possible under the standard conditions employed.

Microsphere size is dependant on the polymer molecular weight and the influence of spray conditions (Rafler & Jobmann, 1994). The viscosity of the polymer solution, the solvent evaporation temperature and the solubility of the polymer and active agent are the main factors controlling particle formation. Due to the limited number of experiments schedules, manufacturing conditions were maintained throughout the experiment where possible. Theoretical drug loading was 10% w/w for all preparations and the polymer concentration was 1% w/v for all DCM solutions and 0.5% w/v for all EthAc solutions, due to a decreased solubility of the polymers in the non-chlorinated solvent. Mean microsphere size is ~2μm for all batches except 5, where the mean size is increased to 4μm due to the presence of aggregates. If spray drying is carried out at temperatures above the glass transition of the polymer, aggregates are produced, with low yields, due to the rubbery nature of the polymer (Park *et al.*, 1994). It is possible that, as the temperature used for microsphere production is close to the Tg of the low molecular weight PDLA (48°C) (see section 3.3.7), that the conditions employed led to formation of aggregates.

Spray dried particles may often develop a shrunken appearance due to collapse of the polymer walls during the very high shear generating process (Wang *et al.*, 1990) but this does not appear to be a problem with microsphere production under these conditions, even with the low molecular weight polymers involved in these studies. However, spray drying of a suspension of BSA particles in the organic polymer solution, under the conditions employed does not allow sufficient coverage of the preformed protein particles. Figure 8.3 is an SEM micrograph of preparation 2 (table 8.1). Large, irregular shaped particles appear to be spray dried BSA while the smooth, spherical spheres are PLLA polymer. The percentage of BSA encapsulated within the microspheres is likely to be low, the protection afforded to the protein by microencapsulation, minimal and the release profile is rapid.

Encapsulation efficiencies of >100% suggest that polymer loss from the system is greater than that of the drug, leading to very high protein loading (>100%). Yields are disappointingly low for all microsphere preparations under the conditions employed. Drug will be located at or near the surface of the microparticles, leading to large burst effects and rapid release rates. Microspheres produced using this method often have imperfect and porous coating of PLA around the protein due to different interfacial energies between the protein and PLA (Wang *et al.*, 1990). Polymer molecular weight

and concentration in the organic phase crucial factors influencing microsphere production by this method. The viscosity of the polymer solution will be low for the low molecular weight polymers used in this study and a higher concentration may be required for optimal microsphere production. Successful production of small PLA microspheres encapsulating lipophilic drugs has been reported (Pavanetto *et al.*, 1993). Particle size, as with other methods, increases with increasing polymer molecular weight and it is anticipated that reducing aspiration would reduce loss of very small microspheres, formed using low molecular weight polymers, from the apparatus, improving the yield to acceptable levels. Therefore, for this method of microsphere production, extensive investigations of process parameters are necessary to reduce loss of polymer and improve encapsulation of the drug.

Generally chloroform or DCM are applied as solvents for microsphere production. In order to minimise toxicity aspects involved with the retention of a small amount of residual solvent, DCM was substituted with EthAc and the effect on microsphere production examined. The microspheres produced (batch 3, table 8.1), like DCM under similar conditions, consist of large, irregular shaped spray dried BSA particles and smooth, spherical PLLA spheres.

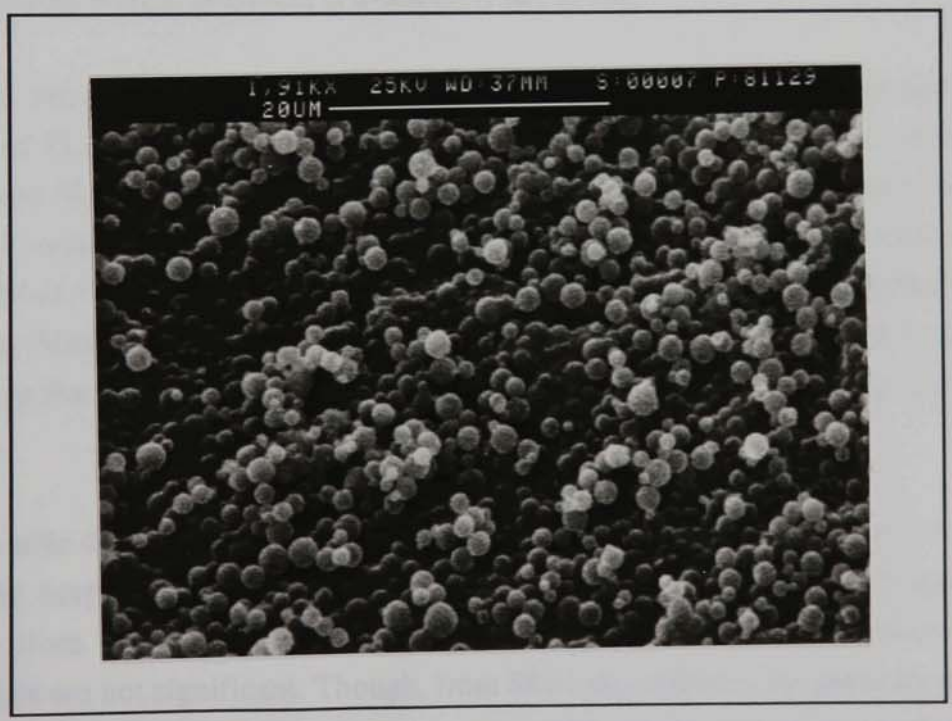


Figure 8.4. Scanning electron micrograph of PLLA microspheres prepared by spray drying method (b) (batch 2)

The production of microspheres by spray drying of w/o emulsions was also studied (method (b)). The particles appear well formed, spherical and few aggregates are present (figure 8.4). From initial morphology investigations, spray drying of a w/o emulsion, containing the protein in the aqueous phase allows the formation of regularly shaped microspheres with smooth surfaces which may permit a sustained release. Using this preparation method, microsphere production was successful using EthAc and PLGA co-polymer and β-Lg, batches 8,7 and 6 respectively, as detailed in table 8.1.

8.3.1 Release of protein from microspheres formed by spray drying methods

Due to the poor yields produced under the conditions employed, washing of the microspheres was not possible to remove unencapsulated BSA. The artificially high "encapsulation efficiency", determined by microsphere digestion (section 2.2) may therefore include unencapsulated BSA. Release profiles were carried out in phosphate buffer (see section 2.3.1). Release from microspheres prepared using spray drying is governed by the same principles as other methods. Release rates are generally faster from smaller particles (Conte *et al.*, 1994) from microspheres of co-polymers and from low molecular weight polymers (Pavanetto *et al.*, 1993).

However, microspheres produced using spray drying often have imperfect and porous coating of PLA around the protein due to different interfacial energies between the protein and PLA (Wang *et al.*, 1990) leading to large burst effects of proteins from the damaged surfaces. For all preparations, burst effects are 65-75% of the total protein. This value is lower for microspheres containing β-Lg formed by spray drying of a w/o emulsion (50%). The encapsulation efficiency is lowest for this preparation (110.7%) suggesting that the method is more suitable for incorporation of lower molecular weight proteins.

There is little difference in the release profiles obtained for BSA from particles formed using the suspension/coating method and the w/o emulsion method (figure 8.5). Release from spheres formed using the emulsion method (b), is slower but the differences are not significant. Though, from SEM observations, the particles prepared using this method appeared to encapsulate more BSA, results suggest that both methods are capable of producing microspheres which can sustain release for up to 100 hours. Substitution of DCM with the non-chlorinated solvent, EthAc, resulted in microspheres

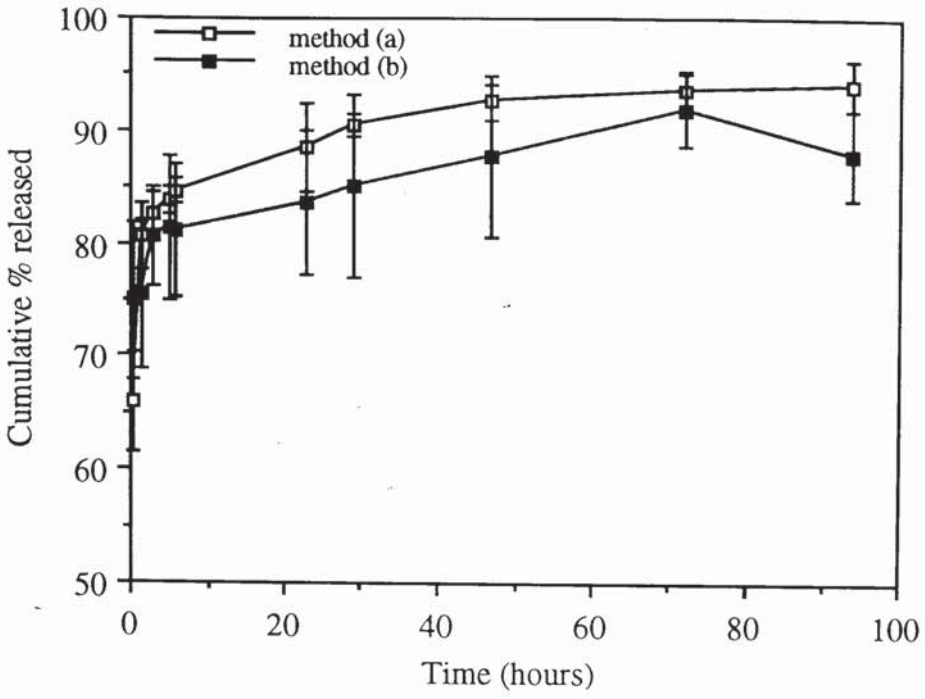


Figure 8.5. Release profiles for BSA from spray dried PLLA microspheres formed using a suspension method (a) (batch 1) or a w/o emulsion method (b) (batch 2), (n=3; mean±s.d.)

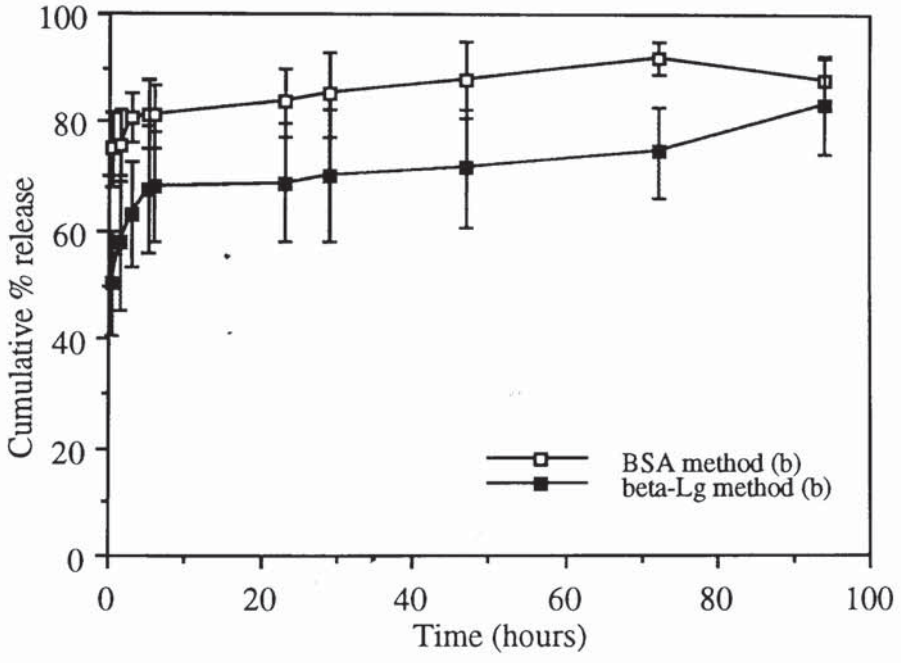


Figure 8.6. Release profiles for BSA (batch 1) and β-Lg (batch 6) from PLLA microspheres produced using w/o emulsion spray drying method (n=3; mean±s.d.)

which gave a similar profile, with a burst of 75% of the protein and 90% released in 100 hours.

Release of the low molecular weight protein, β-Lg, from PLLA microspheres prepared using the spray drying emulsion method, followed a rapid release up to 10 hours. A lag period was then detected, where little protein was released until 80 hours. A similar profile, with an increased burst effect, was obtained for BSA release from a similar microsphere formulation. These profiles are more typical of the three phase release pattern described by Sanders *et al.* (1984) (see section 3.3.3.3) suggesting that the internal structure of microspheres produced using spray drying techniques are different to those produced using solvent evaporation. Gander *et al.* (1995) detected no release of BSA from spray dried microspheres after an initial burst (5-50%) over a two week period. This may be due to the larger particle sizes (1-20μm) and higher molecular weight polymer used in their investigations.

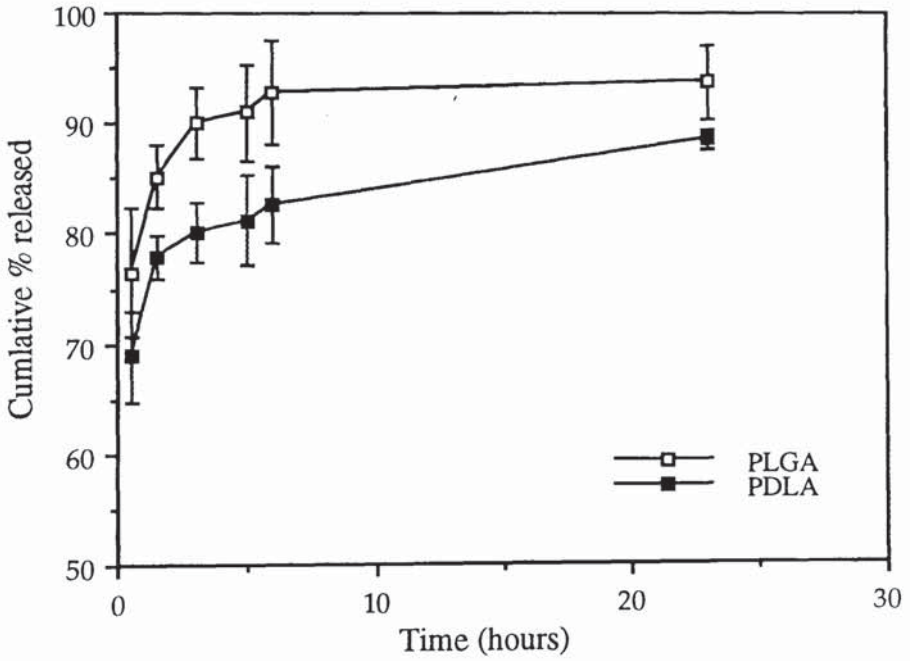


Figure 8.7. Release profiles for BSA from PDLA (batch 5) and PLGA (batch 4) microspheres produced using suspension spray drying method (a) (n=3; mean±s.d.)

Figure 8.7 illustrates the release profiles obtained for BSA from amorphous PDLA and PLGA polymers over a 24 hour period. After this time no further release was detected over the time studied (with ~15% of the protein retained in the microsphere). The release of BSA from PDLA microspheres formed using the suspension/coating method

is slower than from PLGA microspheres. This is the reverse of the situation for microsphere formed by solvent evaporation techniques and may be due to the larger particle size of the PDLA particles, extending the time period required for water penetration into the microsphere matrix.

8.3.3 Stability of spray dried microencapsulated BSA

SDS-PAGE analysis of the microencapsulated BSA was carried out to examine protein degradation. Aggregation of BSA, resulting in bands of higher molecular weight, was visible in all samples, whether produced by the suspension/coating technique or by the emulsion method (figure 8.8). This aggregation may be caused by exposure to the solvent, the high shear processes involved or the temperatures to which the protein is exposed during microsphere production using spray drying but does not appear to occur during microsphere preparation using solvent evaporation techniques (see section 3.3.10). Alternatively, the aggregation may have occurred in the unencapsulated, microsphere-associated BSA during transport from Italy to England. Results by Gander *et al.* (1995) suggest that degradation is not a problem for BSA in microspheres produced using this technique.

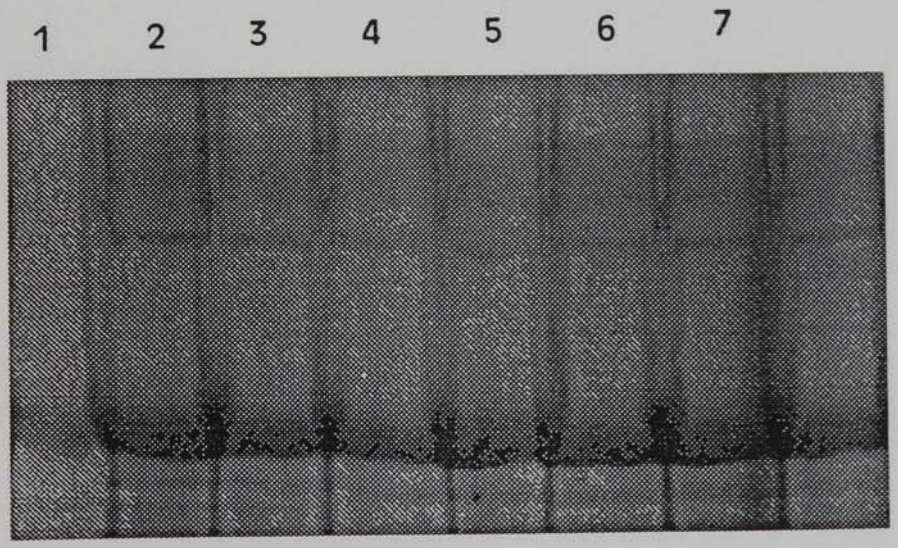


Figure 8.8. SDS-PAGE (12%) pattern of BSA microencapsulated by spray drying. Lanes represent batches according to table 8.1. Lanes (1) BSA prior to encapsulation; (2) batch 2; (3) batch 1; (4) batch 3; (5) batch 5; (6) batch 7; (7) batch (8)

8.4 SUMMARY

Results suggest that the internal structure of microspheres produced using spray drying techniques are different to those produced using solvent evaporation. Further work is required to improve yields and reduce the loss of polymer. Coating of preformed protein microspheres with PLA polymers (Park *et al.*, 1994) may be a more successful approach to producing microspheres with low bursts and sustained release properties but, in this case, the coating procedure requires further modification. A double nozzle spray drying method using mannitol to coat the microsphere surface to reduce agglomeration has been reported for the encapsulation of peptide drugs (Takada *et al.*, 1994) and these approaches, in combination with the results obtained, may allow production of microspheres which can be used for sustained delivery. The relatively rapid protein release from these particles may be of use in the development of a single-step vaccination formulation, providing the initial priming dose, to be followed with boosts from the microspheres with slower-releasing properties.

9.0 CONCLUDING REMARKS

The development of microparticulate formulations and bacterial or viral vectors systems, coupled with appropriate adjuvants, is likely to yield the most effective outcome (Husband, 1993).

A unique feature of polymeric delivery systems is that they can be tailored to meet specific physical, chemical and immunogenic requirements of a particular antigen. The biodegradable delivery system can be manipulated to incorporate variable amounts of antigen and/or adjuvants, used to minimise antigen digestion by enzymes, chemically modified at surface to enhance site specificity and can provide sustained antigen release following mucosal uptake. Although microspheres of 1μm are reported to be advantageous for uptake by macrophages (Tabata & Ikada, 1990) or uptake by PPs and migration to mesenteric lymph nodes (Jani *et al.*, 1989), small microspheres possess a tendency for increased burst effects. Characterisation of size distributions and *in vitro* release are essential to design a vaccine formulation. Particles of less than 6-7μm are effectively phagocytosed by various macrophage populations which can allow delivery of entrapped drugs to the cells responsible for immune response initiation.

The production of microspheres of a suitable size for mucosal delivery was achieved using o/w and w/o/w solvent evaporation techniques, containing protein with high encapsulation efficiencies. Microspheres were also produced by solvent extraction and spray drying techniques.

When developing a novel co-encapsulation product, several criteria must be considered. Firstly, a suitable biodegradable and biocompatible polymer or co-polymer must be selected with the desired degradation kinetics. The optimal ratios between the encapsulated drugs must be considered, the physical and chemical properties of the individual proteins must be considered and the release properties of the system characterised for each formulation. As polymer matrices play an important role in controlling the drug release, other compounds such as the drug itself, or residual solvents and surfactants are also involved and each system must be fully characterised.

Unique antigen packaging and the use of adjuvants for oral administration hold great promise for an efficient antigen delivery to critical tissues, with the oral route having many advantages over systemic delivery. If successfully modulated, GALT and BALT could confer long-term mucosal and systemic immunity against a variety of toxins and pathogens. Antigenic stimulation of IgA precursor B cells in the PP can disseminate to the lamina propria of intestinal, respiratory, genitourinary tracts and a number of secretory glands. Besides providing immunity on mucosal surfaces, it does not require the stringent criteria applied to injection formulations, storage problems may be alleviated and administration to large numbers of people is possible without the need for highly trained personnel. This new technology is likely to substantially influence the design of vaccines for both oral and systemic immunisations.

In the experiments presented, it is shown that microencapsulation of antigens show a clear adjuvant effect when presented *via* the intranasal and oral routes. Advances in immunology and molecular biology have allowed development of synthetic vaccines of antigenic determinants of complex antigens. These and other antigens are being delivered by increasingly sophisticated means which should lead to the provision of long-lasting immune responses. The manipulation of T-cell subsets using adjuvants, antigen packaging or perhaps the addition of individual cytokines to various formulations holds significant promise for optimising immune responses to orally administered vaccines. Co-encapsulation of the *Y. pestis* subunit with an immunostimulant which would elicit a cell-mediated response, such as MDP, would be interesting.

In addition to the improved adjuvant effect due to the tyrosine content of poly(DTH)carbonate, the slow release and higher surface hydrophobicity associated with this formulation may aid the development of single-step immunisation formulations. Currently, studies are underway to study the immune responses to oral delivery of both types of microsphere formulation.

Strategies being developed to increase the oral uptake of vaccine-containing microspheres from the GI tract may lead to the reality of a single step oral immunisation system. At worst, boosting orally with microspheres appears to be a very promising schedule. More recently approaches to develop single dose vaccines include the use of recombinant and especially DNA vaccines.

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