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Formulation of protein and DNA microparticulate vaccine systems

by

Fiona S. Brennan, B.Sc., M.Sc. (Genetics)

Thesis submitted for the degree of

Doctor of Philosophy

in Pharmaceutics

at

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under the direction and supervision of

Majella E. Lane, B.Sc. (Pharm.), Ph.D., M.P.S.I.
and
Owen I. Corrigan, B.Sc. (Pharm.), M.A., Ph.D., F.T.C.D., F.P.S.I.

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Declaration

This thesis is submitted by the undersigned to the University of Dublin for examination for the degree of Doctor of Philosophy. It has not been submitted as an exercise for a degree at any other university. I carried out all the practical work except where duly acknowledged. This manuscript was written by me with the aid of editorial advice from Dr. Majella Lane and Professor Owen Corrigan.

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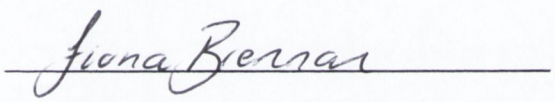

Fiona S. Brennan

To my parents

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Abbreviations and symbols

cDNA	Complementary DNA
cm	centimetre
CD	coefficient of determination
©	copyright
°C	degree centigrade
DSC	differential scanning calorimerty
g	gram
hr	hour
μm	micrometer
mg	milligram
min	minute
MSC	model selection criterion
n	number of replicants
pDNA	Plasmid DNA
±	plus or minus
®	registered trademark
r.p.m.	revolutions per minute
sec	second
™	trademark
λ	wavelength
w/v	weight per volume
w/w	weight per weight

Summary

This study involved investigation of protein and DNA-loaded microspheres manufactured with a range of polymers and under different manufacturing conditions. The polymers used during encapsulation were poly (lactide-co-glycolide) polymers with different ratios of lactic:glycolic acid. Microencapsulation was carried out using a modified double emulsion technique followed by either solvent evaporation or spray drying. Characterisation techniques used to examine the microspheres included scanning electron microscopy, particle size analysis, gel permeation chromatography, confocal microscopy, zeta potential measurement and differential scanning calorimerty. Release of protein or DNA from all batches was studied and fitted to appropriate mathematical models.

Human Serum Albumin (HSA) was encapsulated in PLGA 75:25 and PLGA 50:50 following double emulsion formation and solvent evaporation. Spray-dried protein-loaded microspheres were also manufactured. Emulsification was carried out using either an Ultra Turrax (T25) or a Silverson mixer (L4R). Two emulsification times were used to study the effect of homogenisation time on the physicochemical properties of protein-loaded microspheres

It was found that polymer type and emulsification time had a significant influence on microsphere size and encapsulation efficiency. Release rates and profiles were also dependent on polymer type with PLGA 50:50 exhibiting a higher amount of protein released over the time of the study when compared with PLGA 75:25. Spray dried particles showed the highest encapsulation efficiency results when compared with solvent evaporated microspheres. Protein extracted from solvent evaporated microspheres and microspheres homogenised with a Silverson mixer and spray-dried showed little or no signs of degradation following SDS-PAGE examination.

The influence of temperature, plasmid molecular weight, outer:inner aqueous volumes, DNA loading and manufacture technique were examined in relation to the physicochemical properties of DNA-loaded microspheres. All manufacture techniques were seen to influence encapsulation efficiency and particle size. Lower manufacturing temperatures exhibited higher encapsulation efficiencies and smaller particle sizes when

compared with particles manufactured at higher temperatures. Loading and particle size were both observed to be significantly influenced by the molecular weight of the encapsulated agent. Particle size analysis and encapsulation efficiency increased as the outer aqueous volume was increased. Microsphere morphology was also influenced greatly by manufacturing technique with those batches manufactured following solvent evaporation displaying spherical particles while those manufactured following spray-drying were doughnut shaped. In relation to encapsulation efficiency spray-dried microspheres showed higher encapsulation efficiencies when compared with solvent-evaporated microspheres with approximately 91% of loaded DNA successfully encapsulated. This level of encapsulation was the highest observed for any of the systems studied in the current work.

The biological potency of DNA extracted from microspheres was examined following bacterial cell transformation and COS-7 cell transfection. In was found in both cases that the integrity of DNA was conserved to varying degrees depending on the manufacturing procedure. The interaction of representative batches of microspheres with a macrophage cell model was investigated in order to evaluate how these systems would interact with immune system cells. These indicated that polymer type and manufacturing procedure influenced the degree of uptake into these cells.

Research presentations associated with this thesis

Microencapsulation of plasmid DNA

Proceedings of Annual Research Seminar, School of Pharmacy, Trinity College, Dublin, March 2001, Abstract 11[F.S. Brennan, M.E. Lane and O.I. Corrigan]

Preparation and characterisation of PLGA microspheres containing plasmid DNA

New Trends in Polymers for Oral and Parenteral Administration: From Design to Receptors, Paris, March 2001, Abstract 29 [F. S. Brennan, M.E. Lane and O.I. Corrigan]

Encapsulation of plasmid DNA in biodegradable poly (D,L lactic-co-glycolic acid)

Proceedings of 7th United Kingdom Ireland Controlled Release Society Symposium, London, January 2001, Abstract 3 [F. S. Brennan, M.E. Lane and O.I. Corrigan]

Microencapsulation of plasmid DNA in biodegradable poly (D,L lactic-co-glycolic acid)

Proceedings of the TCD/QUB Joint Annual Research Seminar, School of Pharmacy, Trinity College, Dublin, March 2000, Abstract 12 [F.S. Brennan, M.E. Lane and O.I. Corrigan]

Preparation and Characterisation of Human Serum Albumin Loaded Microspheres

Proceedings of the 12th International Symposium on Microencapsulation, London, 1999, Abstract 48 [F.S. Brennan, M.E. Lane and O.I. Corrigan]

Introduction

Chapter 1

Polymers

1.1. Introduction to polymers

Polymers can be defined as compounds formed by the combination of a number of identical molecules of a simpler substance, monomers, which are linked together through a process known as polymerisation to form polymers (Li and Vert, 1999). They are found in three forms, a natural form, a synthetic form or a combination of both. Table 1-1 lists several natural and synthetic polymers.

Naturally occurring polymers	**Synthetic polymers**
DNA, RNA	Polyesters (poly(lactide), poly(glycolide))
Proteins, Polypeptides	Polycyanocrylate
Polyesters	Polyanhydrides
Collagen	Polyamides
Albumin	Polyvinyls (poly (vinyl chloride))
Carboxymethyl cellulose	Polystyrene
Agarose	Polypropylene
Chitosan	Polyurethanes

Table 1- 1 Examples of natural and synthetic polymers (adapted from Angelova and Hunkeler, 1999)

In their natural form polymers are found in wood, silk and as biopolymers. Examples of these "biopolymers" include DNA, RNA, proteins and enzymes. As proteins they are composed of repeating units of α-amino carboxylic acids and as DNA they are built from alternating nucleotides. Disadvantages of natural polymers include batch-to-batch variation and the possibility of contamination from extraneous sources.

In their synthetic form polymers are produced to form plastics, fibres and gels (Hamley, 2000). The first synthetic polymer was "bakelite", which was produced in 1910 and was not widely used until after the Second World War when the technology for polymerising polyethylene and polypropylene was developed (Martin, 1993). Most of the biodegradable polymers that have emerged over the past 20 years have hydrolysable bonds, namely ester, anhydride, carbonate and urethane in their backbone resulting in polyesters, polyanhydrides, polycarbonates and polyurethanes respectively.

Process/manufacturing conditions can be altered or modified in order to produce polymers with precise biological and physicochemical properties and as a result have been fundamental in the development of a wide range of pharmaceutical products (Mills and Davis, 1987; Pillai and Panchangnula, 2001). Polymers have been used in a variety of different roles in pharmaceutical products as a coating or thickening agent, as drugs themselves such as insulin and heparin (Martin, 1993) and more recently in the area of sustained drug delivery systems (Hamley, 2000).

Polymers are formed by the addition of one monomer to two or more monomer units in a process called polymerisation. There are two main methods through which polymerisation occurs, namely step polymerisation (Hamley, 2000) or chain polymerisation (Li and Vert, 1999). Step polymerisation occurs between species with at least two functional groups in a stepwise reaction (Equation 1-1). Examples of polymers formed following step polymerisation include polyesters and poly(tetrafluoroethane) (Teflon) (Hamley, 2000).

$$HO\text{-}R\text{-}OH + HOOC\text{-}R'\text{-}COOH \longrightarrow HO\text{-}R\text{-}OCO\text{-}R'\text{-}COOH + H2O \quad \textbf{Equation 1-1}$$

Chain polymerisation can be subdivided into

1. Free radical addition polymerisation
2. Ionic polymerisation.

Free radical addition polymerisation involves the addition of a free radical ($R^{\cdot}$, usually generated by the thermal breakdown of a chemical bond or an initiator such as ultraviolet light) to a monomer (Equation 1-2). The identity of the R group is dependent on the polymer involved. For example in proteins the R group represents amino acid radicals such as glycine, alanine and valine (Hamley, 2000).

$$RM\cdot 1 + M1 \longrightarrow RM\cdot 2 \quad \textbf{Equation 1-2}$$

Ionic polymerisation is either cationic (initiated by electrophiles, Equation 1-3) or anionic (initiated by electron deficient species, Equation 1-4).

$$M + I+ \longrightarrow MI+ \quad \text{Cationic} \quad \textbf{Equation 1-3}$$

$$M+I- \longrightarrow MI- \quad \text{Anionic} \quad \textbf{Equation 1-4}$$

Some polymer are composed of identical repeating units and are called homopolymers while some are composed of blocks of repeating sequences or units and are called heteropolymers. An example of a heteropolymer is DNA, which is formed from a repeating sequence of four units of bases, adenine, cytosine, guanine, thymine (Hamley, 2000).

1.2. Biodegradable polymers

Recent approaches in macromolecular biomaterial technologies combine the efforts of scientists in various fields to obtain polymers with well-defined structures with specific chemical, physiochemical, mechanical and biological properties (Angelova and Hunkeler, 1999). Classifying the properties of polymers for their selection as biomaterials is challenging because a wide variety of materials is available for a particular application (e.g. surgery, drug delivery) and no single set of methods can be used to characterise them (Van de Velde and Kiekens, 2002). Figure 1-1 summarises the main issues, which need to be considered in the rational selection of a suitable biodegradable polymer and Table 1-2 outlines choices of polymers, which could be used.

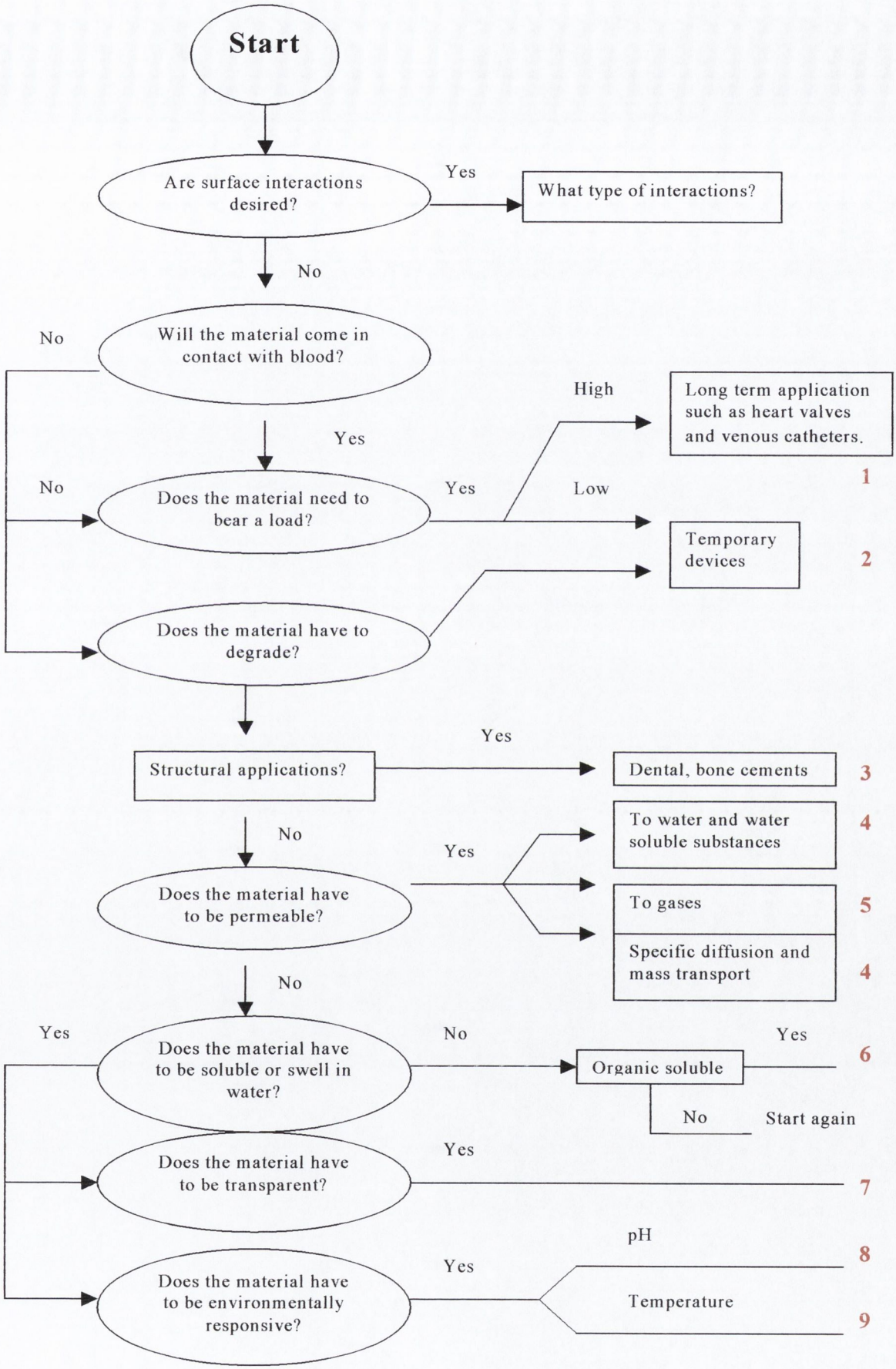


Figure 1- 1 Schematic of rationale behind the choice of a polymer (adapted from Angelova and Hunkeler, 1999)

Number	Polymer type	Polymer	Applications
1	Synthetic	Thermoplastics	Permanent medical devices e.g. prosthetics, catheters and drug delivery systems
2	Synthetic	Polyphosphazenes	Films, hydrogels and drug delivery systems
3	Synthetic	Poly(methyl methacrylate)	Dental and bone replacements
4	Synthetic	Polyethylene Poly(vinyl alcohol)	Sutures, catheters
		Poly(ethylene oxide)	Gels, membranes Biomedical
	Naturally occurring	Carboxymethyl cellulose	Dialysis membranes
		Alginate	Microinjectables
		Chitosan	Controlled delivery
5	Synthetic	Poly(tetrafluoroethylene)(Teflon®)	Sutures, coatings
		Polydimethylsiloxanes	Silicone
6, 7	Synthetic	Poly(hydroxyethyl methacrylate)	Contact lenses, Drug delivery
8	Naturally occurring	Carboxymethyl cellulose	Cell immobilisation
		Chitosan	Controlled delivery
9	Synthetic	Poly(ethylene oxide-*b*- propylene oxide)	In cases where environmentally sensitive polymers are needed
		Poly(vinyl methyl ether)	
		Poly(N-alkylacrylamides)	
	Naturally occurring	Carrageenan	Microencapsulation

Table 1- 2 Examples of choice of polymers (adapted from Angelova and Hunkeler, 1999)

Determination of the molecular weight, degree of crystallinity, size, viscosity and glass transition temperature of polymers and polymer drug delivery systems are important in the characterisation of polymers and their related drug delivery systems (Jacob, 1999; Li and Vert, 1999; Santos, 1999; Souillac and Rytting, 1999; Ward and Tracy, 1999). In examining and predicting the behaviour of drug delivery systems it is essential that the system and the polymer used be characterised fully.

1.2.1. Molecular weight

Due to the process of polymer formation through polymerisation all polymer chains in a sample do not have exactly the same weights. They have a distribution of molecular weights and are therefore termed polydisperse (Hamley, 2000). An ideal polymer would have a polydispersity index equal to 1. For polymers the term molecular weight is more accurately defined as average molecular weight. In order to completely define the molecular weight of a polymer the average molecular weight and the degree of polydispersity or molecular weight distribution (MWD) are required. Analytical techniques, such as gel permeation chromatography, are available to determine the number-average molecular weight (Mn), the weight-average molecular weight (Mw) and the polydispersity (MWD) of a polymer (English et al., 1999).

The number average molecular weight, Mn, is defined by

$$Mn = \sum N_x M_x / N_x \qquad \textbf{Equation 1- 5}$$

where N_x is the mole fraction of molecules of size M_x

The weight-average molecular weight, Mw, is defined by

$$Mw = \sum w_x M_x / N_x w_x \qquad \textbf{Equation 1- 6}$$

where w_x is the weight fraction of molecules whose molecular weight is M_x

The polydispersity (P) or MWD is defined as

$$MWD = Mw/Mn \qquad \textbf{Equation 1- 7}$$

The three determinations can be measured using the techniques of gel permeation chromatography (GPC) or size exclusion chromatography (SEC) and viscometry.

1.2.1.1 Gel Permeation Chromatography

Gel permeation chromatography (GPC) is a form of size exclusion chromatography, which is used to separate molecules on the basis of their size (Santos, 1999). The system typically consists of a solvent delivery system capable of delivering of a constant volume of solvent, an injection port, a column or series of columns, which are packed with a microporous gel, a detector and a system to process the data (Wu and Wang, 2001). A solution of the polymer is passed through the injection port, through the column and finally through the detector which is usually a refractive index detector. The smaller molecules remain on the column longer than the larger ones and fractionation of the polymer molecules occur. The concentration of the eluted polymer is monitored by the detector and the molecular weight distribution is calculated by measuring the elution volume or peak retention volume, V_R. This is a function of the ability of the polymer in solution to diffuse into the pores of the gel. The retention volume is the volume of solution eluted as measured from the beginning of the injection into the column to the peak of the fraction of the particular molecular weight eluted as determined by the chromatogram. High-molecular weights will elute at a low V_R, and low-molecular weight solutes will elute at a high V_R. V_R is related to the interstitial volume, the internal pore volume, V_i, and the equivalent volume of the gel, V_s, by the equation:

$$V_R = V_O + kV_i + k_{LC} V_s \qquad \textbf{Equation 1- 8}$$

where k is the coefficient of distribution, indicating the ease of the polymer solution to move into the pores. If k=0 then the polymer cannot enter the pores at all and if k=1 then the polymer can freely enter the pores. Typically k_{LC}, which is the ratio of the volume of solute in the stationary phase (within the gel) to the volume in the mobile phase, is negligible so the equation becomes:

$$V_R = V_O + kV_i \qquad \textbf{Equation 1- 9}$$

where V_O and V_i are constants for a given column. Thus, V_R is dependent on solute molecular weight and as such a calibration curve can be generated by solutes of known mono-disperse molecular weights. The molecular weight is related to the retention time

and is plotted as a $\log_{10}$ function against elution time (retention volume). From the calibration plot the molecular weight of the unknown sample can be calculated in terms of average molecular weight, Mw, number average molecular weight, Mn, and the *z*-average molecular weight, Mz. The MWD is calculated from these values. GPC is used extensively in the determination of the effect, if any, a loaded agent has on the degradation of a polymer, either increasing the speed of degradation or retarding it (Dunne, 2000; Wu and Wang, 2001) (Steendam et al., 2001).

1.2.1.2 Viscometry

The molecular weight of a polymer can be determined by viscometry. This can be measured using capillary viscometers and yields the viscosity average molar mass M_v. The time for flow of the polymer solution through a given distance is proportional to viscosity and is relative to that of a pure solvent. To determine the molar mass the intrinsic viscosity is required called thus because it relates to the intrinsic ability of a polymer to increase the viscosity of a solvent (Hamley, 2000).

The intrinsic viscosity $[\eta]$ is related to the specific viscosity $\eta_{sp} = 1- \eta/\eta_0$ where η is the viscosity of the polymer solution and η_0 is the viscosity of the solvent. It has been found that for most polymers the Mark-Houwink equation for intrinsic viscosity is obeyed:

$$[\eta] = K_v M^{\alpha}_v \qquad \textbf{Equation 1- 10}$$

K_v and α can be determined following calibration against a polymer with known molar mass; then the viscosity average molar mass, M_v, for a sample of unknown mass is determined by the intrinsic viscosity, $[\eta]$. The exponent α is related to the size of the polymer in solution. The value of M_v lies between Mw and Mn but is closer to Mw.

1.2.2. Characterisation of polymer microstructure

The properties of some polymers are based on their chemical composition and microstructure. These can be determined by a number of different techniques including

infrared spectroscopy, nuclear magnetic resonance (NMR), X-ray diffraction (XRD) and differential scanning calorimerty (DSC).

1.2.2.1. Infrared spectroscopy

Infrared spectroscopy can be used to identify polymers, determine co-polymer composition and provide information on molecular structure (Fieldson, 1999). The degree of polymer branching can be determined if the absorption bands of the branch groups can be identified. Co-polymers can be identified if the different types of the repeat unit have distinct absorption bands. The absorbance of each band can be measured via the Beer-Lambert law:

$$A = \varepsilon c l \qquad \textbf{Equation 1- 11}$$

where ε = the molar absorptivity. c is the concentration of the given species and l is the path length. For a co-polymer with two different types of repeat unit the ratio of absorbencies yields the ratio of concentrations if the molar absorptivities are known.

1.2.2.2. Nuclear magnetic resonance

Nuclear magnetic resonance can also be used to elucidate the same information as infrared spectroscopy. In addition it can be used to determine copolymer ratios and polymer structure based on the absorption of electromagnetic radiation (Burke, 1999).

1.2.2.3. X-ray powder diffraction

The amorphous or crystalline nature of polymeric materials can be confirmed by a number of physical methods of characterisation. The crystalline state of a polymer is characterised by the presence of an order lattice and the amorphous state by a lack of long periodicity of molecules (Rastogi and Suryanarayanan, 1999). The crystalline and amorphous states represent the two extremes of lattice order but there are also intermediate states. The term

used to describe this is the degree of crystallinity and is important in the determination of several polymer properties such as stability, water uptake and dissolution rate.

The technique of X-ray powder diffraction or XRD is a technique which is routinely used in the determination of the degree of crystallinity associated with solid forms such as polymers. XRD is based on the X-ray diffraction pattern of the solid, which is unique to every crystalline form of a given compound. X-rays are electromagnetic radiation which when passed through matter, interact with electrons and are scattered in all directions. Crystalline substances cause diffraction of the x-ray in a unique manner, which is the basis of its identification. Diffraction is caused by the periodicity, or repetitive nature of molecules in a crystalline substance (Rastogi and Suryanarayanan, 1999). The condition under which it occurs is determined by Bragg's law:

$$n\lambda = 2d\sin\theta \qquad \textbf{Equation 1- 12}$$

where d is the distance between the successive planes of a crystal, n is the order of reflection, λ is the wavelength of the X-ray and θ is the angle formed between the x-ray beam and the sample.

1.2.2.4. Differential Scanning Calorimetry (DSC)

DSC is traditionally used to evaluate drug-polymer interactions and to study the thermal nature of polymers and crystal structures. It is a measure of the heat capacity of a system as a function of temperature (Souillac and Rytting, 1999). As the temperature changes so too does the heat capacity of the sample. This allows for the detection of temperature-dependent phase transitions. The sample and reference are kept at the same temperature and the heat flow required to maintain equality in temperature is measured. DSC is an important technique in the study of polymers since most solid polymers are formed by rapid cooling of a melt to low temperatures and are therefore in a glassy state. The transition from a glass to a rubber state, the glass transition, is an example of what is known as a second order phase transition. Such transitions are accompanied by a change in heat capacity but no change in enthalpy (ΔH=0). An important application of DSC is the determination of possible interactions between a drug substance and the polymers used in

its formulation. One approach is to produce binary mixtures of each ingredient and to monitor changes in melting endotherms, which would indicate either a physical or chemical interaction between the two components (Souillac and Rytting, 1999).

1.3. Synthesis and structure of biodegradable polyesters

Biodegradable polymers include polyesters based on lactic and glycolic acids. Both lactic and glycolic acid are eliminated by the body during the Krebs cycle in their monomeric forms as lactic and glycolic acid monomers Varying the ratio of the lactic acid moiety to the glycolic acid moiety in the polymer results in polymers with different molecular weights, crystallinities and related parameters (Wang et al., 2000; Wu and Wang, 2001).

1.3.1. PLGA polymerisation

PLGA is composed of a ratio of two monomers, lactide (PLA) and glycolide (PGA). Lactic acid contains two asymmetric carbon atoms and therefore exists in three diastereoisomeric forms, L-Lactide, D-Lactide or D, L-Lactide (Li and Vert, 1999). Both L-Lactide, which is an optically active stereoregular form and D, L-Lactide, which is an optically inactive racemer, are the two most commonly used isomers of lactide. L-Lactide is semicrystalline due to the high regularity of its polymer chain. D, L-Lactide on the other hand is an amorphous polymer because of the irregularities in its polymer structure (Jain, 2000). Hence the use of D, L-Lactide is preferred over L-Lactide as it enables the homogenous dispersions of drug in the polymer matrix. Glycolide is an achiral molecule and as such only has one form.

The synthesis of lactide and glycolide monomers occurs in two steps. Briefly, the acids of either monomer are polycondensed yielding low molar mass oligomers, which are then thermally depolymerised in the presence of a catalyst such as zinc oxide forming the corresponding cyclic diester. The diester is then recovered following distillation at low pressure (Li and Vert, 1999).

Low molecular weight (molecular weight polymers <10,000) homo- and co-polymers of lactic and glycolic acid can be synthesised by direct condensation of lactic acid and glycolic acid or by azeotropic distillation with an aromatic hydrocarbon.

High molecular weight polymers are produced by ring opening polymerisation of lactides or glycolides (Wang et al., 2000; Okada, 2002). The main method of production of high molecular weight PLGA is polymerisation of either the glycolide or the lactide diester in the presence of a catalyst such as $SnCl_4$, which causes ring opening (Heller, 1993) (Figure 1-2). Polymerisation of lactones can occur via anionic, cationic or insertion coordination polymerisation. Ring opening as a method of PLGA production can be carried out under mild conditions and can produce high molecular weight species in a short time.

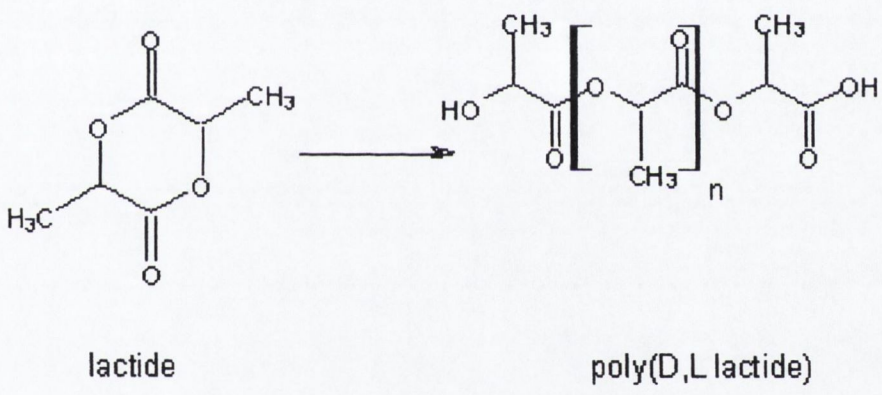


Figure 1- 2 Ring opening of lactide to produce polylactide

1.3.2. Physicochemical properties of PLGA

The physicochemical properties of PLGAs can be varied through the diversification of their composition, which allows for the production and selection of PLGAs with the desired characteristics. This becomes critical in the research and formulation of controlled release drug delivery systems. Wang et al. (2000) carried out work on the production of PLGA polymers. They focused on keeping the molecular weight fixed while varying the composition and on varying the molecular weights of polymers with the same composition. Polymerisation temperatures, polymerisation time, catalyst concentration, the degree of vacuum and the addition of molecular weight controllers have all been used in the past to regulate the molecular weight of polymers. These workers concentrated on controlling the

molecular weight of the polymer through the use of lauryl alcohol. They reported findings, which showed that the amount of lauryl alcohol used correlated with the molecular weight of the polymer. This study showed the extent to which it is possible to produce a range of different molecular weight PLGAs and hence control the biodegradation and release characteristics of a PLGA based drug delivery system.

The T_g's (glass transition temperature) of PLGA copolymers are above the physiological temperature of 37°C and hence they are glassy in nature. As a result of this they have a fairly rigid structure, which confers significant mechanical strength on devices fabricated from these materials (Jain, 2000).

1.3.3. PLGA biodegradation

The rate of decay of PLGA is dependent on a number of factors including microsphere size, the ratio of lactic:glycolic acids (x:y) (Figure 1-3), the polymer molecular weight (x+y), and the nature of the incubating medium such as its pH and ionic strength (Cohen et al., 1991; Wang et al., 2000).

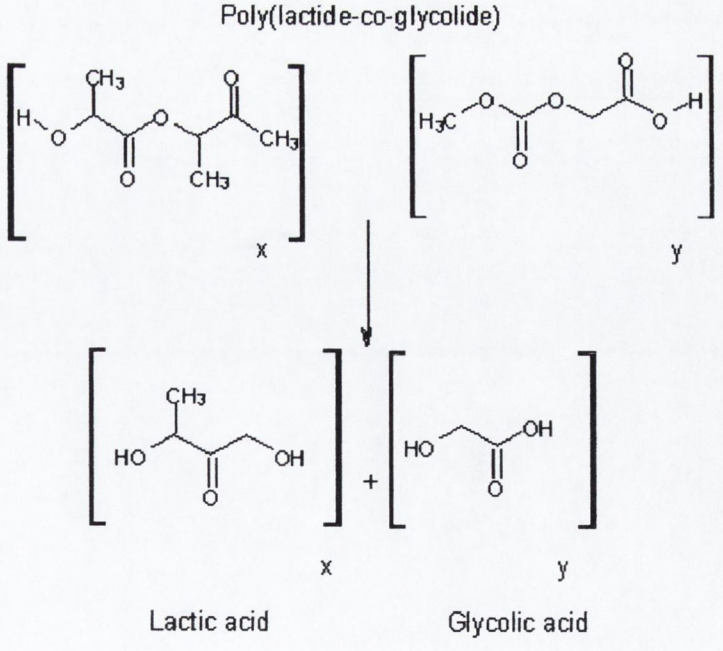


Figure 1- 3 The rate of decay of PLGA is dependent on the ratios of x and y (Adapted from Cohen et al., 1991)

PLGA is composed of a variety of ratios of lactic (PLA) and glycolic (PGA) acids. PGA is more crystalline then PLA because it lacks the methyl side chains of PLA. Lactic acid is more hydrophobic then glycolic acid and hence lactide-rich copolymers absorb less water and degrade slower (Li and Vert, 1999).

The mechanical strength, swelling behaviour and biodegradation of PLGA are also influenced by the polymer crystallinity. The resultant crystallinity is dependent on the type and the molar ratio of the individual monomer components (lactide and glycolide) in the co-polymer chain. Table 1-3 outlines some of the in vitro degradation rates of PLGA polymers. As can be seen L-PLA 100 is the most stable polymer with a half-life of 110 weeks. Increasing the percentage of D, L-PLA serves to decrease the half-life of the polymer. For example, in the case of 75:25 L-PLA: D, L-PLA there is a decrease of 88 weeks when compared with L-PLA 100. With the addition of the glycolide co-polymer (PGA) the rate of degradation increased with increasing concentrations of glycolide (Li and Vert, 1999).

Polymer	**Half-life (weeks)**
PLA 100	110
PLA 75	22
PLA 50	10
PLGA (85:15)	20
PLGA (75:25)	10
PLGA (37.5:25)	3

Table 1- 3 *In vitro* degradation rates of PLGA polymers (adapted from (Li and Vert, 1999))

According to Wang et al. (2000) the biodegradation of PLGA can be broken down into four steps. These are hydration, initial degradation, further degradation and solubilisation. Hydration occurs as the aqueous medium penetrates the polymer matrix which results in polymer relaxation and T_g decrease. The initial degradation starts in the hydrated region of the polymer through ester bond hydrolysis. The hydrolysis cleaves the polymer backbone and leads to molecular weight decrease of the polymers. This hydrolysis/cleavage continues inside the hydrated polymer matrix so that the molecular weight of the polymer continuously decreases. As the molecular weight of the polymer declines so too does the

mechanical strength. At the further degradation stage, the polymer chain continues to be cleaved and the molecular weight of the polymer decreases to a stage when the polymer can no longer maintain its integrity. At this stage the mass loss of the polymer begins. In the final stage the fragments of the polymer are further cleaved to molecules that are soluble in the aqueous incubating medium.

Wang et al. (2000) studied the influence of several variables on the biodegradation of PLGA. These included the composition of PLGA, molecular weight effects and the pH of the medium. They suggested that the biodegradation of PLGA regardless of composition follows first order degradation kinetics, which they expressed as:

$$\frac{-dMw}{dt} \sim k[P] \qquad \textbf{Equation 1- 13}$$

where Mw is the remaining weight -average molecular weight, t is the biodegradation time, k is the biodegradation rate constant (which can be obtained from the slope of the lines) and [P] is the polymer concentration. They concluded that biodegradation of PLGA was higher with high glycolic acid units, high molecular weight and an acidic environment.

1.4. PLGA drug delivery devices

Polymers such as polyesters are widely used in drug delivery systems due in part to their degradation which is dependent on their composition and which can be manipulated to produce the desired rate of release of the active ingredient (Lewis, 1990; Sah et al., 1994; Ravivarapu et al., 2000). Drug delivery systems protect the active ingredient against degradation from the environment and have also been used to reduce the unpleasant taste of certain materials.

The desirable properties of a biodegradable polymer are as follows: it must be biocompatible, produce non-toxic degradation products and be degraded either hydrolytically, enzymatically or microbially with a rate that can be modulated according to polymer properties and environmental conditions, have suitable mechanical and physicochemical properties, be economically viable, sterilisable, suitable for processing and free from elutable impurities that may be toxic (Mills and Davis, 1987).

Over the past 30 years polyesters based on lactic/glycolic acid units have been present in restorable sutures and implants and have emerged as leading biomaterials for a variety of other medical applications (Jones, 2000). The outstanding biocompatibility and the non-toxic and bio-absorbable nature of degradation end products of these polymers are the foundation for their use in the veterinary and human health related areas.

PLGAs have been successfully used as controlled release drug delivery devices over a number of years and have regulatory approval for human use in the U.S. by the FDA for specific applications (Carino, 1999). One of the significant advantages of using PLGA is that as a biodegradable polymer the removal of the device is not a problem. PLGA based polymers have been used to entrap several commercially available drugs in the preparation of controlled release systems, e.g. Zoladex® (Zeneca) and Decapeptyl® (Ipsen Biotech) both of which are licensed for use in humans in both Europe and the U.S.A. (O'Hagan, 1998). Table 1-4 lists some of the commercial PLGA devices used.

Commercial product	**Producer**
Zoladex®	Astra Zeneca
Sandostatin®	Norvartis
Somatinline®	Ipsen
Decapeptyl®	Ipsen
Trenatone®	Takeda-Abbot
Enatone®	Takeda-Abbot
Suprefact®	Aventis
Arestin®	Oropharm
Neutropin-Depot®	Genetech

Table 1- 4 Examples of commercial products employing PLGA and their producers (information supplied by Boehringer Ingelheim)

There are a number of different PLGA delivery devices which are outlined below most of which are not confined to PLGA alone but have also been used for the production of delivery devices based on other polymers. However since the scope of the thesis is the production of PLGA microparticles, the discussion will be confined to devices fabricated using PLGA.

1.4.1. Implants

Implantable drug delivery devices include polymeric cylinders, disc, pellets and slabs (Li and Vert, 1999). These are usually more then 0.1mm in thickness and are a compression of the polymer-drug mix. These types of systems provide a prolonged release of drug and ease of manufacture.

1.4.2. In situ formed devices

In situ formed devices are relatively novel devices, which allow for the injection of a polymer-drug solution through an intramuscular or subcutaneous route. The PLGA polymer is heated and then homogenised with the drug. Once injected *in vivo* the polymer-drug comes in contact with physiological fluid and the polymer entraps the drug by forming a gel-like substance. The *in situ* device now behaves like an implant as the polymer hardens and allows the drug to be released in a controlled manner (Jain et al., 2000).

1.4.3. Microparticles and nanoparticles

A microsphere is a structure made of a continuous phase of one or more miscible polymers in which particulate drug is dispersed (Mathiowitz, 1999). A number of microencapsulation processes have been developed and the process used is dependent on the polymer used, the type of drug to be encapsulated and the intended route of administration. Jain et al. (2000) outline some guidelines governing the encapsulation process of a given drug which include:

1. That the process should not in any way affect the stability and integrity of the encapsulated drug.
2. That the route of administration, target cell and size of the resultant microspheres should be taken into account before encapsulation. For example, target mucosal cells requires microspheres to have a size of less then 10μm, which can only be achieved following certain encapsulation procedures.
3. That the resulting microspheres should be a free flowing powder and not exhibit aggregation.

There are several techniques of microsphere production and the most commonly used of these are detailed below.

1.4.3.1. Single emulsion techniques

In the formulation of a single emulsion the polymer is first dispersed in a water miscible organic solvent, to which the drug is then added. The polymer-solvent/drug solution is then added to and emulsified with a larger volume of aqueous fluid with stabiliser. The resultant emulsion is then subjected to solvent removal or evaporation (Mathiowitz, 1999).

1.4.3.2. Double emulsion techniques

The most commonly used method in the preparation of drug-loaded microspheres is through the use of a water/oil/water emulsion (Figure 1-4) (O'Hagan et al., 1994). The core or drug material is dissolved in an aqueous solution, which is added to an organic solution containing the polymer. These two solutions are emulsified together using either a homogeniser or a sonicator. Once the primary emulsion is formed, it is added to a larger aqueous phase containing a stabiliser. This is then further emulsified to produce a water/oil/water double emulsion and is subjected to solvent removal to produce particles. Once formed, the particles are generally centrifuged down and washed to remove residual solvent. The particles are then dried under a vacuum, nitrogen or are freeze dried.

The choice of a polymer will depend on the desired physicochemical characteristics for the delivery system required (Singh and O'Hagan, 1998). The solvent in which the polymer is dissolved is an important choice especially in the case of pharmaceuticals. The decision is based both on the desired microparticulate characteristics and on the toxicity of the solvent. Various solvents are used including ethyl acetate and dichloromethane. Ethyl acetate is lower in toxicity when compared with dichloromethane but many polymers including PLGAs are much more soluble in dichloromethane than in ethyl acetate (Singh and O'Hagan, 1998). Homogenisation can be carried out using a variety of different instruments and various emulsification speeds have been used to produce particles with the desired size (Uchida et al., 1994).

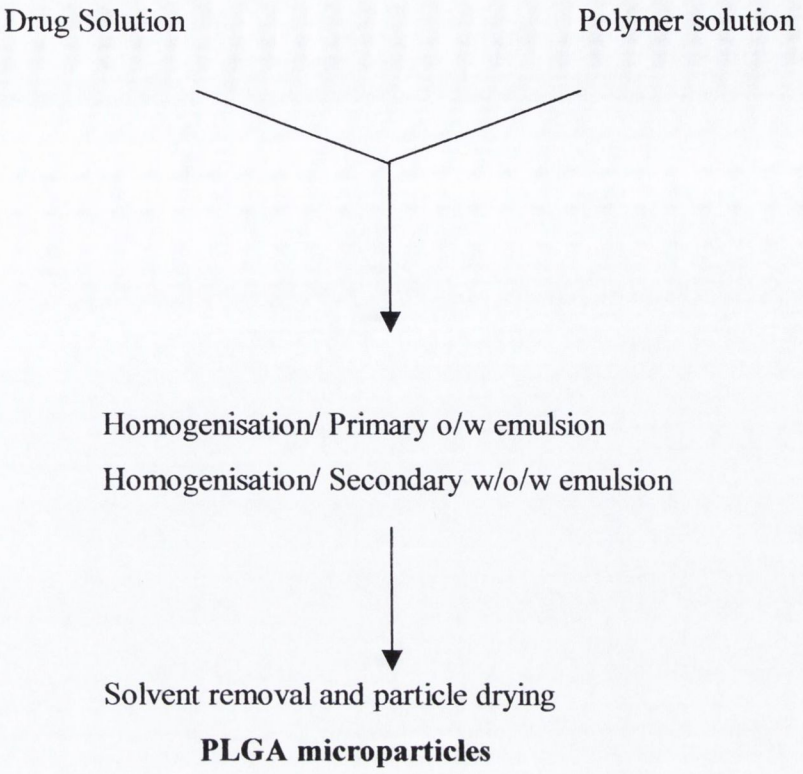


Figure 1- 4 Schematic of microparticulate formation

The secondary aqueous phase generally contains a stabiliser. The choice of type and concentration of stabiliser is dependent on desired microparticulate size, type of solvent used, porosity of microparticles and entrapped drug (Sahoo et al., 2002). Examples of stabilisers include polyvinyl alcohol (PVA) which is popular due to its low toxicity, polyvinyl pyrrolidone (PVP) and methyl cellulose. Concentrations of stabiliser range from 0.1% w/w to 10% w/w. Lower concentrations of stabiliser have been reported to result in microspheres with low loading and entrapment efficiency (Hariharan and Price, 2002). Solvent removal is generally carried out by one of two methods, solvent evaporation or solvent extraction (Singh and O'Hagan, 1998). The relative merits of these techniques are summarised in Table 1-5.

Technique	Method	Advantages	Disadvantages
Solvent evaporation	Evaporation of solvent through stirring of double emulsion	Production of smooth and uniform microspheres	May take several hours depending on solvent, temperature and stirring speed
Solvent extraction	Dilution of double emulsion into large aqueous solution until all solvent is extracted from the microspheres into aqueous phase	Relatively quick – may take minutes	Cracks and pores may appear on microsphere surface due to rapid solvent removal

Table 1- 5 Advantages and disadvantages associated with solvent removal techniques

1.4.3.3. Coacervation or phase separation

Coacervation or phase separation relies on the phenomenon of decreasing solubility of a polymer in response to the addition of a component to the polymer solution in an organic phase (Jain et al., 2000). The polymer is firstly dissolved in an organic solvent, which is then added to the drug/ water solution. Hydrophobic drugs are added to the polymer solution. The polymer is extracted through the addition of an organic non-solvent, which is added to the polymer-drug-hydrophobic drug solution. This results in the phase separation of the polymer, which as a result, forms soft coacervate droplets entrapping the drug. The droplets are hardened following the addition of an organic solution and are collected through washing and filtration.

1.4.3.4. Other production techniques

Other techniques commonly used in the production of microspheres include hot melt encapsulation which avoids the use of solvents. Another technique which reports production without the use of organic solvents is based on polymer-polymer immiscibility (Franssen and Hennink, 1998). Researchers reported the development of a novel encapsulation procedure based on the immiscibility of poly(ethyleneglycol) and methacrylated dextrans. Above a certain concentration it was reported that the polymers

separated into two phases and following emulsification, the polymer in the dispersed phase was cross-linked at high temperatures forming spherical non-porous microspheres. Limited organic solvent residues were desirable where the integrity of the drug was compromised by the presence of organic solvents or where the microspheres were destined for human delivery.

1. 5 Drug release from PLGA microspheres

In addition to the proven track record of PLGA in the microencapsulation of several agents, the rate of PLGA disintegration can be controlled hence allowing for control of release of the active material from the microsphere. Following careful selection of the appropriate polymer, the desired molecular weight agent and suitable experimental conditions it is possible to prepare a drug delivery system which releases the entrapped active agent at a predetermined rate (O'Hagan, 1994). Several investigators have examined the effect of varying conditions such as the molecular weight of the encapsulated agent and the polymer molecular weight on the morphology and release from PLGA microspheres (Gallagher and Corrigan, 2000).

Microspheres release small molecules as a function of time (Fitzgerald and Corrigan, 1996). Depending on the composition of an erodible device such as the type of polymer and drug loading and the geometry, the release kinetics from the polymer can be affected by the following factors (Siepmann and Göpferich, 2001):

1. Water intrusion into the device
2. Drug dissolution
3. Diffusion of drug through a water swollen matrix
4. Release following erosion and/or bulk degradation
5. Creation of aqueous pores
6. pH changes inside the matrix by polymer degradation products
7. Crystallisation of polymer degradation products
8. Adsorption/desorption processes.

The incorporation of an active agent into PLGA systems has been reported to result in a variety of drug release profiles (Gallagher and Corrigan, 2000). These profiles suggest either a two stage or three stage process of drug release. The method of release of the active core from the microsphere can be classified as an initial burst release followed by a second or third release phase. The sustained release period is dependent on several factors including:

1. Polymer molecular weight (Mehta et al., 1996; Steendam et al., 2001)
2. Molecular weight of encapsulated agent (Sandor et al., 2001)
3. Loading of encapsulated drug (Sandor et al., 2001)
4. Temperature of encapsulation (Yang et al., 2000 a)
5. pH of the dissolution media (Jiang et al., 2002)
6. Homogeneity of the drug dispersion in the matrix
7. The stability of the drug in the polymeric matrix

Diffusion can be defined as a "process of mass transfer of individual molecules of a substance brought about by random molecular motion and associated with a concentration gradient" and was described by Higuchi (1963) in an equation which describes the release of an active agent from a planar slab. This equation was developed further by Sinclair and Peppas in 1984 who developed the Sinclair and Peppas general power law expression (Equation 1-14) (Peppas and Sinclair, 1984).

$$\frac{Mt}{M\infty} = Kt^n \qquad \textbf{Equation 1-14}$$

Where K is the diffusional constant, and n is the diffusional exponent. For diffusional release from a planar slab $n = 0.5$ and this model becomes equivalent to the Higuchi model for release from a planar slab. At $n = 0.432$ the equation describes the diffusional release from a sphere (Peppas and Sinclair, 1984).

The passage of matter through a barrier may occur by simple molecular permeation, by dissolution of the permeating molecules in the bulk membrane, or by movement through solvent-filled pores and channels. There are several devices or mechanisms of diffusion of a drug from a polymer matrix. These include diffusion of the drug through the polymer

matrix and through solvent-filled pores. The release of small molecules from a polymer matrix appears to be controlled by diffusion of the drug through the polymer (Fitzgerald and Corrigan, 1996).

Multiple mechanisms have been attributed to the initial burst release from PLGA systems (Wang et al., 2002). Several studies have attributed the initial burst to the rapid release of drug from the surface of microspheres however Wang et al. (2002) believe this to only contribute partially to the burst release observed by many authors. Octreotide acetate was encapsulated in PLGA 50:50 microspheres following a double emulsion-solvent evaporation technique. The initial burst release was attributed to the increased permeability of the microspheres and to the numerous pores observed on the microsphere surface. The drug release during this time was attributed to the diffusion of the drug from the surface of the microspheres. However between 20 minutes and 5.5 hours of incubation in buffer the morphology of the microspheres were reported to have changed leading to observations that the internal structure of the microspheres showed a porous core and a much less porous surface. This observation was reported to correlate with the reduced permeability of the microspheres. Results reported by these researchers indicated that a decreased permeability of microspheres resulted from spontaneous polymer chain rearrangements and caused the cessation of the burst effect. However due to the dependency of drug release on the physicochemical characteristics of the encapsulated drug and the polymer, further studies would be necessary to indicate whether these observed polymer rearrangements were universal.

In some cases, subsequent phases of drug release has been attributed to the degradation of the polymer. Drug release, which is dependent on polymer biodegradation and dissolution, can be described by the Prout-Tomkins (1944) model, which was applied to the release of levamisole from PLGA microspheres and co-evaporated discs (Gallagher and Corrigan,, 2000).

$$F_{TOT} = F_B(1 - e^{-k_B t}) + (1 - F_B)\left(\frac{e^{kt - kT\,\max}}{1 + e^{kt - kT\,\max}}\right) \qquad \textbf{Equation 1- 15}$$

where F_B is the fraction of drug available for surface release, F_{TOT} is the fraction of drug released at time t, k is the rate constant and T_{max} is the time when the extent of drug release is equal to 50% of the final drug concentration.

Chapter 2

Vaccines and the Immune System

2. 1 The immune system

Our environment contains a great number and variety of infectious microbes – viruses, bacteria, fungi and muticellular parasites. These can cause disease if allowed to remain and multiply in the body but are predominantly short lived and leave little permanent damage. This is due to our immune system which is powerful enough to combat most infectious agents and adaptive enough to deal with the wide spectrum of infectious agents in the environment (Roitt and Male, 2001).

An immune response involves firstly, recognition of the invading foreign material followed by a reaction to eliminate it. Broadly speaking the immune response falls into two categories, which are the innate and adaptive immune response (Ada and Ramsey, 1997). A specific immune response, such as the production of antibodies in response to a particular pathogen is known as adaptive immunity. This produces a memory effect within the body and confers life long immunity against the same pathogen. This is distinct from innate immunity which relies on phagocytotic cells to overcome certain bacterial cells and which do not require prior exposure, but do not confer life long immunity to the host. Every immune system response is mediated by a variety of cells which originate in the bone marrow.

2.1. 1 Cells of the immune system

The cells of the immune system originate in the bone marrow from pluripotential haematopoietic stem cells. Pluripotential can be defined as cells which have the potential to become several different cell types. These cells first appear in the foetal liver of the developing embryo. After birth they are found in the bone marrow where they remain. The pluripotential haematopoietic stem cells divide into two specialised types of stem cells, a lymphoid stem call and a myeloid stem cell (Figure 2-1) (Janeway and Travers, 1996). The lymphoid stem cell gives rise to T and B lymphocytes and the myeloid stem cell gives rise to erythrocytes (red blood cells which carry oxygen), leukocytes and megakaryocytes (which give rise to platelets).

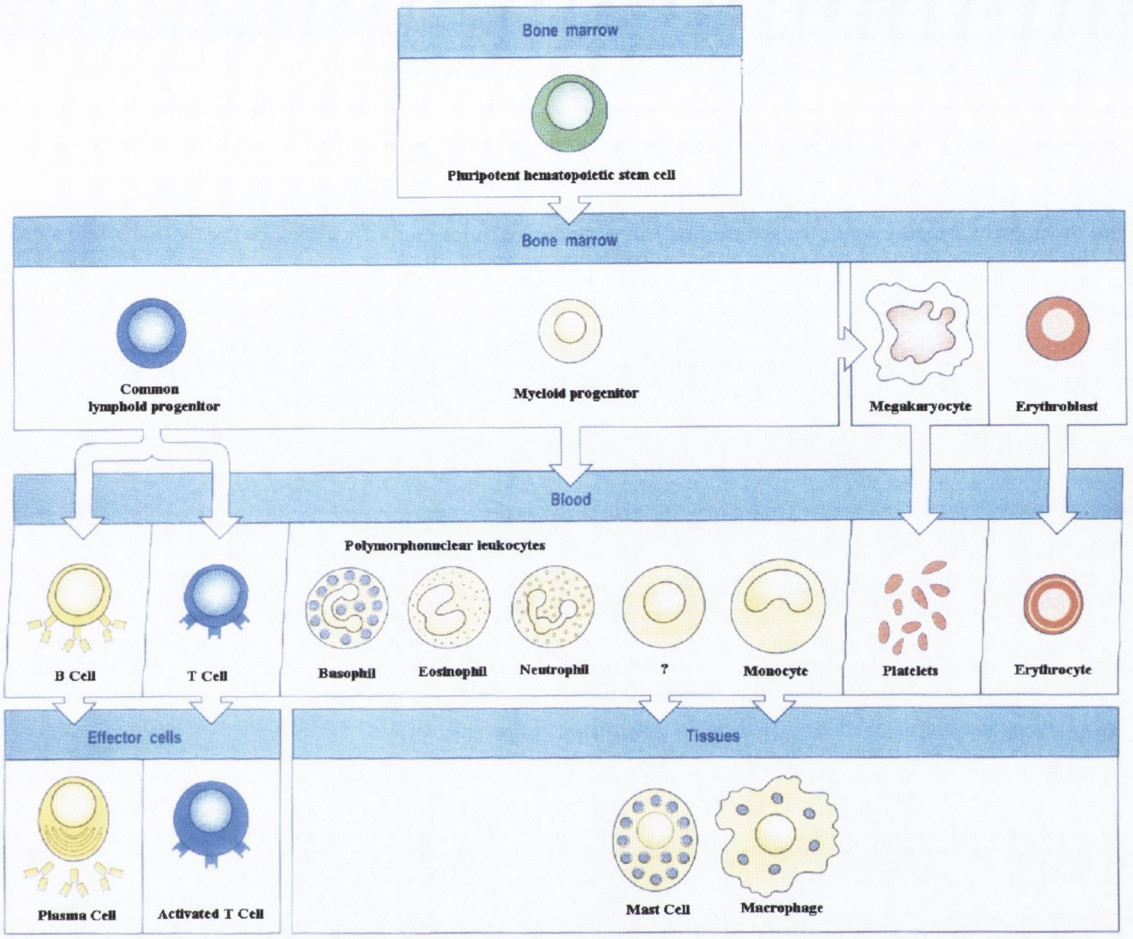


Figure 2- 1 Cells of the immune system (adapted from (Janeway and Travers, 1996))

The cells which recognise antigens and initiate immune responses are the lymphocytes. They are the most numerous and widely dispersed cell type in the human body with approximately 10^{12} lymphocyte cells in an adult human (Eisen, 1980). Gut associated lymphocytes are spread along the inside of the intestinal walls and form clusters which, if large enough, may form distinct follicles with germinal centres. Some may become so confluent that they interdigitate with the overlying epithelium forming lymphoepithelial structures and remain associated with the intestinal wall. These structures include the tonsils (found in the pharyngeal wall), appendix (at the juncture of the small and large intestine) and the Peyer's patches (in the later part of the small intestine).

Circulating lymphocytes belong to one of two classes: B and T lymphocytes. Some B cell lymphocytes differentiate into plasma cells when activated and secrete antibodies. Others

function as memory cells. There are two types of T lymphocytes. One includes the cytotoxic T cells (CD-8) which kill infected cells and the second type includes the helper and inflammatory T cells (CD-4) which activate other immune system cells such as B cells and phagocytotic cells (Ada and Ramsey, 1997).

Both T and B lymphocytes bear highly diverse receptors on their surface each of which is specific for a particular antigen, and which together are capable of recognising a wide diversity of antigens. The actions of both types of lymphocytes are evident in the two main types of immune response; Cell-mediated and humoral immune responses. B cells and T cells do not have separate roles within the immune system; instead they are responsible for activating each other to achieve an optimal response.

2.1. 2 Cell mediated immunity

Cell mediated immunity is generated through the activation of T cells. T cells recognise antigens which may be derived from pathogens, such as bacteria or viruses that replicate within the cells, or from pathogens and their products which cells internalise by endocytosis from the extracellular fluid (Janeway and Travers, 1996). T cells can detect cells infected with intracellular pathogens because such cells display on their surface, peptide fragments derived from the pathogen.

These foreign peptides are delivered to the cell surface by specialised cellular glycoproteins, called the major histocompatibility complex molecules or MHC molecules (Figure 2-2). These MHC molecules are encoded by a large cluster of genes called the major histocompatibility complex (MHC). There are two types of MHC molecules, class I and class II. Both have similar structures and functions but have distinguishable subunits which allow them to recognise surface peptides in different cell types (Raychudhuri and Rock, 1998). In both cases peptides derived from the invading pathogen are complexed with the appropriate MHC molecule and are transported to the cell surface where they are presented to specific T cells.

Briefly, MHC class I molecules recognise microorganisms which replicate in the cell cytosol. These microorganisms are usually viruses. The MHC class I molecules deliver peptides from the microorganisms to the cell surface where the MHC class I: peptide complex is recognised by killer T cells (CD-8), which kills the infected cell (Figure 2-2 A).

MHC class II complexes recognise pathogenic bacteria and eukaryotic parasites, which replicate in the cell's endosomes or lysosomes. They activate CD-4 T cells which in turn activate two other cells, T_H1 cells – inflammatory T cells which activate macrophages to kill the intravesicular bacteria or T_H2 cells - helper T cells which activate B cells into producing antibodies against the specific pathogen (Figure 2-2 B), thus activating the humoral immune system.

2.1. 3 Humoral immunity

Many infectious strains of bacteria utilise the extracellular spaces in the body for multiplication (Ada and Ramsey, 1997). This space is also traversed by pathogens as they move from cell to cell. The role of the humoral immune system is in the destruction of these extracellular micro-organisms. There are three main ways in which the humoral immune system achieves this.

Firstly, antigens stimulate the B lymphocyte cells of the humoral immune system which in response differentiate into antibody-secreting plasma cells (Figure 2-2 C). Certain types of bacteria and viruses bind to surface receptors on host cells. Antibodies can bind to these and neutralise them thus preventing their adherence onto cell surfaces (Figure 2-3).

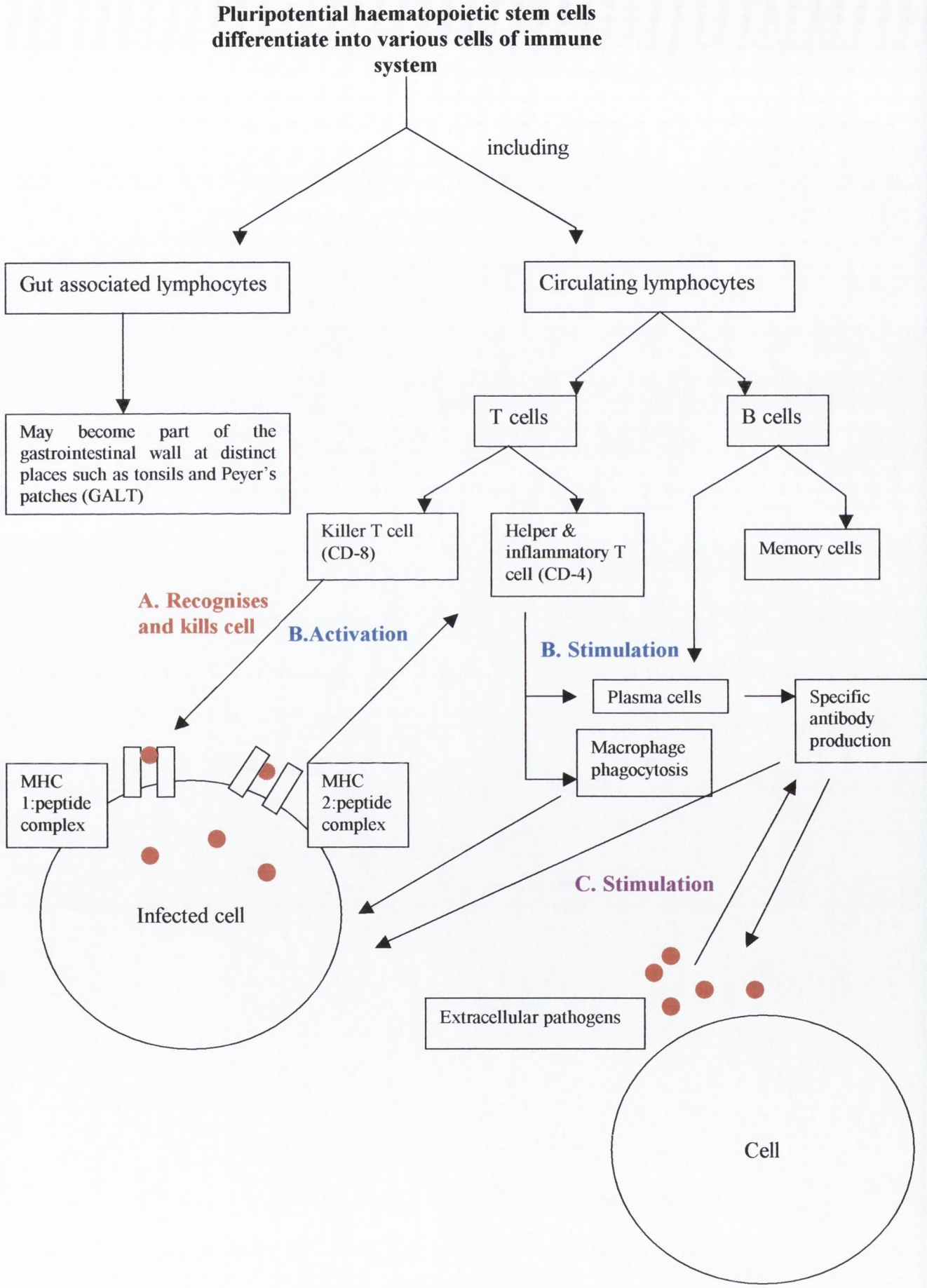


Figure 2- 2 Overview of cellular (A and B) and humoral (C) immune system

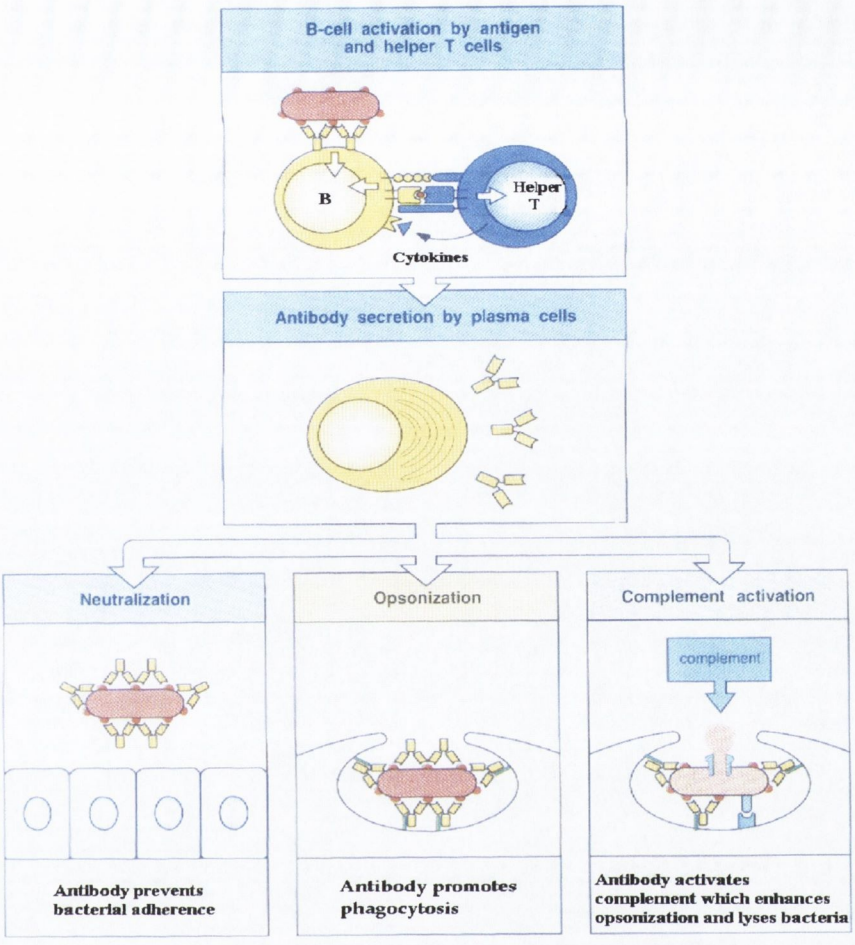


Figure 2- 3 B cell activation and its role in immune response (adapted from Janeway and Travers, 1996)

Antibodies produced by B cells can also facilitate the uptake of viral or bacterial cells into phagocytotic cells which are designed to destroy ingested bacteria. This is achieved through the opsonisation of particles (coating of the surface of the micro-organism with certain receptors) or through the activation of the complement system (Figure 2-3). The complement system is a detailed system through which the activation of a series of factors results in the uptake of a pathogen by a phagocytotic cell (Janeway and Travers, 1996).

2.1. 4 Antibody diversity

Antibodies are produced following B cell maturation and differentiation following antigen stimulation. Some B cells become memory cells which react quickly to a second challenge by the same pathogen and produce the appropriate antibodies. There are five different

classes of antibody or immunoglobulin molecules: IgM, IgD, IgE, IgG and IgA. Members of the same class are said to have the same isotype. All antibody molecules are similar in overall structure, each composed of four polypeptide chains, which accounts for certain common physicochemical features such as charge and solubility. All antibodies have a common core structure of two identical light chains (about 24kDa) and two identical heavy chains (about 55 or 70kDa). One light chain is attached to each heavy chain and the heavy chains are attached to each other. Both the heavy and light chains are composed of a series of repeating homologous (or identical) units each about 110 amino acid residues in length. Antibodies belonging to the same isotype contain regions of homology in their amino acid sequence while antibodies of different isotypes do not. Heavy chains are designated by the isotype of the antibody they belong to. IgA, IgD, IgE, IgG and IgM contain α, δ, ε, γ and μ heavy chains respectively. The shared region of amino acid sequence is responsible for the shared physicochemical properties of antibodies of the same isotype and also for their ability to bind to certain cell surface receptors and other molecules activating certain immune responses. Hence the amino acid sequence of the heavy chain is responsible for the role and activities of each antibody in response to specific antigens.

There are more then one billion different antibodies in each individual, each with unique amino acid sequences in their antigen binding domains. This remarkable feat is made possible through a process known as isotype switching. Each chain, heavy or light, contains distinct amino acid sequence domains which are called variable domains (V) and constant domains (C). There are two isotypes of light chain, these are κ and λ. These do not interchange and therefore the light chains are not responsible for the functions of the antibody. In the case of the heavy chain there is only one type of variable region therefore antibodies of different isotypes contain exactly the same variable region. However the constant region of the heavy chain is composed of a series of constant region genes which when spliced (joined) to the variable region genes creates the heavy region of the antibody. However the constant region genes can undergo a process in which the constant region genes rearrange themselves producing different sequences of genes. The association of the variable region with different constant region genes enables antibodies of a given specificity to change their functions. This is known as isotype switching and results in the production of 10^9 different antibodies in each individual.

2.1. 5 The mucosal immune system

The mammalian mucosal immune system is an integrated system of tissues, lymphoid cells and effector cells which protect the host from infection via the mucosal membranes (O'Hagan et al., 1994). The mucosal system includes the bronchial associated lymphoid tissue (BALT) and the gut associated lymphoid tissue (GALT, includes the tonsils, adenoids and appendix) (Figure 2-4). The mucosal immune system is anatomically and functionally divided into sites where foreign antigens are encountered and selectively taken up for initiation of the immune response. It is also the site of B and T lymphocytes, plasma cells macrophages and antigen presenting cells. Pathogens most commonly enter the body via the mucosal surfaces which include the respiratory, digestive and urogenital tissues.

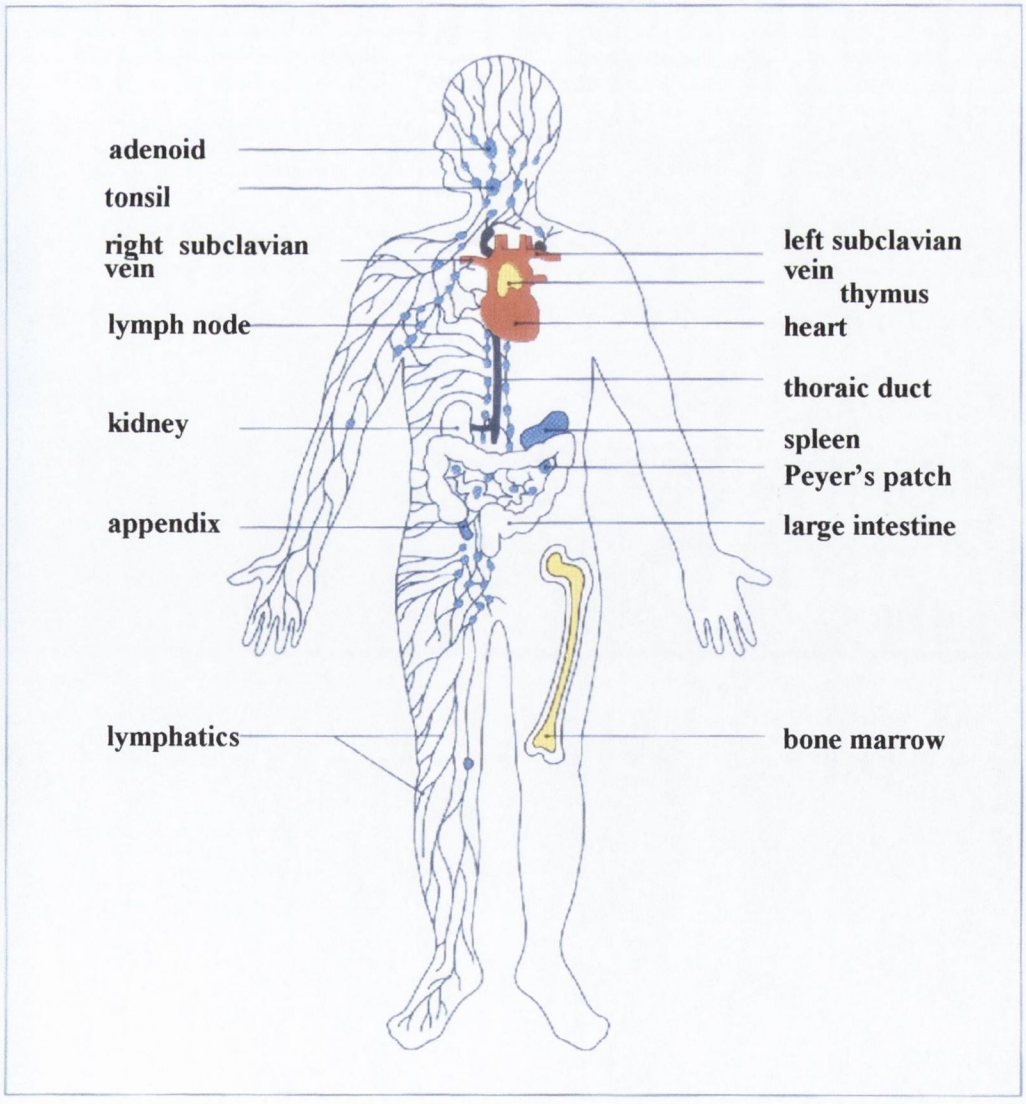


Figure 2- 4 Distribution of the lymphoid tissues in the GALT (adapted from (Janeway and Travers, 1996))

Mucosal surfaces account for a huge surface area of over 400 m^2 in humans and are semi-permeable in structure (Janeway and Travers, 1996). These properties require mucosal surfaces to be protected from infection. The use of vaccines which induce mucosal immune responses is important when one considers that most invading pathogens enter the body through mucosal membranes (Kiyono et al., 1996). The mucosal tissues of the body possess secretory components and secretory IgA (sIgA) whose daily synthesis exceeds that of any other isotype of antibody (Eldridge and Staas, 1991).

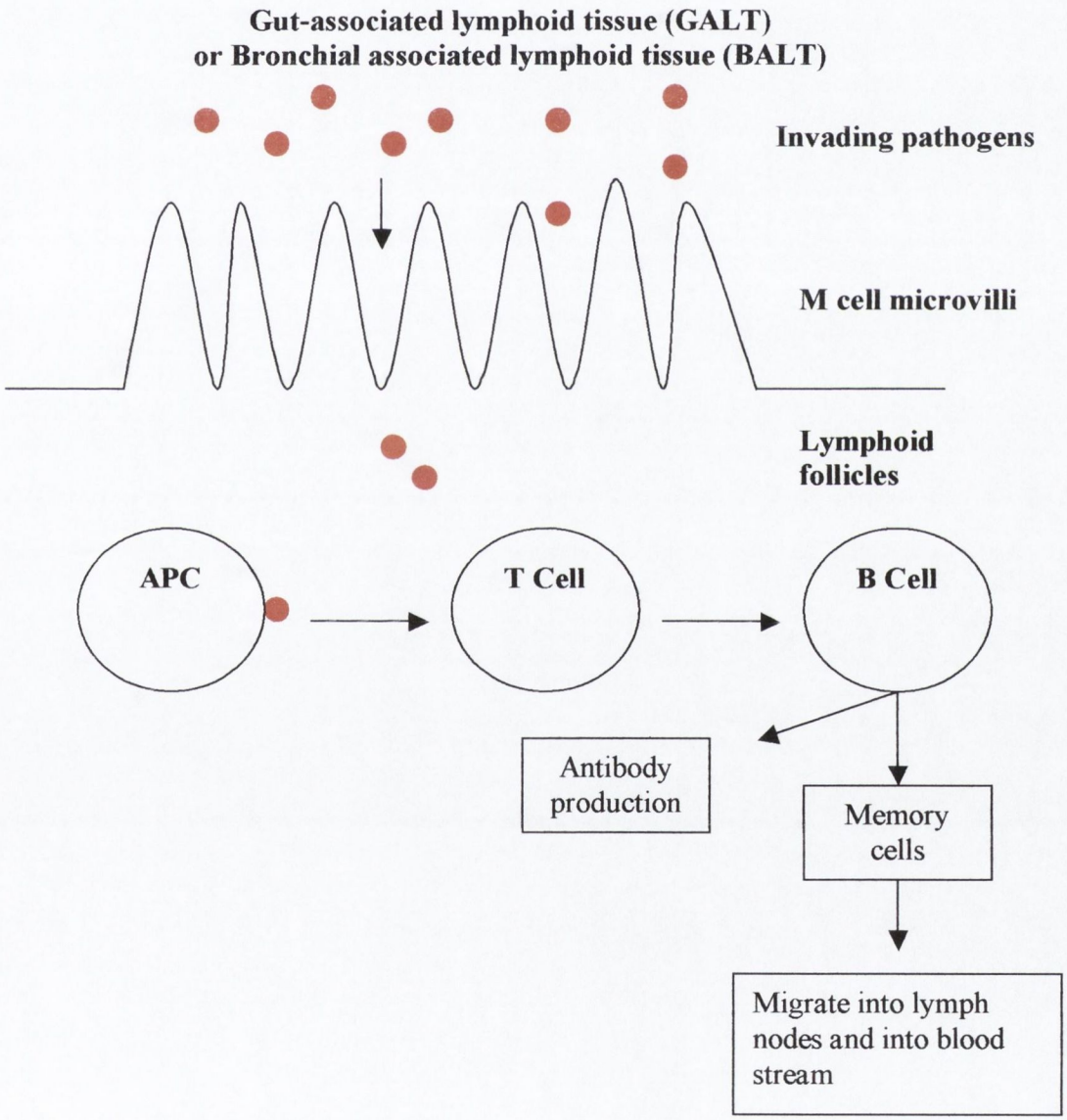


Figure 2- 5 Diagram of presentation of pathogen from antigen presenting cell (APC) via T cells to B cells

Following in vivo stimulation of the mucosal tissues IgA, B lymphocytes and T cells begin to circulate to other sites in the body. T cells and B cells migrate to lymphoid aggregates, such as the Peyer's patches in the gastrointestinal tract. The lymphoid follicles lie below a specialised layer of epithelial cells called membranous or microfold (M) cells (Figure 2-5). M cells have short microvillii which are adapted to the uptake of particles such as antigens (Clark et al., 2001). These cells then transport the foreign particle by luminal transport across their membranes. Once the antigen passes though the M cells, it is presented to regulatory T cells by the antigen presenting cells (APC). This action stimulates the B cells within the follicles to begin the production of specific antibodies against the antigen. In this way any invading pathogen is detected by antigen presenting cells, recognised by T cells and dealt with through the actions of B cells. The role of B cells is also to confer protective immunity against a second invasion by the same pathogen through the production of memory cells.

2. 2 Formulation of vaccines

One of the greatest medical achievements in the combating of diseases is the development of the vaccine. A vaccine can induce an immunological resistance to a disease but is not necessarily infectious. As a direct result of the widespread use of vaccination the incidence of disease has declined considerably, particularly in developed countries (O'Hagan, 1998). By the late 1990s, eight percent of infants worldwide (up from about 5 percent in the mid 1970s) had been immunised against five devastating diseases (diphtheria, pertussis, polio, tetanus and tuberculosis) and the annual death toll from these infections was reduced by approximately three million (Langridge, 2000). The first vaccine was developed in 1798 by Edward Jenner and was based on the homology between the bovine vaccinia virus (cowpox virus) and the human vaccina virus (smallpox) (Moss, 1991). Vaccines contain antigens which are found in either the structure or the secretions of an infectious agent. Upon primary exposure to the antigen the immune system, within the first 2- 4 weeks increases levels of serum antibody. These high levels then drop away rapidly but leave the immune systems with a recognition pathway which enables a more rapid, larger and specific response upon subsequent exposures to the same antigen (Carino, 1999). In order for a vaccine to be effective it can be presented first as an initial dose followed by booster shots which allow for long lasting immunity. There are several different vaccine designs

which include live or inactivated organisms, immunoglobulins and other more novel forms.

2.2. 1 Live vaccines

The first vaccine developed was a live vaccine and employed the cowpox virus which was similar enough to the human small pox to establish immunity against small pox while being unable to produce the clinical disease (Carino, 1999). Other live attenuated (non-pathogenic) forms of viruses or bacteria include the measles or rubella vaccine in the case of viral vaccines and the BCG in the case of bacterial derived vaccines (Davis and Whalen, 1995). Attenuation is achieved by growing the virus in non-human cells and through the course of selection the virus becomes less able to grow in human cells. Immunisation in these cases is generally produced following a single inoculation and immunity is long lasting (British National Formulary, 2001).

2.2. 2 Inactivated or subunit vaccines

Entire viruses or bacteria can be killed, rendering them non-infectious and then delivered as vaccines. Inactivated forms of vaccines require several initial inoculations followed by booster injections (Davis and Whalen, 1995). Subunit vaccines often include surface proteins or polysaccharides or the inactivated toxins of the bacteria if the disease is caused by these toxins. They also require several inoculations for adequate immunity to be recorded. Subunit vaccines include vaccines against diphtheria, tetanus and cholera. There is great potential in this type of vaccination because the development of recombinant DNA technology can allow for the synthesis of desired antigenic epitopes in large and well-defined quantities (Carino, 1999).

2.2. 3 Immunoglobulins

Immunoglobulins derived from human serum are used in vaccination and confer immunity which lasts several weeks. Immunoglobulins can be classified into two groups. These are normal immunoglobulins and specific immunoglobulins. Normal immunoglobulin is pooled from approximately 1000 donations of plasma and confers immunity in the form of antibodies against viruses, which are prevalent in the general population. This type is

mostly commonly administered via the intramuscular route and is successful in providing immunity against a wide range of diseases such as hepatitis A, measles and rubella. They can also be used intravenously in certain cases in the treatment or prophylaxis of infection following bone marrow transplantation. Commercial brands include Gammabulin ® (Hyland Immuno) and Kabiglobulin® (Pharmacia) (British National Formulary, 2001).

Specific immunoglobulins are prepared through the selection of plasma containing high levels of required antibody. These include hepatitis B, rabies, tetanus and cytomegalovirus immunoglobulin. Other immunoglobulins include the Anti-D (Rh_0) immunoglobulin which is used to prevent a rhesus-negative mother from forming antibodies to foetal rhesus-positive cells which may pass into the maternal circulation. The objective being to protect any subsequent child from the hazard of haemolytic disease of the newborn. Commercial brands include Anti-D (Rh_0) Immunoglobulin (Hyland Immuno) and WinRho SDF ® (Octapharma).

2.2 4 Commercially available vaccines

Table 2-1 outlines some of the diseases for which vaccines are currently available, the type of vaccine used, the agent it is derived from, the route of administration and the commercial name and manufacturer. Combined vaccines are also available such as in the case of hepatitis A and B and diphtheria, tetanus and pertussis (whooping-cough) nad polio (British National Formulary, 2001).

Target diseases	Type of vaccine	Agent	Route of injection	Commercial name and producer
BCG	Live bacterial strain	*Mycobacterium bovis*	Percutaneous - Young infants	
Botulism Antitoxin	Antitoxic globulins	*Clostridium botulinum* Types A, B & E	Intramuscular	
Cholera	Inactivated strain	*Vibrio cholerae*		
Diphtheria	Purified antigen	*Corynebacterium diphtheriae*	Intramuscular or Deep subcutaneous as primary immunisation	**Infanrix ®** (SmithKline Beecham)
Haemophilus influenzae type B	Purified polysaccharide	*Haemophilus influenzae* type B	Intramuscular	**HibTITER®** (Wyeth)
Haemophilus influenzae type B	Purified polysaccharide	*Haemophilus influenzae* type B	Intramuscular Subcutaneous	**ACT-HIB®** (Aventis)

Table 2-1 Examples of diseases and currently available vaccine strategies

Target disease	Type of vaccine	Agent	Route of injection	Commercial name and producer
Hepatitis A	Inactivated virus	Hepatitis A Virus	Intramuscular	
Hepatitis A & B combined	Inactivated virus A & inactivated virus surface antigen B	Hepatitis A Virus & Hepatitis B Virus	Intramuscular	**Twinrix ®**
Hepatitis B	Inactivated virus surface antigen	Hepatitis B Virus	Intramuscular	**Energix B®** (SmithKline Beecham) **HB-Vax 11®** (Aventis)
Influenza	Various	Influenza viruses A & B	Deep subcutaneous or Intramuscular	Inactivated Influenza Vaccine 1. **Split Viron** (Aventis). **Surface antigen** (Evans Vaccines) **Begrivac ®** (Wyeth) **Fluarix ®** (SmithKline Beecham) **Fluvirin ®** (Evans Vaccines) **Influvac Sub-unit ®** (Solvay)
MMR	Live virus		Deep subcutaneous Intramuscular	**MMR 11®** (Aventis Pasteur) **Priorix ®** (SmithKline Beecham

2.2. 5 Limitations associated with conventional vaccines

Disadvantages associated with current vaccine strategies include side effects which are usually related to the type of vaccine given and to the individual receiving it. For example, several live viral vaccines contain antibacterial agents and thus cause side effects in those individuals with hypersensitivity to such agents (Ada, 1990). Also with inactivated vaccines there is the need to ensure the product does not contain any live organisms while with attenuated vaccines the risk is that advantageous agents in the cells or medium may revert to virulence (Kabanov et al., 1998). They should for example not be administered to pregnant women because of possible side effects in the foetus. Routes of administration can also be on a patient-to-patient basis as well as a vaccine-to-vaccine basis. For example patients with bleeding disorders should not be inoculated via the intramuscular route.

Further disadvantages associated with current vaccine protocols include the need in some cases to deliver two or three doses in order to provide immunity (O'Hagan, 1998). In developing countries, where successful immunisation is often most needed, this process of repetitive immunisation is often inadequate. For example in the case of the tetanus vaccine two or three inoculations are required to achieve full immunity. In many developing countries there are difficulties in achieving this due to low patient compliance which results in thousands of deaths every year from tetanus. The World Health Organisation (WHO) estimates this level of non-compliance to be approximately 70% of people in the developing world (Carino, 1999).

Another widespread limitation with current vaccines is the lack of suitable adjuvants as the only adjuvants which have been licensed for human use are those based on insoluble aluminium compounds. These are not universally effective and can induce IgE antibody responses. They may also fail to induce cell-mediated immunity. In order for vaccines based on adjuvant compounds to achieve protective immunity two or three doses of the vaccine is required. The spread of additional diseases such as HIV and hepatitis is also a danger in areas where sterile syringes are not freely available (O'Hagan, 1994).

There are currently 50 vaccine preparations available in the United States however these are aimed at a small minority of infectious diseases (Carino, 1999). Novel immunisation techniques are currently being pursued in an effort to produce vaccines which rely on

lower concentrations of antigen to produce effective immune responses and which are stable, heat insensitive and which ultimately would confer immunity to a wide range of infectious agents (O'Hagan, 1998).

2. 3 Novel delivery strategies for vaccines

The objectives of the World Health Organisation (WHO) in the development of novel vaccines include improving the design and delivery of vaccines, the development of successful vaccines where previous therapies have been unsuccessful, the development of increased potency and improved delivery routes and the targeting of populations at risk such as neonates and immunodeficiency patients. New techniques in the area of molecular biology and genetics offer the opportunity to overcome the shortcomings of traditional vaccines and attempt to address the objectives of the WHO (Ada, 1990). This is most evident in the area of mucosal vaccination.

Much of the early work on vaccine development has concentrated on the induction of antibody-based humoral immunity toward specific pathogens (Hilleman, 2000). This type of immunity is most effective against organisms that have a significant presence in the blood stream. However, a great deal of recent work has focused on the development of new vaccines that induce mucosal immunity (Carino, 1999). Mucosal surfaces (gastrointestinal, respiratory, vaginal etc) are the leading routes of infection into the body but these tissues are also the biggest source of immunocompetent cells in the body (Kiyono et al., 1996). The cells present in the mucosal surfaces and the surfaces of the respiratory and gastrointestinal tracts are able to detect, present and process a range of antigens and mount specific local immune responses in reply.

Mucosal vaccine delivery systems such as those which use the intranasal, oral, inhalation, rectal and ocular routes offer many advantages over conventional parenteral vaccine routes. These advantages include ease of administration, low cost, vaccine stability and patient compliance. Mucosal delivery of vaccines would avoid the pain and discomfort associated with injections and would also eliminate the possibility of infections caused by inadequately sterilised needles or re-use. Mucosal immunisation would appear also to be very attractive in the elderly and in the very young as, unlike the systemic immune system,

the mucosal immune system does not suffer age related dysfunctions as in the elderly and in the very young the mucosal immune system develops before the systemic immune system (O'Hagan, 1998). Consequently there is considerable interest in the development of novel delivery systems for mucosal and especially oral administration of controlled release vaccines and most work in this area has been focused on trying to immunise the GALT or BALT by delivering the antigenic material in liposomes or in biodegradable microspheres (Carino, 1999).

2.3. 1 Liposomes as vaccines

Liposomes are composed of concentric lipid membranes containing biodegradable lipids such as phospholipids separated by an aqueous compartment (Ada and Ramsey, 1997). Antigens may be adsorbed onto the surface of a liposome or incorporated into it depending on the nature of the liposome. Liposomes are not generally amenable to oral delivery as they are rapidly degraded by bile and other enzymes present in the gastrointestinal system. The use of a polymerised liposome in which the phospholipid molecules of the lipid bilayer are cross-linked by covalent bonds has been reported (Clark et al., 2001). This renders the liposome amenable to oral vaccination while at the same time degradable within the phagocytotic cells of the immune system. Most oral vaccines are targeted to phagocytotic cells, most notably M cells, which, following the endocytosis of these particles, would stimulate the host immune system. The development of a polymerised liposome structure was reported by Clark et al. (2002) to target DNA directly to the M cells of the Peyer's patches. Limitations associated with liposomes include their instability during storage and the relatively high cost of manufacture when compared with oil-in-water emulsions.

2.3. 2 Microparticulates as vaccines

PLGA and related polymers have been used in pharmaceutical products for several years particularly in the areas of resorbable sutures and other materials (Jones, 2000). They have also been used in controlled release drug delivery systems because of their physicochemical properties (Eldridge and Staas, 1991; Jones, 1997; Singh et al., 2000; McKeever et al., 2002).

PLGA polymers have also been used in the production of a wide range of immunostimulating vaccines. Table 2-2 outlines just a few examples of the wide range of antigens and other molecules which are amenable to PLGA encapsulation.

Antigen	**PLGA resomer**	**Route of delivery**	**Reference**
Ovalbumin	RG 502	Subcutaneous	(Uchida et al., 1994)
Tetanus Toxoid	RG 502	Subcutaneous	(Raghuvanshi et al., 2001)
Synthetic malarial peptides	RG 502 RG 752	Subcutaneous	(Men et al., 1996)
Ovalbumin	RG 503	Intraperitoneal Oral	(O'Hagan et al., 1993)
Cholera toxin		Oral	
Staphylococcal enterotoxin B		Intraperitoneal	(Eldridge and Staas, 1991)
Luciferase plasmid		Oral	(Jones et al., 1997)
Beta galactosidase Plasmid	RG 502	Intramuscular Intravenous	(McKeever et al., 2002)
HIV-1 pCMVkm p55 gag plasmid	RG 502	Intramuscular	(Singh et al., 2000)
Luciferase plasmid			
HIV-1 gag plasmid	RG 502	Intranasal	(Singh et al., 2002)

Table 2-2 Examples of proteins and antigens in PLGA microspheres evaluated for immune system response

The properties of PLGA as a vaccine adjuvant were demonstrated as early as 1991 by Eldridge and Staas. They used PLGA to construct a toxoid vaccine of staphylococcal

enterotoxin B which was administered intraperitoneally to mice. The study involved administration of free toxoid vaccine of staphylococcal enterotoxin B or an encapsulated vaccine of the same. Following primary inoculation with the same dose they observed IgG titres which were approximately 80 fold higher in the mice inoculated with encapsulated vaccine when compared with those inoculated with free vaccine. This response fell after 40 days post inoculation in the mice with the free vaccine but was maintained through to day 90 post inoculation in those mice inoculated with the encapsulated vaccine. Following secondary immunisation both vaccines elicited an immune response but the microencapsulated form maintained its level at 32-64 times that of the free vaccine. They also found that higher levels of toxoid could be delivered in an encapsulated form.

Eldridge and Staas (1991) also examined the effect of microparticulate size on the release characteristics of the toxoid vaccine and the resulting immune response upon intraperitoneal administration. They used particles of less than 10μm and reported antibody titres of plasma IgG from day 10 which rose maximally on days 30 and 40 and then fell through day 60. This was in contrast to the response produced by particles of greater than 10 μm which was not detected until day 30 and rose after this on days 50 and 60. Co-administration of the two sizes of particles produced antibody titres up to day 30 which were similar to the response with particles of less than 10μm. After this time however antibody titres rose higher then the additive amount of responses of both particles suggesting a synergistic effect. Uchida et al., (1994) also studied the effect of microparticulate size on the immune response. The immune system response in relation to particulate size was greatest when particle sizes fell between 3.5 μm and 7 μm. Hence it was concluded that the response of the immune system was enhanced by smaller particle size.

More recent studies have reported enhanced immune response following subcutaneous injection of nanoparticulates containing tetanus toxoid (TT) in combination with alum (Raghuvanshi et al., 2001). Alum has long been used in conventional vaccines as an adjuvant and was used in this study in conjunction with PLGA (50:50) loaded nanoparticles. Greater immune responses were noted when animals were inoculated with the combination vaccine when compared with those inoculated with controls. Raghuvanshi

et al., (2001) suggested that the presence of the alum played a significant role in boosting the adjuvant effect of TT nanoparticles.

The benefits associated with polymeric delivery systems include the range of polymers from which they can be prepared which can be designed to delay the gastric transit of the vaccine or to target the vaccine to the mucosal associated tissues (MALTs) of the Peyer's patches (O'Hagan, 1998).

Mucosal administration and in particular oral delivery continues to be the most attractive route for the delivery of all medicinal agents (Singh and O'Hagan, 1998). However this route has not been used extensively in vaccination despite oral delivery of other pharmaceuticals constituting 60% of drug administration. Only a few current vaccines are administered orally including the polio vaccine and a live typhoid vaccine (Ty21a) (British National Formulary, 2001). The ultimate aims of orally delivered vaccines are (1) the protection of the antigen against degradation form gastric enzymes (2) the enhanced uptake of the particles in to the mucosal tissues and (3) the reduction of the concentration of antigen required to stimulate an immune response.

2.3. 3 Microparticulates as oral vaccines

The ability of polymeric microparticles to induce enhanced antibody responses to a number of different antigens following administration via a number of different routes has been known for some time (Uchida et al., 1994; Men et al., 1996; Johnstone and Barry, 1997; Raghuvanshi et al., 2001). However, mucosal delivery of vaccines is an attractive prospect since delivery by this route would stimulate mucosal immunity at the sites where pathogens initially infect hosts. The successful exploitation of microparticles as oral vaccines is dependent on their uptake by the M cells of the intestinal Peyer's patches following oral administration (O'Hagan, 1994). The first determination of the possible uptake of a microparticle is dependent on its size.

Sanders and Ashworth (1961) demonstrated that following oral administration of a polystyrene latex particles solid particles of 2200 Å in size could be absorbed through the intestinal wall. This was achieved following the oral administration of a concentrated emulsion of polystyrene latex particles of 2200 Å in size (220 μm).

Eldridge et al. (1989) studied the size-dependent uptake and reported that orally-administered PLGA microparticles of $\geq$ 5 μm were taken up and remained in the Peyer's patches for 35 days stimulating the mucosal immune system through the release of antigen in the Peyer's patches. It was also reported that microspheres of less then 5 μm extravasated the Peyer's patches in macrophage cells and stimulated the systemic immune system. This data suggests that the type of immune response stimulated could be controlled via the particle size of the administered microspheres which in turn is taken up by particular cell types.

Further studies by Jenkins et al. (1994) studied the effect of range of particle sizes on uptake of particles across the intestinal wall. A range of fluorescent polystyrene particles 0.15, 0.5, 1, 3, and 10 μm in size were used. Polystyrene samples were administered intraduodenally in the region of the Peyer's patches of the test animals. Mesenteric lymph fluid was collected at various intervals following administration. The Peyer's patches and the mesenteric lymph nodes (adjacent to the cannulated lymph duct) were surgically removed from the animal and were digested with 1% potassium hydroxide. Microspheres from the lymph fluid and digested Peyer's patches and lymph nodes were recovered and detected using flow cytometry. Results indicated that absorption through the intestine to the Peyer's patches and lymph nodes into the lymph fluid was rapid with microspheres of all sizes being detected 5 minutes post administration. Smaller particles sizes of 0.15, 0.5 and 1 μm displayed a biphasic adsorption pattern across the intestinal epithelium into the lymph nodes. The first phase of this was reported to occur between 5 and 30 minutes post administration followed by a later period of uptake between 60 and 70 minutes. The larger particles, 3 and 10 μm, only presented early phase absorption. It was suggested that the absorption pattern through the Peyer's patches was mediated by a transcellular process, which involved membrane bound vesicles (possibly involving receptors) or vacuoles. They also reported that the larger particles (3 and 10 μm) were retained in the Peyer's patches for longer then the smaller ones, which were extravasated to other organs. This data could be used in the specific targeting of particles to certain organs for retention or for systemic delivery via the lymph duct depending on the size. Microparticles of less than 10 μm have been used to target antigens to the GALT or Peyer's patches while microparticles greater than 10 μm are generally used to target antigen to the gut and protect them from gastric enzymes (Singh and O'Hagan, 1998).

Desai et al., (1996) investigated the role of particle size on uptake of biodegradable microparticles. PLGA (50:50) microspheres of various sizes were manufactured containing bovine serum albumin following a double emulsion/solvent evaporation technique. The particles produced were 100 nm, 500 nm, 1 μm and 10 μm in size. It was reported that microparticulate uptake by the Peyer's patches was 15 to 250 fold higher for the 100 nm particles than any other and that uptake by the Peyer's patches was 2 to 200 fold greater than into non-Peyer's patch tissue. Mucosal structures such as the Peyer's patches can be distinguished from surrounding epithelium by the presence of specialised antigen sampling cells called M cells (Clark et al., 2001).

O'Hagan (1998) reported two main implications for the uptake and transport of antigens by M cells. Antigens transported by M cells would escape degradation and would be released into the immune inductive environment of the Peyer's patches. Jepson et al. (1993) carried out experiments in which the binding of polystyrene and PLGA microspheres to M cells was compared. Results showed that although binding of the PLGA microspheres to M cells was lower then that seen with the polystyrene microspheres, the proportion of bound particles, which were translocated across the membrane, was higher in the case of PLGA particles.

Uptake of microparticles by the Peyer's patches through M cell translocation appears to involve the microvillii on the surface of the M cells (Ermak and Giannasca, 1998). Contact of microparticles with these microvillii is followed by a rapid phagocytosis through the apical cell membrane. This is then followed by transport through the cell to the basolateral cell membrane where the phagosome fuses with the membrane and its contents are "emptied" into the surrounding intraepithelial pocket. Ermak and Giannasca (1998) reported transport times of approximately 30-60 minutes from the apical surface of M cells to the basolateral pockets containing lymphocytes. The immune response following immunisation with ovalbumin encapsulated in PLGA and the soluble antigen by itself were compared. Animals were inoculated via intraperitoneal and oral routes with a primary inoculation followed by a booster injection. Marked differences were reported following oral immunisation in salivary IgA response in encapsulated vaccine versus free vaccine. This finding was supported by findings following oral immunisation of cholera toxin in microparticles versus immunisation of cholera toxin alone. It was reported that following oral immunisation with cholera toxin alone there was no production of antibody cells.

However following immunisation with encapsulated cholera toxin antibody secreting cells were produced. Uchida et al. (1994) reported similar findings in antibody response in studies carried out with ovalbumin –loaded PLGA microparticles which was reported to be significantly higher then the saline/ovalbumin control. The group also reported findings of higher antibody response with increasing concentrations of antigen.

Uchida et al. (1994) also compared the immune response following the inoculation of test animals with PLGA microspheres containing ovalbumin via two different routes of administration. Administration was by subcutaneous injection or via oral administration. The microspheres were produced following a double emulsion -solvent evaporation process. They reported that administration via the subcutaneous route was more efficient at producing an immune response when compared with oral administration. They also related that as the numbers of microparticles increased during subcutaneous administration, so too did the immune response until it reached a maximal level, whereas, following oral administration, the immune responses were similar until 4.8 $x10^8$ microspheres were delivered, upon which the immune response increased.

The use of microparticulates in oral vaccination is of major interest in the area of pharmaceuticals because it holds considerable promise for the development of new vaccines and may overcome some of the shortfalls of conventional vaccination (O'Hagan, 1994). However the successful exploitation of microparticles as oral vaccines is dependent on several factors. Firstly the size of the particle should be such that it can be effectively taken up by the Peyer's patches. This has been documented by several researchers and it has been reported as a general rule that the smaller the particle the more likely it is to be taken up by the Peyer's patches (Desai et al., 1996). Eldridge and Staas (1991) showed that particles of less than 5 μm were taken up by the Peyer's patches. Secondly, the encapsulation procedure should be such that it maintains optimal integrity of the encapsulated agent. This is of particular importance to microparticles formulated using a double emulsion technique as during the encapsulation procedure antigens are usually exposed to potentially denaturing conditions such as high shear speed, high temperatures and organic solvents (Ada and Ramsey, 1997). The benefits associated with the microencapsulation of antigenic material over conventional vaccines are numerous and include protection of the antigenic material against degrading enzymes found in the gut, the ability, through the selection of suitable

polymeric material, to develop controlled release of the encapsulated material and the potential to target particles to different areas in the body.

2.3. 4 Miscellaneous techniques

Novel vaccination techniques include the use of subunit vaccines, synthetic peptides and the generation of vaccine antigens by mutagenesis, chemical conjunction and genetic reassortment to name but a few (Table 2-3).

Approach	**Candidate vaccine**
Subunit vaccine	*H. influenza, N. meningitidis, S. pneumoniae.*
Synthetic peptides	*V. cholerae* toxin, HIV V_3 loop, Group A *Streptococcus.*
Nucleic acid vaccines	Influenza A, Hepatitis B.
Mutagenesis	*V. cholerae*
Reassortments	Influenza, rotavirus

Table 2-3: Examples of novel biotechnology approaches to vaccine development (adapted from Kiyono et al. (1996))

Other novel approaches to vaccination have been successfully exploited, such as the use of interferons especially interferon gamma-1b in chronic granulomatous disease. Commercial products include Immukin® (Boehringer Ingelheim) (British National Formulary, 2001).

Polysaccharide products of culture grown viruses have been used in the preparation of vaccines against H. influenza and N. meningitidis along with other purified products. According to Kiyono et al. (1996) these products have not been used in mucosal immunisation prior to 1996. Synthetic peptides do not require live organisms for their synthesis and are therefore of significant importance in novel techniques in vaccination.

Site directed mutagenesis employs restriction enzymes which cleave DNA at restriction sites in order to facilitate the removal of a portion of DNA and its replacement with mutant DNA. Nucleic acid mediated immunisation refers to the induction of an immune response to

a protein expressed *in vivo* subsequent to the introduction of its encoding polynucleotide (DNA or RNA) (Davis and Whalen, 1995).

The most promising area in the development of novel immunisation techniques involves the encapsulation of DNA and other nucleic acids to form a nucleic acid vaccine. This type of vaccine should preserve all the positive aspects of existing vaccines while avoiding their risks (Weiner et al., 1999).

Chapter 3

Gene Therapy

3. 1 Introduction

Long before the discovery of the gene our species practised genetic manipulation through the breeding of certain animals or plants for particular purposes. These methods of selective breeding were the first studies in the science of modern genetics. The First International Congress of Genetics was held in London in 1899 but was called the International Congress of Hybridisation and Cross-Breeding of Varieties. Wolff and Lederberg (1994) define gene therapy as the "application of genetic principles to the treatment of human disease" and they go on further to say that "more specifically the term gene therapy unites pharmacotherapeutic with genetic principles, implicating the use of polynucleotide to treat the disease state".

Several key dates are important in the evolution of gene therapy. The first of these is the conception of a protein receptor in 1878 by Langley. This was then extended and the molecular understanding between protein:receptor interactions was elucidated bringing about the development of rational drug design based on targeting of the receptor. Another important milestone in gene therapy was the discovery that a gene could be transferred within nucleic acids in 1944 by Avery, MacLeod and McCarthy. Another important date in gene therapy was the elucidation of the structure of DNA by Watson and Crick in 1953. Since then the area of gene therapy has been moving steadily towards the production of the optimum drug, a concept which is well summarised by Aposhian in 1970 " if one considers the purpose of a drug to be to restore the normal function of some particular process in the body, then DNA would be considered to be the ultimate drug".

More recently gene therapy has been defined as the alteration of a phenotypic expression through the alteration of the cell's genotypic content. A phenotype can be defined as the appearance or other characteristic of an organism, resulting from the interaction of its genetic constitution or its genotype with the environment (Lewin, 1994). Through the addition of a functional gene or the deletion of a pre-existing gene the behaviour of a target cell can be altered in order to achieve a desired therapeutic effect (Taneja et al., 1995) (Blakenstein, 1999).

There are two current approaches to gene therapy, which can be classified as follows.

Viral mediated gene transfer includes retroviruses, adenovirus and adeno-associated viruses. Gene therapy involving viruses involves the use of defective viruses as vector or "carrier" molecules for DNA. These defective viruses retain their ability to infect cells but instead of carrying viral genes they contain therapeutic DNA that can be introduced into cells. These viruses should not be capable of replication or of infecting the host cell with viral genes (Ledley, 1995). Non-viral mediated gene transfer encompasses naked DNA vaccination (DNA in a suitable carrier solution), polymer mediated DNA delivery and liposome mediated DNA delivery.

Traditional gene therapy is designed to lead to genetic modification of cells affected by disease but other approaches have been designed to target non-diseased cells such as the immune system cells constituting a form of nucleic acid vaccine (Kabanov et al., 1998).

3.1. 1Deoxyribonucleic Acid (DNA)

As human gene therapy and the use of DNA as a therapeutic agent becomes a potential reality in pharmaceutics greater emphasis must be placed on the characterisation and stability of nucleic acid based agents (Middaugh et al., 1998). Using DNA as a pharmaceutical agent poses a number of issues with regard to DNA structure, sequence, concentration and stability. The sequence of DNA used is of particular importance. The sequence of DNA is the backbone in the formation of proteins and polypeptides and even minor alterations in the sequence can produce vastly different proteins. DNA and RNA are composed of strands of nucleic acids. In the case of ribonucleic acid (RNA) there is only one strand and in the case of deoxyribonucleic acid (DNA) there are two.

Deoxyribonucleic acid is composed of two strands of nucleic acids joined together by hydrogen bonds (Lewin, 1994). Each strand is composed of a chemically linked sequence of subunits. Each subunit contains a nitrogenous base, which is a heterocyclic ring of carbon and nitrogen atoms, a pentose sugar and a phosphate group. The nitrogenous bases belong to one of two types, pyrimidines and purines. Pyrimidines are composed of a six membered ring of carbons and nitrogens while the purines are composed of fused five and six member rings.

In DNA the purines are adenine and guanine and the pyrimidines are cytosine and thymine and are referred to by their initial letters A, G, C and T. Two types of pentose sugars are present in nucleic acids and distinguish DNA from RNA. In DNA the sugar is 2-deoxyribose and in RNA it is ribose (Figure 3-1).

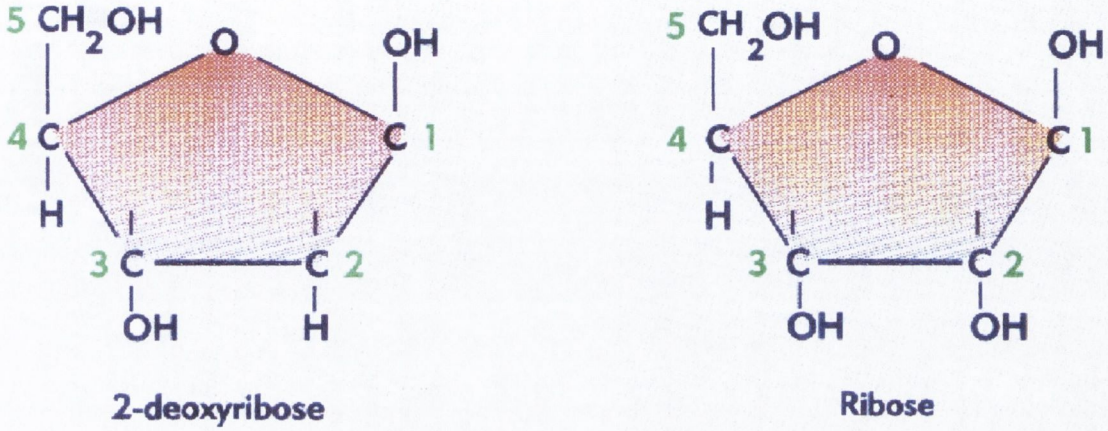


Figure 3- 1 Sugar groups present in DNA and RNA (adapted from Lewin, 1994)

A base linked to a sugar is called a nucleoside, when a phosphate group is added the base-sugar-phosphate is termed a nucleotide. Nucleotides constitute the building blocks from which nucleic acids are constructed. The nucleotides are linked together into a polynucleotide chain by a backbone consisting of alternating series of sugar and phosphate residues. The 5' position of one pentose ring is connected to the 3' position of the next pentose ring via a phosphate group. At one end of the nucleotide chain there is a free 5' group and the terminal nucleotide at the other end has a free 3' end (Lewin, 1994). When the two strands of nucleotides line up to form a DNA strand they run antiparallel to each other (in opposite directions) their bases bonding through hydrogen bonds (Figure 3-2). There are also specific rules concerning the pairing of the bases – guanine can only bond with cytosine.

DNA is most commonly found in what is known as the B-form, which is composed of double-stranded DNA with 10.5 base pairs per turn which has a 3.4° angle between bases. Other forms of DNA include a Z-form DNA, plasmid or circular pieces of DNA.

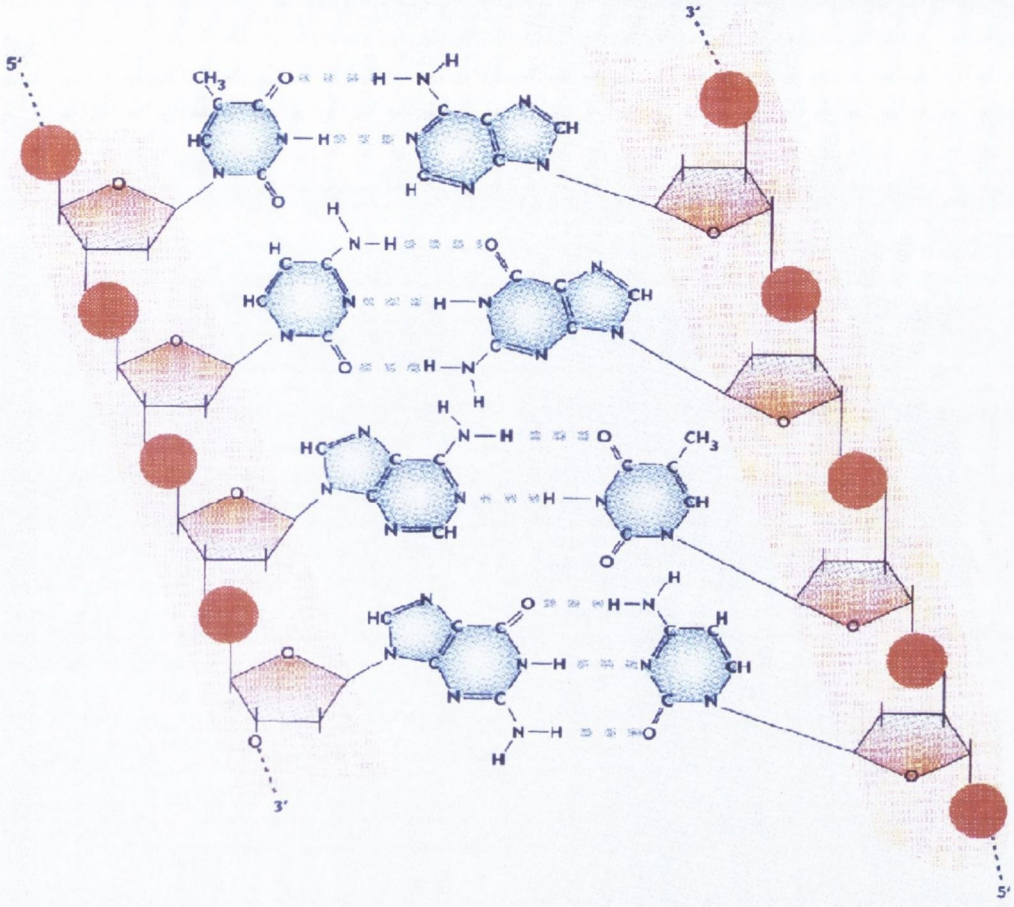


Figure 3- 2 Schematic of complementary base pairing (adapted from Lewin, 1994)

The central dogma, which defines the paradigm of molecular biology, is that "genes are perpetuated as sequences of nucleic acids but function by being expressed in the form of proteins" (Lewin, 1994). There are three types of processes that are responsible for the maintenance of DNA and for its conversion into a protein:

1. Information is perpetuated by replication: this is a process through which a double-stranded nucleic acid is duplicated to give identical copies.

Information is expressed by a two-stage process:

2. Transcription generates a single-stranded RNA identical in sequence to one of the strands of the duplex DNA.
3. Translation converts the nucleotide sequence of the RNA into the sequence of amino acids comprising a protein.

3.1. 2 Preparation of plasmid DNA (pDNA)

DNA in a plasmid form is one of the most common vectors used in gene therapy (vector in this instance referring to DNA which can carry pre-selected genes) (Hasan et al., 1999). Bacterial plasmids are isolated as covalently closed circular DNA molecules in negatively supercoiled forms. They contain restriction sites, which are sites where specific restriction enzymes can cut the plasmid DNA allowing for the addition of therapeutic genes into the plasmid. Plasmids are naturally occurring circular duplex DNA ranging in size from two-kilo bases to several hundred-kilo bases. They are extrachromosomal (not part of the organism's chromosomes) and are capable of independent replication. In many *in vitro* models the plasmids, are used as reporter vectors which contain genes (Figure 3-3), which code for a product, which can be easily detected. Examples of these are luciferase vectors, which once translated produce luciferase proteins, which in turn can catalyse a luminescent reaction, which can be measured using a luminometer. Other examples include β-galactosidase vectors, which produce a β-galactosidase enzyme whose expression can also be measured using a spectrophotometric assay.

Reporter vectors can be cleaved at a variety of sites by restriction enzymes allowing for the insertion of desired genes (Promega Handbook, 1996). They also often carry genes conferring antibiotic resistance (Hasan et al., 1999). For example some luciferase vectors contain genes which confer ampicillin resistance on the vector. Selective propagation in bacterial cells is achieved through the addition of the antibiotic into the growing medium and in antibiotic containing agar plates. This ensures that only those bacterial cells containing and expressing the antibiotic resistant plasmid will grow.

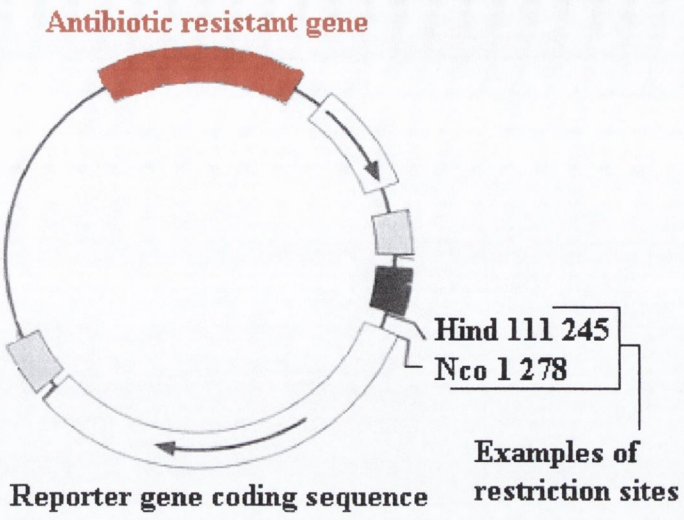


Figure 3- 3 Example of a typical plasmid DNA with an antibiotic resistant gene, restriction sites and a reporter gene coding sequence.

Plasmid DNA is typically prepared by the insertion of the desired plasmid into bacterial cells and the plating out of the bacteria onto antibiotic selective plates, which ensures the propagation of only those cells, which contain the desired DNA. The bacterial cells are then propagated overnight in agar broth, which results in the replication of the plasmid DNA (QIAGEN Handbook, 2000). This is followed by a purification step in which the bacterial cells are lysed open to release the DNA. In general the plasmid DNA is then separated from chromosomal DNA using an ion-exchange column. The DNA is then washed from the column and precipitated following the addition of isopropanol and ethanol, which binds to the DNA and condenses it. The resultant pellet is then resuspended in a suitable medium and stored at -80°C.

Since DNA is prepared using bacterial cells, contamination of the DNA by bacterial products is also an issue that must be considered before the DNA can be used in any delivery system. Endotoxins, also known as lipoplysaccharides, are found in the cell membrane of Gram-negative bacteria such *E.coli,* which are commonly used to propagate DNA. Bacterial cells shed large amounts of endotoxins into the surrounding environment as they die strongly influencing the transfection efficiency of DNA in cells. Endotoxins have also been reported to cause fever, endotoxic shock syndrome and activation of the complement cascade in humans and animals (QIAGEN Handbook, 2000). It is therefore essential that they are removed if the DNA is to be used for gene therapy or in any preparation destined for human or animal use. In non-endotoxin free kits the DNA is co-

purified with the endotoxin however, in endotoxin-free plasmid preparation kits there is an endotoxin-removal step integrated into the purification step which prevents the binding of the endotoxins to the wall of the ion-exchange column and allows purification of the DNA with minimal levels of endotoxin (QIAGEN Handbook, 2000).

Preparation of the plasmid is of great importance because cutting one or two of the DNA strands would cause the loss of supercoiling generating an open circular form or the formation of a linear form of DNA (Middaugh et al., 1998). Plasmids themselves once formed can also become entangled with other plasmids, which can cause further breakages.

The manipulation of plasmid DNA for use in pharmaceutical systems incurs certain restrictions. These include the ability to determine concentration, structure, physicochemical properties, stability of oligonucleotides and risk of contaminants since they are grown up in bacterial cells. As with any other pharmaceutical agent the formulation of DNA based drugs needs to be designed in such a way that the DNA remains stable and in its active form for as long as possible. A time-dependent alteration in the structure or stability of DNA is undesirable therefore the DNA should be formulated with an agent that can protect it from common processes such as oxidation and deamination (Wang et al., 1999). This can be overcome by using polymers such as polyesters during formulation of DNA based drugs (Hao et al., 2000).

3.1. 3 Qualitative analysis of DNA

Visualisation of DNA is possible using dyes such as ethidium bromide or 4', 6-diaminidino-2-phenylindole (DAPI) dye. Ethidium bromide is highly carcinogenic and toxic but can be used successfully in conjunction with confocal microscopy to view the position of DNA within microspheres. Ethidium bromide is a double stranded DNA intercalator, which binds with little or no sequence preference at a ratio of one dye per 4-5 base pairs of DNA (Figure 3-4) (Haughland, 1996). Excitation of bound ethidium bromide occurs at 518 nm and is 20 to 30-fold higher then non-bound ethidium bromide. The emission wavelength of DNA-bound ethidium bromide is 605 nm and when viewed under these conditions the bound ethidium bromide fluoresces.

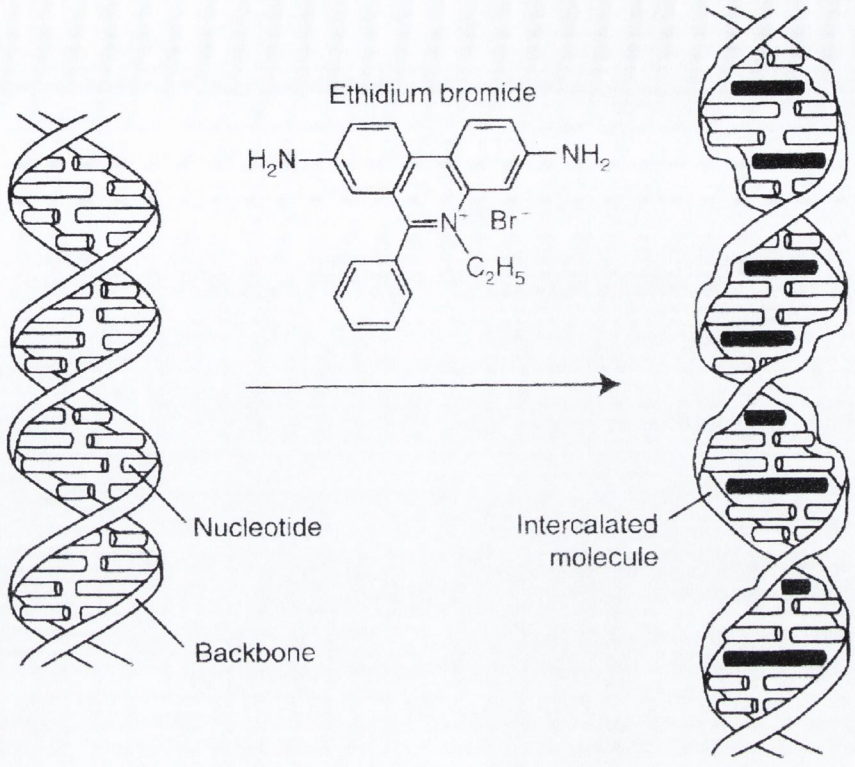


Figure 3- 4 Schematic of ethidium bromide as a double stranded DNA intercalator

3.1. 4 Quantitative analysis of DNA

DNA concentration can be estimated spectrophotometrically since it shows good absorbance at 260 nm but this is not specific to DNA as RNA and certain proteins also absorb around this wavelength. Absorption can be affected by contaminants such as by-products of polymer degradation and is therefore not entirely accurate. Commercially available methods of quantifying DNA include the use of dyes, which fluoresce. Examples of these include PicoGreen (Molecular Probes) and a Hoechst fluorescent dye which when used with a fluorometer can detect levels of double stranded DNA (dsDNA) as low as 25 pg/ml and are specific for double stranded DNA (Singer et al., 1997).

Another method for semi-quantitative measurements of DNA concentration and structure is polymerase chain reaction (PCR) which amplifies minute quantities of DNA into thousands of copies of the same sequence hence allowing the detection of even the smallest traces of DNA and the determination of their configuration following agarose gel electrophoresis.

Another semi-quantitative method of DNA analysis is the use of mammalian cells which can be used as a host for the expression of foreign genes. They are commonly used to confirm that genes which are cloned into vectors maintain their ability to produce the reporter protein. The plasmid DNA is introduced into the mammalian cell line either through a process of electroporation or through chemical mediated transfection. The mammalian cell line then translates the foreign DNA and produces the protein of interest which is generally easy to detect, for example luciferase or β-galactosidase whose production can be measured by simple techniques as described above. The expression of such proteins is transient and lasts a few weeks before the reporter DNA is lost from the cells. However this technique is invaluable in determining the efficacy of the DNA which is based on its structure and concentration.

3. 2 Viral mediated gene therapy

Viruses are natural carriers of DNA from cell to cell and are composed of proteins and nucleic acids. Viral nucleic acids are tightly packed and are protected by proteins in what is known as a viral envelope. The mechanisms of DNA delivery into host cells vary depending on the viral family they belong to and may also vary within members of the same family (Kabanov et al., 1998). Some steps are common to all viruses although the method through which these steps occur may vary. The first step in viral infection is to attach itself to the host cell membrane through cell surface receptors. The presence of specific receptors on the cell surface is important in determining the host range and cell tropism of a virus. Subsequent steps include membrane penetration and nuclear targeting and translocation across the nuclear pore complex. These initial steps employ not only different surface receptors but also different cellular components depending on the virus involved and the structure of the virus is altered following each process.

3.2. 1 Retroviruses as vectors for gene therapy

Retroviruses were among the first viruses to be used in gene therapy (Kabanov et al., 1998) (Figure 3-5). They are RNA viruses, which propagate via conversion into DNA (Lewin,

1994). Following infection retroviruses permanently insert their genes into host genomes and only infect replicating cells.

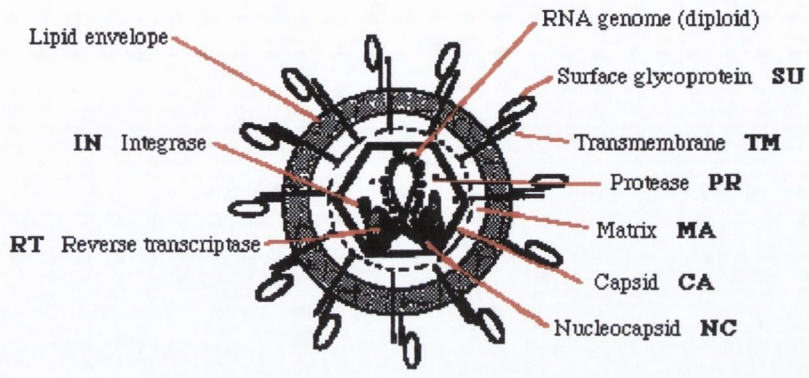


Figure 3- 5 Diagram of a retrovirus (reproduced from www-micro.msb.le.ac.uk/335/Retroviruses.html)

Early studies using retroviruses as DNA vectors include one carried out by Wolff et al., (1987). Wolff used a retroviral vector expressing a mutagenised but fully functional human hypoxanthine guanine phosphoribosyl transferase (HPRT) to transfect hepatocyte (liver) cells. He reported human HPRT levels in transfected cells of 10-25% exogenous levels. It was noted that hepatocyte infection with the retroviral vector was possible only at certain stages of the cell cycle. Wolff also reported that previous attempts at insertion into hepatocytes with other therapeutic means such as DNA mediated electroporation led to inefficient gene transfer. Complications associated with retroviruses include the inability to modify or terminate treatment in response to any side effects or cure and random integration of the therapeutic gene leading to the possibility of oncogene activation or to the inhibition of tumour suppressor gene (Tomlinson et al., 1996).

3.2. 2 Adenoviruses as vectors for gene therapy

Adenoviruses are nonenveloped, linear, double stranded DNA viruses which are responsible for a variety of different clinical diseases in humans including respiratory infections, conjunctivitis, hemorrhagic cystitis and gastroenteritis (Figure 3-6) (Wolff, 1999). Their role in gene therapy is based on the observation that adenoviruses infect non-dividing cells and do not integrate their genes into the host genome. The genes are maintained in the host genome and are only expressed for a finite period (Ledley, 1995).

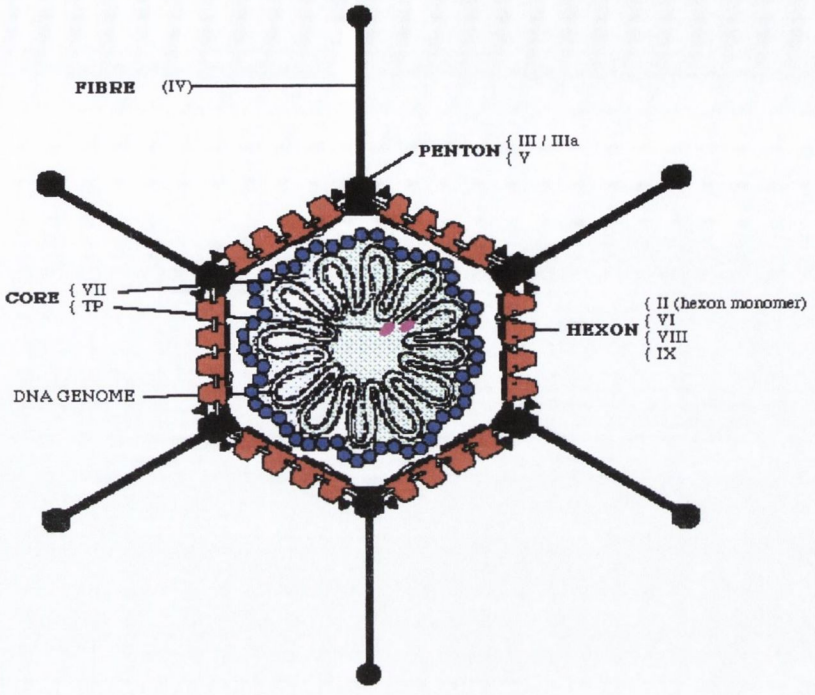


Figure 3- 6 Schematic representation of an adenovirus (reproduced from www-micro.msb.le.ac.uk/335/Adenoviruses.html)

Adenoviruses were first used in gene therapy in the search for a therapy for cystic fibrosis. Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein which functions as a chloride channel in epithelial cells. Mutations in this protein lead to defects in chloride transportation across these cells. Mutants of two different adenoviruses were constructed carrying a complementary copy of the sequence of DNA needed to produce a normal CFTR protein. Both were used in phase 1 clinical trials but have so far failed to confer any benefits to the patient (Wolff, 1999).

Other examples of adenoviruses include adenoviruses 2 and 5 which enter host cells by attaching through an immunoglobulin gene family protein and it enters the cell using clathrin dependent endocytosis. The final steps of viral integration involve the shedding of protruding fibres from the capsule head, which contains the viral DNA. The capsule then penetrates endosomes and is weakened by the presence of endosomal enzymes. This weakening allows for the release of the viral DNA and its importation into the host cell nucleus (Kabanov et al, 1998). Other viruses such the hepatitis B virus which is an enveloped virus, enters the host genome through a more complicated process. The viral particle attaches to a specific surface receptor and is internalised into the cell and

transported to the cell nucleus. Its DNA is transcripted through the enzyme RNA transcriptase and the newly formed viral RNA is packaged into nucleocapsids in the cytoplasm. Through a process know as reverse transcriptase viral RNA is copied into double stranded DNA. Virus particles bud through internal membranes and are released into the extracellular membrane by vesicular transport (Kabanov et al., 1998). As the examples of viral infection of host cells shows, viruses are extremely efficient at host cell penetration. In conjunction with their own inherent mechanisms viruses are also able to exploit the environment and molecules present in the host cell. The use of adenoviruses in clinical trials has triggered immune and inflammatory problems due to their toxicity, which may also inhibit the level and persistence of gene expression (Ledley, 1995). Even though these viruses have been designed to be non-replicating there is a potential risk of generating infectious, replication-competent virus during the production or use of viral vectors for gene transfer (Tomlinson et al., 1996).

3.2 3 Adeno-associated viruses as vectors for gene therapy

Adeno-associated viruses are capable of permanent integration into a host genome and target both dividing and non-dividing cells. Wild-type adeno-associated viruses are capable of site-specific recombination but modified viruses used in gene therapy are not and therefore randomly insert their genes into the host chromosome (Miller, 1992).

3. 3 Non-viral mediated gene transfer

There are several current approaches to non-viral delivery. These include *in vivo* and *in vitro* naked DNA transfer, polymer mediated DNA delivery, liposome mediated delivery of DNA, microinjection of DNA, particle bombardment and electroporation.

3.3. 1 Naked DNA transfer

Injections of naked DNA intramuscularly have been successful in expressing viral antigens and establishing humoral immune responses. This might indicate a role for simple intramuscular DNA injection in the creation of genetic vaccines (Wolff, 1997).

Wolff et al. (1990) confirmed the expression of reporter gene following direct injection of naked DNA and RNA in mouse muscle. They used expression vectors with genes for chloramphenicol acetyltransferase, luciferase and β-galactosidase and found significant levels of gene expression in the muscle tissue following staining and examination of tissue sections. Following DNA injection substantial amounts of luciferase expression was found for at least 60 days. It was reported that extraction of injected DNA from the host showed it to be present either in a circular extrachromosomal form or to be integrated into the host chromosome.

Further studies into the expression of plasmid DNA following intramuscular injection were carried out and it was reported that significant expression from plasmid DNA persisted in mouse muscle for at least 19 months post injection (Wolff et al., 1992). Polymerase chain reaction (PCR) studies were carried out, which is a more sensitive method or the detection of trace quantities of DNA, and showed that in some of the muscle DNA samples, plasmid DNA persisted for at least 19 months.

Because of the possible deleterious effects from DNA integration into host genomes a more sensitive method of DNA detection was used (Danko et al., 1994). In subsequent studies bacterial electroporation was used to determine whether the β-galactosidase vector had been successfully integrated into the host genome. The loss of β-galactosidase expression would have indicated disruption in the coding sequence of the gene. Plasmid DNA from muscle cells was transformed into bacterial cells following electroporation and colonies were plated out on agar plate. Cloned plasmid DNA was then purified from the resultant bacterial colonies and it was reported that the integrity of the plasmid was conserved and the plasmids were not altered in size. However, when injected into other tissues such as brain, liver, spleen, uterus, stomach lung or kidney no significant levels of gene expression were observed (Danko et al., 1994). However myocardial muscle did show expression levels comparable with skeletal muscle indicating that cardiac muscle is able to take up and express plasmid DNA. However this expression was only stable for two weeks.

This was in agreement with an observation forwarded by Jiao et al. (1992) in which it was hypothesised that an intrinsic feature of muscle such as its multinucleated fibres enables

foreign DNA to persist once it enters the cell. This hypothesis was tested in 1992 by Jiao et al. who used brain cells, which share "the non-dividing or slowly dividing property of skeletal myofibers but are not multinucleated". Three plasmids vectors each expressing the firefly luciferase gene but under the control of different promoters and a plasmid expressing β-galactosidase were used to monitor transfection efficiency. A cationic lipid-DNA formulation and a novel lipid: cholesterol (1:1)-DNA formulation was used to transfect cultured cells from foetal brain cortex. The transfected cell suspension (25×10^4 to 50×10^4 cells) of cerebral cortex was injected into the host rat brain. Results showed that cells transfected with the novel transfection agent had significantly higher expression levels then the original lipid: DNA formulation. The reasons underlying this result were not clear. It was reported that foetal neurons were successfully transfected *in vitro* and were able to survive in host brains. Expression levels in adult brains, which were transplanted with the transfected cells, were dependent on the reporter vector and the transfection agent used. Luciferase activity was still detectable 60 days post transplantation with foetal cells transfected with a pRSVL vector. The presence of these transfected cells was suggested to be due to the stability of transfected plasmid DNA in the neurons. A further possibility was that the non-dividing nature of the neuronal cells allowed the plasmid DNA to persist in these cells for long periods (Jiao et al., 1992). This suggested that it is the slow or non-dividing nature of cells such as skeletal, cardiac and now neurons, which allows for the persistence of DNA expression following naked DNA injection.

Further studies which evaluated DNA administration via the parenteral route reported that treatment of the muscle with 0.75% bupivacaine (mg/ml) prior to DNA injection resulted in expression levels which were 40-fold that of DNA injection alone (Danko et al., 1994). Bupivicaine is a local anaesthetic, which selectively destroys myofibers in muscle tissue and thus induces muscle regeneration.

In vivo injection of naked DNA into different tissue types and using a number of different DNA vectors and promoter genes has become more successful over the past few years. However despite studies which report gene expression nineteen months post injection, generating a sustained expression appears to be a problem. Herweijer et al. (2001) studied direct injection of DNA into hepatocyte cells and explored different explanations for the rapid loss of expression. These explanations included damage or death of cells due to the presence of intracellular DNA, increased cell cycling due to intravascular delivery of

plasmid DNA, loss of cell replication, promoter gene expression inactivation and stimulation of antigen-specific immune responses. It was reported that the loss of expression is a biphasic process in which the first phase lasts from day 1 until about day 7 with the second beginning thereafter. Firstly the delivery procedure itself damages the liver and results in increased cell cycling. This is coupled with transcriptional down regulation from immune system responses. After day 7 it is believed that the immune system again is responsible for loss or reduction in expression.

The effect and distribution of plasmid DNA following non-invasive nasal delivery of naked DNA was studied (Oh et al., 2001). It was reported that the liver was the site of highest biodistribution of nasally administered DNA and that localised plasmid in the brain was found at higher levels following this route of administration when compared with intravenous administration. This led Oh et al. (2001) to the conclusion that nasally administered DNA could prove to be a promising non-invasive route of DNA delivery.

3.3.1. 1 Cellular uptake of naked DNA

DNA expression in cells requires entry into the cell and transport to the nucleus (Budker et al., 2000). Wolff (1997) carried out studies using gold-labelled DNA which was found to be near or in the nuclear pore complex indicating that this was its entry point into the nucleus. Budker et al. (2000) presented three hypotheses in an attempt to explain cellular uptake of naked DNA. The first hypothesis involved large membrane disruption, which was initially suggested on the basis of early studies, which employed intraparenchymal injection to deliver the DNA. β-Galactosidase was administered and was found located around the site of injection. Muscle tissues, also in early studies, expressed the reporter gene at higher levels then any other tissue, which was reported to be due to the ability of the myofiber cells in muscle (where levels of expression are highest) to be able to withstand gross disruption of their membranes. However this hypothesis was rejected following intravascular injections of naked DNA where expression levels were found to be higher then those following intraparenchymal injection.

The second hypothesis is that DNA transport into the cell occurs through membrane pores which occurs naturally or as a result of the injection process. However following studies with Cy3-labeled plasmid DNA and hepatocyte cells it was reported that most of the DNA was located outside the hepatocytes immediately after injection and that cellular uptake took longer. Cy3 is a DNA intercalator and is used as a DNA fluorescence marker (Budker et al. 2000).

The third hypothesis that DNA is taken up into cells by a receptor-mediated process is based on several observations. Namely that there are a number of cell membrane receptors, which are postulated to be involved in the uptake of oligonucleotides and these could play a role in DNA uptake. "Scavenger" receptors are also potential candidates for DNA uptake following studies, which indicated that DNA uptake was inhibited by ligands of the macrophage scavenger receptor which include dextran sulphate. However researchers noted that further and definitive proof of this hypothesis would require identification of the receptor followed by the deletion of the receptor in knockout mice (animals with specific gene deletions) (Budker et al., 2000).

3.3. 2 Polymer mediated DNA delivery

Polymer mediated DNA delivery is employed in the development of sustained release drug systems for DNA. These systems have been used for various types of therapeutic molecules including recombinant proteins and peptides, vaccines and oligonucleotides (Heller, 1993). Encapsulating the naked DNA in a polymer confers a protection on the DNA against degradation from enzymes. It also allows the development of a time-dependent release mechanism on the DNA through the rate of degradation of the polymer, which in turn is controlled by the ratio of lactic to glycolic acid present in a PLGA polymer. PLGA also has the benefit that it degrades into lactic and glycolic acid moieties, which are naturally occurring in the body.

The therapeutic molecule is surrounded with a polymer and is released gradually as the polymer degrades. These systems, in addition to providing sustained effects, protect the active ingredients from enzymatic and hydrolytic degradation. Formulation of these systems depends upon the physicochemical and structural properties of the agent to be

encapsulated. The most commonly used polymers in this system are polyesters that include polylactides and polyglycolides which are usually formulated by an emulsion solvent evaporation technique. Other commonly used polymers include cationic polymers such as PEI and poly-lysine.

3.3.3 Lipid-mediated DNA delivery

Lipids are molecules which contain a hydrophilic and hydrophobic unit and therefore form ordered structures in aqueous solutions (Kabanov et al., 1998). These structures are organised in such a manner that they maximise hydrophilic and minimise hydrophobic interactions. The solvation of the polar head groups and dispersive interactions of the hydrocarbon chains of the lipid molecules provide for the negative contribution to the free energy during the formation of those structures. The positive contributions to the free energy result from the repulsive interactions of the polar head groups, including steric and electrostatic repulsion. As a result lipid molecules self assemble into different microscopic structures such as bilayers and micelles (spherical, rod-like or disk-like), which, especially at high concentrations, can pack into macroscopically ordered phases such as lamellar, hexagonal and cubic. In addition, the morphology of these macroscopic phases depends on interparticle interactions, which can be repulsive or attractive. The latter forces are van der Waals while the major repulsive forces are electrostatic and steric forces. These amphiphilic systems can be macroscopically homogeneous and continuous or can be dispersed into colloidal suspensions.

The isotropic colloidal solutions of self-assembled lipid particles are most frequently used for complexing with nucleic acids in medical applications (Kabanov et al., 1998). Liposomes are not in a thermodynamically stable phase but rather a kinetically trapped system; this is inferred because liposomes must be prepared through homogenisation or extrusion methods thereby requiring energy.

3.3. 4 Further models of gene delivery

Microinjection of DNA into the cell nucleus results in random integration of DNA and occasional expression of recombinant genes. Particle bombardment with DNA involves the precipitation of DNA onto microparticulate projectiles, which are shot into the cell. The

"gene gun" is a result of studies carried out using microprojectiles and will be discussed in the section below on nucleic acid vaccines. Electroporation is a technique in which cells are exposed to a high voltage. In many cases >90% of cells in many cell lines are transiently transfected with the DNA (Kabanov et al., 1998).

3. 4 Nucleic Acid Vaccines

Nucleic acid mediated immunisation refers to the induction of an immune response to a protein expressed *in vivo* subsequent to the introduction of its encoding polynucleotide (DNA or RNA). The choice of tissue for gene transfer dictates the route of administration. There are many possible routes of administration of a nucleic acid vaccine. These include intramuscular (im), intradermal (id), intravenous (iv) and oral administration. Several factors including efficiency of delivery, stability of gene expression, targeted cell type and ease of administration all of which influence the choice of target tissue. Another factor influencing formulation of nucleic acid vaccines is that the easiest route of administration in terms of cost and patient compliance is oral administration. All of these considerations must be taken into account if nucleic acid vaccines are to function optimally. The development of DNA as a pharmaceutical tool, which would express therapeutic proteins *in vivo* and hence possibly generate immune responses is one of the potential advantages of using DNA as a pharmaceutical product and a potential vaccine (Middaugh et al., 1998).

Briefly plasmid DNA is "spliced" with DNA from a potentially antigenic source (Hasan et al., 1999). The plasmid acts as a carrier vehicle for the antigenic DNA which once inside the host cell is translated into antigenic peptides. The antigenic peptides are then delivered to the cell surface via the major histocompatibility complex molecules (MHC) I and II (Chapter 2), which in turn activate either a cell-mediated immune response and T cells in the case of MHC I or the humoral immune system and antibody production from B cells in the case of MHC II.

The first paper demonstrating the efficacy of using DNA as a vaccine was published in 1993 (Ulmer et al., 1993). It was reported that intramuscular injection of expression DNA vectors resulted in the uptake of DNA into host cells and the translation of this DNA into

proteins. It was suggested that this technique could be a way of introducing viral proteins into the antigen-processing pathway, which would result in the generation of cytotoxic T lymphocytes (CTL). The ability of injected complementary DNA (cDNA) encoding the viral protein influenza A nucleoprotein (NP) to generate specific CTL responses and to protect against subsequent challenges of influenza was studied. The cDNA construct was prepared and was injected intramuscularly into mice. Ulmer et al. (1993) reported the production of high-titre IgG antibodies in spleen cells of mice injected with NP DNA. This is thought to require helper T cells and they suggested that both MHC class 1 and MHC class11 processed NP expressed from the injected plasmid. It was also reported that NP DNA immunisation resulted in decreased lung titres and increased survival following exposure of the inoculated animals to sub-lethal lung infection with influenza when compared to non-inoculated controls.

3.4. 1 Advantages and disadvantages of nucleic acid vaccines

DNA vaccines have several advantages over more classical forms of immunisation. DNA vaccines have the possibility of resulting in life long expression of the encoded protein which would remove the need for successive and booster injections. They also would remove the need to use live or attenuated forms of micro organisms thereby removing the risk of side effects through infection by these organisms (Davis and Whalen, 1995). DNA stability is not affected by high temperatures making it a viable option in Third World countries where the reduced cost of nucleic acid vaccines would also be of great benefit (Ban et al., 1997). It was also pointed out that nucleic acid vaccines could bypass a phenomenon in which vaccine efficiency in infants is affected because of the presence of maternally derived antibodies (Siegrist, 1997). Siegrist suggested that maternal antibodies reduce the amount of antigen which is taken up by antigen presenting cells and that nucleic acid vaccines should be able to circumvent this decrease in antigen uptake and hence offer a more successful vaccine then traditional ones. Other benefits, which would be conferred with the introduction of nucleic-acid vaccines, would be the ability to treat several diseases with one injection by combining the DNA needed.

3.4. 2 Immunisation with naked DNA

Immunisation with naked DNA has been carried out through a variety of routes and through the use of a number of techniques. Routes employed with naked DNA include injections via intramuscular, intra-venous, intra-nasal (in) and subcutaneous (sc) routes. DNA can also be introduced into the body through a syringe or through gene gun techniques. Immunisation using nucleic acids is putatively a very promising and novel technology however little is known about the complex biological signalling mechanisms underlying this. Fynan et al. (1993) evaluated how different routes of inoculation with naked DNA affected the efficiency of DNA vaccines in terms of cell transfection and presentation to immune system cells. A route that is generally believed to take up and express DNA more effectively then others is the intra-muscular route. Tissues which present particles to the immune system cells more readily then others include those which are part of the mucosal system and which are also highly immunocompetent. Fynan et al. (1993) studied inoculation via intra-muscular (im), intra-venous (iv), intra-nasal (in), subcutaneous (sc), intraperitoneal (ip) and intradermal (id) routes. Plasmid DNA which encoded for an influenza virus hemaglutinin glycoprotein was diluted in saline as a vehicle for injection. This method of inoculation was compared with the same plasmid DNA delivered via gene gun. The subject animals were challenged with lethal levels of influenza one to two weeks after a second inoculation. It was reported that, with the exception of ip routes of injection, levels of survivors ranged from 67% to 95% of total animals in the case of DNA in saline. The route which produced the highest levels of survivors was the intramuscular route with 95% of animals surviving lethal challenge, with the group which were inoculated subcutaneously demonstrating the highest mortality (with the exception of those inoculated via the intraperitoneal route where survival rate was 0%). The group also studied inoculations via gene gun and reported levels of inoculation which relied on 250-2500 lower levels of DNA than inoculations of DNA in saline alone.

Johnstone and Barry, (1997) investigated several variables influencing genetic immunisation. These variables were the type of antigen used, the host animal, the method of immunisation and the concentration of DNA used in each injection. It was reported that the expression level of the DNA used was dependent on the antigen used. In terms of the variable of age they compared the gene expression in 4 week, 6 week and 10-week-old mice and reported that as the age of the animal increased the immune response, which was

directly correlated with the gene expression, decreased. When comparing inoculation method a gene gun delivery system and intramuscular injection of the DNA were employed. Two different DNA plasmids were used and it was reported that overall, the concentration of DNA needed to produce equivalent levels of gene expression was greater for the intramuscular route then for the gene gun method. This was not directly dependent on the type of DNA used but the degree to which the levels differed depended on the type of plasmid used. This difference in expression levels from one method to the other was attributed to the enzymatic degradation of the DNA following intramuscular injection which delivers the DNA extracellularly when compared with the gene gun method which delivers the DNA intracellularly. However the intramuscular route of immunisation allowed for the inoculation of a higher concentration of DNA when compared with the gene gun which was limited to 2.5 ug per shot due to clumping of higher concentrations of DNA. From this study factors that appear to affect DNA vaccination are (a) the type of response studied (b) the age of the subject (c) the route of inoculation and (d) the type of DNA used.

In more recent studies Hevey et al. (2002) compared the merits of naked DNA vaccination with more classical vaccine technology. They studied a filovirus known as the Marburg virus (MBGV), which, because of its high virulence and potential aerosol infectivity, can only be studied under maximum biocontainment. This virus has emerged from an unknown natural reservoir in Africa where there have been recent outbreaks of the disease. One incident occured in the Democratic Republic of Congo where it was estimated that 70-80% of infections were fatal. The vaccines used were a subunit vaccine, an inactivated whole virus, an attenuated virus strain, a recombinant alphavirus replicon and naked DNA. Classical vaccine approaches with formalin–killed MBGV have been shown to confer immunity to 40% of immunised guinea pigs and 50% of non-human primates. The DNA used coded for MBGV glycoproteins and was cloned from MBGV infected cells. The DNA was cloned into a plasmid with a cytomegalovirus promoter region and was precipitated onto gold beads, which were then delivered to skin cells of guinea pigs via gene gun. The other vaccine preparations such as the inactivated whole virus were administered subcutaneously to guinea pigs. All animals were then challenged with a plaque picked variant of MBGV lethal for guinea pigs. Of the animals which received the live virus it was reported that in certain strains of guinea pig the virus proved nonlethal, while in others it proved to be fatal to 2 out 10 animals. None of the animals treated with

the killed vaccine showed any clinical signs and none were reported to show any anti-MGBV-GP antibodies one month after infection. The recombinant virus vaccine only partially protected the animals. In animals inoculated with naked DNA antibody production was slow and minimal in comparison with other techniques. These researchers further noted that MBGV-GP delivered as a DNA vaccine via gene-gun prevented death in 8 of 10 guinea pigs. It was also reported that there was a 10-fold increase in anti-MGBV antibody responses 1 month after infection. The development of a feasible vaccination strategy must take into account the virulent nature of this disease and the future of a DNA vaccine to combat it would have to be studied further before it was considered as a method of MBGV vaccination.

3.4. 3 Immunisation with liposomes

Liposomes have been used in studies where DNA incorporated into liposomes constituting a vaccine, was targeted to mucosal tissue. Klavinskis et al. (1997) studied the effects on the mucosal immune system in response to immunisation with a DNA-liposome complex. A plasmid DNA was used with enhanced luciferase expression in nasal tissue in an attempt to establish a model immunisation strategy against the human immunodeficiency virus (HIV). The main sites of infection of the HIV virus in the body are the mucosal surfaces from where the virus disseminates to the lymph nodes. The main targets of an effective vaccine against HIV would be mucosal tissues with the aim of stimulating the immune system at these sites. This study was an attempt to elicit humoral responses at the cervical, vaginal and rectal mucosa in response to intranasal immunisation using a DNA-liposome complex. A reporter gene vector was used and it was found that immunisation via this route, elicited a proliferative T cell response which was antigen specific in the spleen of mice immunised with the luciferase expression construct. It was reported that immune responses at the vaginal mucosa and lymph nodes were detected, which led to the suggestion that there was a stimulation of the common mucosal immune system.

3.4. 4 Immunisation with microparticulates

Several polymer-mediated drug delivery systems have been developed over the past few years, which employ a wide range of biodegradable polymers, which encapsulate an even wider range of active material as vaccines (O'Hagan, 1994). In more recent years these microparticulate delivery systems have been extended into the realm of gene therapy and the related field of nucleic acid vaccine delivery systems. These delivery systems typically include DNA microencapsulated in a biodegradable polymer such as poly (lactide-co-glycolide) acid (PLGA).

Jones et al. (1997) reported the development of an encapsulation method for plasmid DNA in a poly (D, L-lactide-co-glycolide) polymer. Previous studies had shown that administration of plasmid DNA into the body via naked DNA injection or particle bombardment followed by the transcription of the encoded protein would induce humoral and cellular immune responses. Since most pathogens gain access to the body via the mucosal surfaces of the respiratory, gastrointestinal and urinogenital tracts it would be advantageous if immunity at these surfaces could be elicited by DNA vaccination. These workers used a plasmid encoding for firefly luciferase under the control of a cytomegalovirus promoter. This was encapsulated in a poly (D, L-lactide-co-glycolide) polymer through a double emulsion technique. Microencapsulated DNA was administered to mice via intraperitoneal (ip) injection or through oral gavage. Control animals received unencapsulated DNA via identical routes and standards received intramuscular injection of naked DNA. It was reported that relatively strong IgM and IgG responses were detected following ip injection with encapsulated DNA while injection of naked DNA showed weaker results. Orally administered, encapsulated DNA also showed detectable IgG and IgM readings. It was also reported that local mucosal immune responses were highest following oral administration of encapsulated DNA.

Chen et al. (1998) studied immune responses following oral immunisation with a microencapsulated DNA vaccine against Group A rotavirus. Current immunisation techniques against the disease have included the development of live oral vaccines but these have not been effective in developing countries where the need for a vaccine is greatest (O'Hagan et al. 1993). Rotavirus virons contain a ribonucleic acid (RNA) core which is coated by a protein called VP6. Chen et al. (1998) used PLG encapsulated

rotavirus VP6 DNA to induce antibody responses and protect against subsequent challenges by rotavirus. The plasmid DNA used encoded for the rotavirus VP6 and was encapsulated using a modified solvent extraction procedure. The resultant microparticles were administered following oral gavage to adult mice. Mice were subsequently challenged with the rotavirus and stool samples were examined for rotavirus–specific IgAs. When compared with previous studies using gene gun immunisations the researchers found that a higher concentration of DNA was needed in encapsulated vaccines to induce immune responses. But when compared with oral administration of naked DNA, encapsulated DNA resulted in enhanced responses. Significant production of faecal IgA was also detected in the PLG-encapsulated VP6 DNA-vaccinated animals and following subsequent challenges these inoculated animals had higher levels of rotavirus-specific IgAs when compared with mice inoculated with control microspheres.

More recently studies have been carried out using modified versions of polymer delivery systems. McKeever et al. (2002) used a modified DNA vaccine to elicit immune responses in mice. Lipophilic agents such as taurocholic acid (TA) and monomethoxy polyethylene-glycol-distearoylphosphatidylehanolamine (PEG-DSPE) were added to the vaccine formulation along with a DNA plasmid in an attempt to enhance the activity of beta-galactosidase in an encapsulated DNA vaccine. They reported that the inclusion of such agents increased the number of animals with a positive antibody response. Other studies include those carried out by Singh et al. (2000) in which cationic microspheres were formulated and the DNA adsorbed onto the surface. Cationic microspheres were formed through the inclusion of cetyltrimethylammonium bromide (CTAB) 0.5% w/v in the primary emulsion. Enhanced levels of antibody response were reported following im administration of these particles. Further studies by Singh et al. (2002) studied the immune responses induced following intra nasal immunisation with DNA encoding a HIV-1 protein gag following the adsorption of the DNA onto the surface of cationic microparticles. Enhanced levels of local and systemic cell mediated and humoral immune responses were detected following administration of the adsorbed microparticles when compared with naked DNA.

3.4. 4.1 Targeting of microspheres to immune system cells

While the delivery of DNA as a vaccine has been extensively studied there have been few studies on how vaccine formulation may contribute to the desired immune response. For a vaccine to be most effective in inducing an immune response it must be targeted to those cells, which are involved in immune system responses. Wang et al. (1999) studied the phagocytosis of DNA loaded microspheres in a murine macrophage cell culture. The DNA plasmid, which encoded for β-galactosidase, was encapsulated in a PLGA (50:50) polymer. It was reported that the microspheres were phagocytosed by only the macrophage cell line and not by any control cell lines and that DNA-loaded microspheres did not differ with respect to the numbers of particles taken up when compared with empty particles. The addition of a lipid (monophosphoryl lipid A), which is an immunomodulator, did significantly increase the rate of phagocytosis.

More recently Walter et al. (2001) investigated how the extent of phagocytosis of spray dried DNA loaded microspheres varies with polymers of different molecular weights. Several different molecular weight PLGAs were employed in an effort to compare the rate of phagocytosis with respect to polymer molecular weight and charge. Similar levels of phagocytosis by macrophage cells were reported for all polymer molecular weights. The effect of DNA loading on phagocytosis was studied and was reported not to have any relationship with the rate of uptake. The attachment of particles to macrophage cells has been described as a physicochemical rather then an active energy–dependent biological reaction. Tabata and Ikada (1990) reported that phagocytosis was enhanced as the absolute values of the zeta potential increases for both the negatively charged and the positively charged surfaces of microspheres. It was also noted that the lowest phagocytosis was found for the microspheres with a surface zeta potential of zero.

3.4. 5 Key issues in nucleic acid vaccines

The critical issue in the development of any new drug technology and especially in the area of nucleic acids is one of safety. The biotechnology revolution, which allowed the development of novel vaccines, has led to significant changes in regulations governing the

safety issues of such vaccines. Over the last few years regulatory bodies such as the FDA in the USA and in Europe the EU have brought out directives and guidelines governing the assessment of efficacy, immunogeneticity and safety both during pre- and post-licensure periods

Origin and Scope

Particulate systems based on lactic and glycolic acid have been extensively studied, particularly in the area of drug delivery. Several different ratios of lactic acid to glycolic acid, with varying molecular weights have been investigated. PLGA polymers were initially developed by the pharmaceutical industry for use as degradable sutures (Frazza and Schmitt, 1971). Subsequently characteristics such as biocompatibility, controlled degradation kinetics, ease of fabrication and established regulatory approval for human applications have attracted interest in these polymers for alternative biomedical applications (O'Hagan et al., 1998). These polymers have since been developed as controlled release systems for narcotic antagonists, contraceptive hormones, conventional drugs and antibiotics (Wise et al., 1976; Beck et al., 1979). PLGA polymers have been used in the preparation of several commercially available drugs including Zoladex™ (Zeneca) and Decapeptyl™ (Ipsen Biotech), which are licensed for use in humans in Europe and the USA.

This study investigates the effect of several processing parameters and their effect on the physicochemical properties of encapsulated DNA. PLGA polymers with a composition of 50:50 lactic acid:glycolic acid with varying molecular weights have been extensively studied (Barman et al., 2000; Hao et al., 2000; Singh et al., 2002). Other investigators have used PLGA 75:25 but systems involving the use of this polymer have not been extensively studied especially in the field of DNA encapsulation (O'Hagan et al., 1994; Mehta et al., 1996). Polymers with a PLGA composition of 50:50 lactic acid:glycolic acid degrade faster relative to polymers with a PLGA composition of 75:25 due to the higher ratio of glycolic acid, which is more hydrophilic than lactic acid. Recently a number of papers have studied the encapsulation of DNA in PLGA 50:50 (Capan et al., 1999; Wang et al., 1999; McKeever et al., 2002). Current work involving PLGA 75:25 based delivery systems includes studies which examine the influence of polymer physicochemical properties on the characteristics of encapsulated systems such as release, degradation and surface morphology (Prior et al., 2002).

The objectives of the present work were:

- To examine the influence of polymer type on the physicochemical properties, such as particle size, release profiles and encapsulation efficiencies of Human Serum Albumin-loaded microspheres.

- To use the techniques examined in relation to the protein–loaded microspheres to develop a protocol for the encapsulation of plasmid DNA.

- To examine the influence of manufacturing temperature, plasmid molecular weight, outer:inner aqueous volume ratios, DNA loading and manufacturing technique on the physicochemical properties of DNA-loaded microspheres.

- To evaluate these microspheres in terms of DNA potency and relative uptake using a macrophage cell model and to develop a model for the assessment of nucleic acid vaccines.

4. 1 Materials

4.1. 1 Polymers, protein and general materials

Material	*Supplier/Manufacturer*
Ammonium persulphate	Sigma, St. Louis, USA
Formaldehyde	Sigma, St. Louis, USA
Human Serum Albumin (HSA) protein	Sigma, St. Louis, USA
Micro BCA Protein Assay Kit	Pierce, USA
Poly (D, L-lactide-co-glycolide) $((C_3H_4O_2)\ n\ (C_2H_2O_2))$	
Resomer 502 mw.14,500, (Batch 241846) mn. 6,539	Boehringer Ingelheim, Germany
Resomer 752 mw. 20,900, (Batch 15056) mn. 6,359	Boehringer Ingelheim, Germany
Polystyrene molecular weight standards MW: 95,800, 9,300, 12,860, 2,500	Aldrich, U.K.
MW: 4,750	Fluka, Switzerland
Protogel™	National Diagnostics, Georgia, USA
Polyvinyl alcohol	Sigma, St. Louis, USA
Silver nitrate	Sigma, St. Louis, USA
Sodium azide	Sigma, St. Louis, USA
Sodium carbonate	Sigma, St. Louis, USA
Sodium chloride	Sigma, St. Louis, USA
Sodium hydroxide	Sigma, St. Louis, USA
Sodium dodectysulphate	Sigma, St. Louis, USA
Sodium thiosulphate	Sigma, St. Louis, USA

Experimental

Chapter 4

Materials and Experimental Methods

4. 1 Materials

4.1. 1 Polymers, protein and general materials

Material	*Supplier/Manufacturer*
Ammonium persulphate	Sigma, St. Louis, USA
Formaldehyde	Sigma, St. Louis, USA
Human Serum Albumin (HSA) protein	Sigma, St. Louis, USA
Micro BCA Protein Assay Kit	Pierce, USA
Poly (D, L-lactide-co-glycolide) *($(C_3H_4O_2)$ n $(C_2H_2O_2)$)*	
Resomer 502 mw.14,500, (Batch 241846) mn. 6,539	Boehringer Ingelheim, Germany
Resomer 752 mw. 20,900, (Batch 15056) mn. 6,359	Boehringer Ingelheim, Germany
Polystyrene molecular weight standards MW: 95,800, 9,300, 12,860, 2,500	Aldrich, U.K.
MW: 4,750	Fluka, Switzerland
Protogel™	National Diagnostics, Georgia, USA
Polyvinyl alcohol	Sigma, St. Louis, USA
Silver nitrate	Sigma, St. Louis, USA
Sodium azide	Sigma, St. Louis, USA
Sodium carbonate	Sigma, St. Louis, USA
Sodium chloride	Sigma, St. Louis, USA
Sodium hydroxide	Sigma, St. Louis, USA
Sodium dodectysulphate	Sigma, St. Louis, USA
Sodium thiosulphate	Sigma, St. Louis, USA

Material contd.	***Supplier/Manufacturer contd.***
Sørensen modified phosphate buffer solution (pH 7.4)	
$NaH_2PO_4,2H_2O$ 2.1g/L	Riedel-de-Haein, Germany
Na_2HPO_4 $12H_2$ 19.1g/L	Riedel-de-Haein, Germany
NaCl 4.4g/L	Riedel-de-Haein, Germany
TEMED (N,N,N',N',- Tertramethylethylene diamine)	Sigma, St. Louis, USA
Tris-HCl (pH8)	Sigma, St. Louis, USA
Zeta potential transfer standard	Malvern, UK

4.1. 2 DNA Materials

Material	***Supplier/Manufacturer***
Firefly luciferase pGL3-Promoter Vector	Promega, Madison, USA
pSV ß -galactosidase Control Vector	Promega, Madison, USA
Renilla luciferase pRL-CMV Vector	Promega, Madison, USA
Agarose	Sigma, St. Louis, USA
Ampicillin	Sigma, St. Louis, USA
Bromophenol blue	Sigma, St. Louis, USA
Conical Pyrex Flasks	Fisher, Ireland
Dual-luciferase reporter assay system	Promega, Madison, USA
Ethidium bromide	Sigma, St. Louis, USA
JM109 Competent cells	Promega, Madison, USA
L-Agar	Sigma, St. Louis, USA
LB Broth EZmix	Sigma, St. Louis, USA
LB Broth	Sigma, St. Louis, USA
Magnesium sulphate	Sigma, St. Louis, USA
Molecular Markers (M.W. range: 23,130 base pairs to 125 base pairs)	Promega, Madison, USA
Petri dishes	Sigma, St. Louis, USA
PicoGreen dsDNA Quantitation kit	Molecular Probes, Netherlands
Plasmid Kit	Qiagen, UK

Material contd.	*Supplier/Manufacturer contd.*
Polaroid camera film	Sigma, St. Louis, USA
Qiagen® Mega Endofree™ plasmid purification kit	Qiagen Ltd, UK
Restriction enzymes (Hind 111)	New England, USA
Sterile loops	Lennox, Ireland
Sterilins	Labplex, UK
Tris Borate EDTA	Sigma, St. Louis, USA
Tris-EDTA buffer	Sigma, St. Louis, USA
Ultracentrifuge tubes	Sorvall, UK
Virkon	Lennox, Ireland

4.1. 3 Cell Culture Materials

Material	*Supplier/Manufacturer*
COS-7 cells	European Collection of Animal Cell Cultures (E.C.A.C.C), UK
Cytoplasm stain RAPI-DIFF 11	Diagnostic Developments, UK
DOTAP	Boehringer Mannheim, Germany
Dulbecco's modified Eagle's medium	Sigma, St. Louis, USA
Enzyme free dissociation buffer	GibcoBRL, Scotland
Foetal calf serum	Sigma, St. Louis, USA
Gentamicin	GibcoBRL, Scotland
Glutaraldehyde	Agar Scientific, UK
HBSS	GibcoBRL, Scotland
Hepes	Sigma, St. Louis, USA
IPTG (β- D-isopropyl-thiogalactopyranoside)	Promega, Madison, USA
J774A.1 Macrophage cells	ATCC, USA
L-Glutamine	Sigma, St. Louis, USA
Lysis Buffer	Promega, Madison, USA
OptiMEM	GibcoBRL, Scotland
Osmium tetroxide	Agar Scientific, UK
Propylene oxide	Agar Scientific, UK
Trypsin	Sigma, St. Louis, USA

Material contd.	***Supplier/Manufacturer contd.***
X-Gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside)	Promega, Madison, USA
10ml sterile pipettes	Grenier
75cm ^{3} flask	Grenier

4.1. 4 Solvents

Solvents	***Supplier/Manufacturer***
Acetic acid reagent grade	Chemical Stores, TCD
Chloroform ($CHCl_3$) reagent grade	Lab-Scan, Ireland
Dichloromethane (CH_2Cl_2) reagent grade	Chemical Stores, TCD
Ethanol (C_2H_5OH) (100%) reagent grade	Chemical Stores, TCD
Methanol reagent grade	Chemical Stores, TCD
Tetrahydrafuran (C_4H_8O) HPLC grade	Lab-Scan, Ireland

4.1. 5 Equipment

Equipment/Instrument	***Supplier/Manufacturer***
ATTO electrophoresis unit AE 6450	MSC, Ireland
Bransonic 220 sonic bath	Lennox, Ireland
Büchi Mini Spray Dryer B190	Masons, Ireland
Camera RTS	Contax, UK
Cell scrapers	Sigma, St. Louis, USA
Centrifuge - 4236	AGB, Ireland
CO_2 Water-Jacketed Incubator	Nuaire, USA
Confocal Microscope	Biorad, UK
Cytospin 3	Shandon, USA
Differential Scanning Calorimeter 821^e Mettler Toledo	Mettler, Switzerland
Finn pipette p200	Labsystems, Finland
Gilson Pipetteman p1000	Gilson, France
Gilson Pipetteman p20	Gilson, France
Freezer -86°C	Forma Scientific Inc, USA

Equipment/Instrument contd.	***Supplier/Manufacturer contd.***
Haemocytometer	Sigma, St. Louis, USA
Heidolph stirrer RZR 1	AGB, Ireland
Hetofrig water bath	AGB, Ireland
Hetosicc freeze-drier	AGB, Ireland
HP 8452A Diode Array spectrophotometer	Hewlett Packard, USA
IKA Ultra-Turrax T 25 Disperser	IKA-Labortechnik, Germany
Incubator	AGB, Ireland
Incubator – Shaker G25	New Brunswick Scientific,USA
Luminometer	MSC, Ireland
Malvern Particle Sizer 2600C	Malvern, UK
Mettler AE 240 Analytical Balance	Mettler, Switzerland
Mettler Toledo Differential Scanning Calorimeter 821	Mettler, Switzerland
Mettler Toledo STAR® Version 5.1	Mettler, Switzerland
Microtome	Leica, Austria
Midigel apparatus – Hybaid PS 250	MSC, Ireland
Orion pH probe model 8103 Ross®	Orion, USA
Phase Contrast Microscope	Zeiss, Germany
Precision Scientific shaking water bath	MSC, Ireland
Scanning Electron microscope S4300	Hitachi, Japan
Silverson Mixer L4R	Silversons Machine Ltd, UK
Sorvall refrigerated centrifuge RC5C	Sorvall Instruments, Du Pont, USA
Spectrofluorophotometer RF-1501	Shimadzu
Spectrofluorophotometer Sipper 1500	Shimadzu
Spectrophotometer Genesys 5	AGB, Ireland
Techne water bath	AGB, Ireland
Transmission scanning microscope H7000	Hitachi, Japan
Ultracentrifuge 113	Sigma, St. Louis, USA
Waters Millennium Chromatography Manager	Waters
Waters Novapack C18 HPLC Column	Waters
Waters Refractive Index Detector 410	Waters
Waters Styragel HPLC Column	Waters
Zetasizer 3000	Malvern, UK

4. 2 Experimental Methods

4.2. 1 Formation of Protein Loaded Microspheres

Human serum albumin (HSA) was microencapsulated in a modified double emulsion process (Jeffery et al., 1993; Uchida et al., 1994; McGee et al., 1997). The first stage involved formation of a water/oil/water emulsion and the second stage was a solvent evaporation process of the resulting emulsion to form microparticles. Emulsification of the protein was carried out using either a Silverson mixer (L4R) or an Ultra Turrax mixer (T25). Drying processes evaluated in this work involved either freeze-drying or spray drying technologies. The separate manufacturing stages will now be further detailed.

4.2.1. 1 Emulsification of HSA

PLGA microspheres containing HSA were prepared using a modified double emulsion technique. Two polymer grades were used; these were PLGA Resomer® RG 752 and PLGA Resomer® RG 502. Emulsions were manufactured using either a Silverson Mixer L4R or an Ultra Turrax T25.

Ultra Turrax (T25)

40 mg HSA was dissolved in deionised water and emulsified with 40 ml of dichloromethane containing dissolved 2g PLGA at 2,000 rpm. This primary emulsion was then added to 200 ml of 1% w/v PVA and emulsified with at 8000 rpm for either 90 seconds or for 120 seconds. The resulting emulsion was then diluted to 0.27% w/v PVA following the addition of deionised water. The resulting preparation was further emulsified at 8,000 rpm for either 90 seconds or for 120 seconds.

Silverson mixer (L4R)

40 mg HSA was dissolved in deionised water and vortexed with 40 ml dichloromethane containing 2g PLGA. This primary emulsion was then added to 20 ml 1% w/v PVA and emulsified at 2,000 rpm for 120 seconds. The resulting emulsion was then diluted to 0.27% w/v PVA following the addition of deionised water. The resulting preparation was further emulsified at 2,000 rpm for 120 seconds.

4.2.1.2 Microparticulate solidification

Solvent evaporation

The organic solvent was evaporated following mixing overnight with a Heidolph mixer at 300 rpm. Microspheres were recovered by centrifugation and were subsequently washed three times in deionised water and freeze-dried. Particles composed of both polymer types were freeze-dried using a Hetofrig water bath to freeze the samples in a bath of propylene glycol and were then attached to a vacuum overnight.

Spray drying

Spray drying of emulsions was carried out using a Büchi Spray Drier. Spray drying conditions were as follows: an inlet/outlet temperature of 80 °C/50 °C; an aspirator rate of 75 %; a pump rate of 10 % and an airflow rate of 600 NL h^{-1} (Baras et al., 2000).

4.2. 2 In vitro release of HSA

Release studies were carried out in a phosphate buffer solution (Sϕrensons PBS pH 7.4, Pharmaceutical Handbook). A quantity of protein-loaded microspheres (50 mg) was suspended in 50 ml of PBS buffer in 100 ml conical flasks (Sah et al., 1994; Coombes et al., 1998). The samples were incubated at 37°C in a shaking water bath. The release tests were set up in triplicate and were sampled at predetermined intervals. 2 ml samples were removed and centrifuged at each sampling time. 1 ml of the buffer was removed and assayed for protein release using a protein assay kit (Pierce) and samples read with a Genesys 5 spectrophotometer. After each sample collection, the sample PBS was replaced with fresh buffer. Analyses were carried out in triplicate. Micromath® SCIENTIST® for Windows™, version 2.1 was used to fit drug release profiles. The coefficient of determination (CD) and the model selection criteria (MSC) were used as the measures of goodness of fit when evaluating the suitability of a model. The CD is a measure of the total variance accounted for by the model (Scientist User Handbook, 1995). MSc is a normalised modification of the Akaike Information criteria and gives the same rankings between models but is independent of the scaling of the data points. The appropriateness of a particular model to fit a set of data is judged based on the MSC. A model that gives the largest MSC is the best model to fit that particular data set.

4.2. 3 Loading

Sharif et al (1995) compared two methods for the determination of the levels of proteins entrapped in microspheres. The researchers compared protein loading values attained following extraction of the protein into dichloromethane (DCM) and following alkaline hydrolysis by sodium hydroxide (NaOH) and extraction into sodium dodecyl sulphate (SDS). Results showed that while extraction with DCM gave underestimation of protein levels, extraction via the alkaline hydrolysis method resulted in accurate extraction and determination of protein levels when compared with radiolabelled bovine serum albumin protein. In the current study 50 mg of microspheres was dissolved in 10 ml of 0.1M NaOH containing 5% (w/v) SDS. Following centrifugation at 4000 g for 10 minutes, 1 ml was removed and assayed to determine HSA concentration. All determinations were carried out in triplicate.

4.2. 4 Analysis of released protein

Aqueous samples of protein extracted from microspheres were analysed on an SDS-polyacrylamide gel (Jeffery et al., 1993). SDS-PAGE was carried out as described by Laemmli (1970), using an ATTO electrophoresis unit with 5% stacking gels and 10% resolving gels for the detection of the HSA protein. The following table details the contents of the various gel solutions (Table 4-1). The volumes quoted are those required for the pouring of one gel.

Solution components	**5% stacking gel (ml)**	**10% resolving gel (ml)**
Protogel™ (Ultrapure 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide)	0.83	3.3
Deionised water	3.4	4.0
1.5M Tris-HCL (pH 8.8)	-------	2.5
1M Tris-HCL (pH 6.8)	0.63	-------
10% SDS (w/v)	0.05	0.10
10% APS (w/v)	0.05	0.10
TEMED	0.005	0.004

Table 4- 1 Components of SDS-PAGE

Polyacrylamide gels were run for approximately 2 hours at 80 V and protein bands were detected using a silver staining technique (Pandey et al., 2000). Briefly following electrophoresis the gel was fixed in a fixative solution for 20 to 30 minutes. It was rinsed with water and incubated in water for 1 hour on a shaking platform. It was then incubated in a sensitising solution for 1 to 2 minutes after which time it was washed with water. The gel was then stained following incubation in silver nitrate for 30 minutes at 4°C and was then developed in developing solution. When a suitable degree of staining was achieved the developing solution was replaced with 1% acetic acid. The gel was then dried and the bands analysed. Table 4-2 lists the components of the solutions used during this process.

Fixation solution	Methanol:Acetic acid:water	50:5:45 (v/v/v)
Sensitising solution	Sodium thiosulphate	0.02% (w/v)
Staining solution	Silver nitrate	0.10% (w/v)
Developing solution	Formaldehyde	0.04% in 2% sodium carbonate

Table 4- 2 Components of silver staining technique

4. 3 Preparation of DNA Loaded Microspheres

4.3. 1 Plasmid material

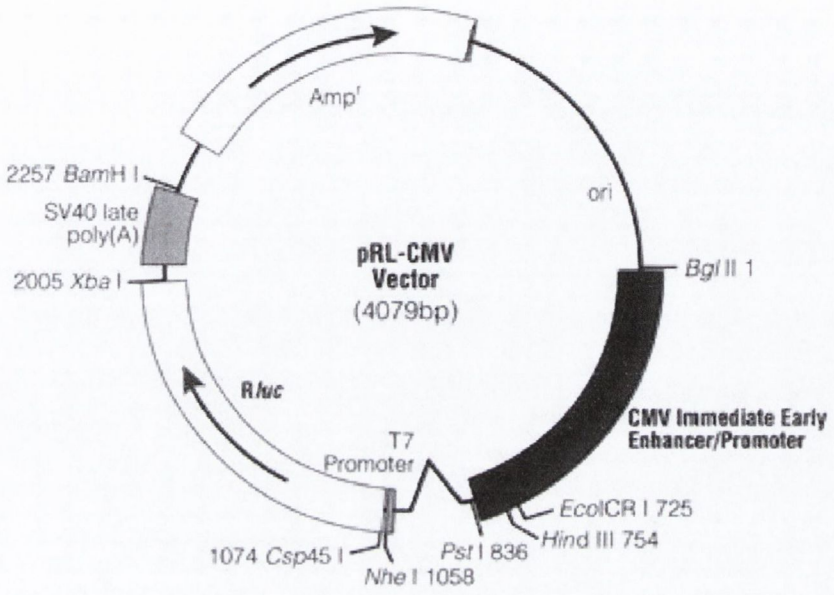
Three types of plasmid DNA were used (see Appendix for DNA sequence). These plasmids encode for renilla luciferase, photinus luciferase (firefly luciferase) and ß-galactosidase enzymes. DNA is composed of a double helix of bases, which line up to form base pairs with complementary bases. Therefore DNA is measured as a composite of base pairs. The size of a plasmid vector is generally described in terms of base pairs and can be converted to daltons since 1-kilo base of plasmid DNA is 6.5 x 10^5 daltons of duplex DNA (Mathowitz, 2000).

The *Renilla* luciferase plasmid contains the cDNA sequence encoding for R*luc*. It is cloned from the anthozoan coelenterate *Renilla reniformis*, the sea pansy and is 4079 base pairs in size (Figure 4-1). The *Renilla* luciferase protein is a 36 kDa monomeric protein. Both plasmids, *photinus pyralis* and *renilla reniformis*, have separate evolutionary origins and hence the luciferase enzymes produced by both have dissimilar enzyme structures and substrate requirements. The firefly luciferase enzyme produces light through a reaction using luciferin and ATP in the presence of oxygen and magnesium while the renilla luciferase enzyme utilises oxygen and coelenterate luciferin to generate light.

The photinus luciferase vector contains the complementary DNA (cDNA) sequence of firefly luciferase, which is cloned from the North American firefly (*Photinus pyralis*). This sequence, *luc+*, is a modified version of the native *luciferase* and encodes for the enzyme luciferase which is detected using a luminometer. It is commonly used because of the ease of detection and the high levels of luciferase enzyme produced. The firefly luciferase protein is a monomer of 60.7 kDa and the plasmid is 5010 base pairs in size (Figure 4-2).

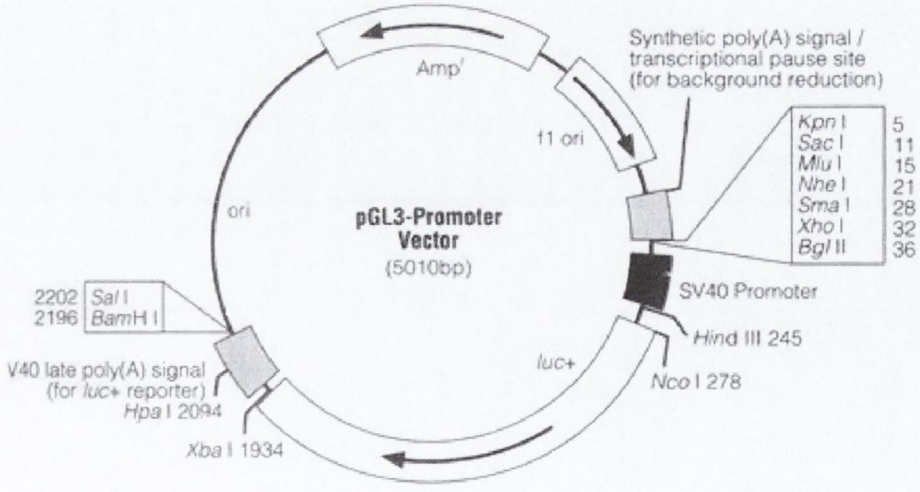
The third reporter gene sequence used was the pSV-ß-galactosidase control vector. It is an easily monitored plasmid due to the enzyme it transcribes, which is the *lac Z* gene present in the pSV-ß-galactosidase control vector. The *lac Z* gene encodes for the ß-galactosidase enzyme, which can be assayed directly and easily. The pSV-ß-galactosidase control vector

is 6821 base pairs in size with the encoded enzyme having a molecular weight of 116 kDa (Figure 4-3).



The pRL-CMV Vector map.

Figure 4- 1 Renilla luciferase Vector map



pGL3-Promoter Vector map.

Figure 4- 2 Firefly luciferase Vector map

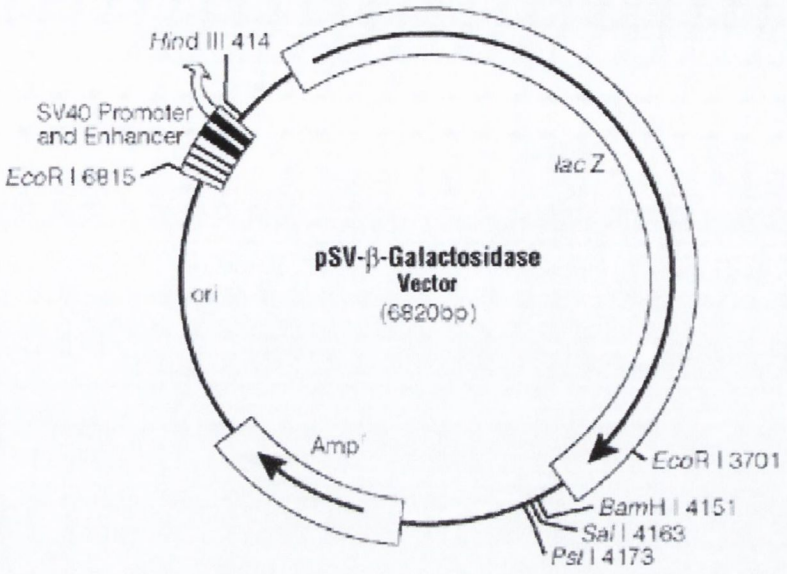


Figure 4- 3 PSV-β-Galactosidase Vector map

4.3. 2 Preparation of plasmid DNA

The plasmids were propagated in Escherichia Coli cells. They were then purified using an endotoxin-free plasmid purification kit (Qiagen) according to the manufacturer's instructions and re-suspended in Tris-EDTA (TE) buffer. Briefly, 10 ng of DNA was transformed into 100 µl of *E. Coli* cells and grown up on ampicillin (amp+) agar plates in the case of cells transformed with pRL-CMV or pGL3 as both plasmids contain ampicillin resistant genes. Agar plates for use with β-galactosidase transformed *E-Coli* cells were plated with β-D-isopropyl-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-gal). The plates were incubated overnight at 37°C. A single colony was picked and inoculated into a starter culture of 5 mls of LB broth. This was then incubated at 37°C for 8 hours with shaking (300 rpm). The culture was then diluted to 1/500 with 500 mls of LB broth. This was then grown at 37°C for 12 hours with shaking (300 rpm). The resulting bacterial suspension was centrifuged and the bacterial cells harvested. The pellet was resuspended in Buffer P1 (see Appendix), which causes cell lysis, and incubated in Buffer P2 (see Appendix). 50 ml Buffer P3 (see Appendix) was added and the lysate poured into a QIA filter Mega-Giga Cartridge. A vacuum source was applied and 50 ml of Buffer FWA (see Appendix) added and the liquid again pulled

through the filter. 12.5 ml of Buffer ER (see Appendix) was added to the filtered lysate, which contained the DNA. The lysate was added to a Qiagen-tip column and allowed to empty by gravity flow. The DNA was eluted with 35 ml of Buffer QN (see Appendix) and precipitated with isopropanol. The DNA pellet was washed with 70% ethanol and resuspended in endotoxin-free TE buffer (see Appendix).

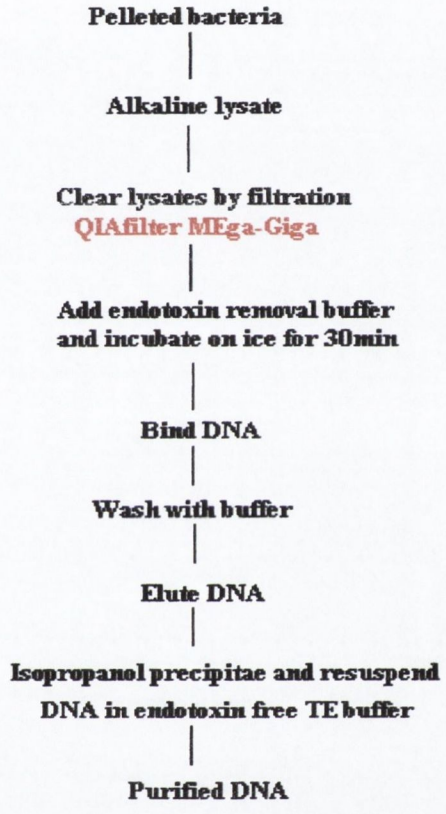


Figure 4- 4 Schematic of DNA purification process (reproduced from Qiagen handbook)

Agarose gel electrophoresis was used to analyse DNA yield and structure (Jong et al., 1997; Capan et al., 1999). DNA quality was determined following a restriction digest of the plasmids using Hind 111 and samples were subsequently checked using a 1% agarose gel. DNA yields were also determined by UV absorbance at 260 nm (Barman et al., 2000).

4.3. 3 Formation of DNA loaded microspheres

Plasmid DNA microspheres were manufactured following a modified double emulsion technique. Microencapsulation of plasmid DNA took place in two stages. Briefly, after formation of a water/oil/water emulsion the solvent was evaporated to harvest the microparticles. Emulsification of plasmid DNA was carried out using either a Silverson mixer (L4R) or an Ultra Turrax (T25) mixer. Drying of the emulsion involved either freeze-drying or spray drying the resulting emulsion.

4.3.3.1 Emulsification of DNA

Ultra Turrax (T25) and Silverson (L4R)

PLGA microspheres containing DNA were prepared by a modified double emulsion technique (McKeever et al., 2002). Tris-EDTA (TE) buffer containing 1 mg/ml DNA was added to a quantity of ethanol and vortexed with 40 ml dichloromethane containing 40 mg RG 752. The resulting primary emulsion was then added to 200 ml 1% w/v polyvinyl alcohol and emulsified for 120 seconds with either the Ultra Turrax (T25) or the Silverson mixer (L4R). The resulting emulsion was then diluted to 0.27% w/v PVA with deionised water and emulsified for 120 seconds with either the Ultra Turrax (T25) or the Silverson (L4R).

4.3.3.2 Microparticulate solidification

Solvent evaporation

This was carried out as described in Section 4.2.1.2.

Spray drying

The resulting emulsion was spray dried with a Büchi 190 Spray Drier under the same conditions as in Section 4.2.1.2.

4.3. 4 In vitro release of DNA

The release profiles of all batches of microspheres were analysed. Release profiles were set up in triplicate containing 20 mg of microspheres in 2 ml of PBS buffer (phosphate buffer solution, pH 7.4, containing 0.02% sodium azide) (Barman et al., 2000; Perez et al., 2001). The microcentrifuge tubes were incubated at 37°C in a shaking water bath. At predetermined intervals the samples were centrifuged, the supernatant removed and replaced with fresh PBS buffer. The DNA released from the microspheres was quantified using a PicoGreen® double stranded DNA quantitation kit. The resulting fluorescence of the reagent when bound to double stranded DNA was measured by a spectrofluorophotometer according to the manufacturers instructions (Molecular Probes, The Netherlands).

4.3. 5 Analysis of DNA extracted from PLGA microspheres

DNA was extracted from the microspheres using a modified chloroform extraction method (Barman et al., 2000). A quantity of DNA-loaded microspheres were weighed out and dissolved in 2 ml of Tris-EDTA buffer (pH 8). An equal volume of chloroform was added and the tubes were rotated end-over-end for 120 minutes at ambient temperature. The two phases were separated following centrifugation for 10 minutes at 13,000 rpm and 100 µl of the supernatant was removed. From the remaining supernatant released DNA was precipitated following the addition of 0.1 volume 3 M NaOAC and 2 volumes of ice-cold ethanol. The samples were incubated at –80°C for 20 minutes. The samples were then centrifuged at 13,000 rpm for 20 minutes. The pellet was rinsed with 70 % ethanol and resuspended in an appropriate amount of TE buffer (Hao et al., 2000).

Both the aqueous supernatant and the resuspended DNA were analysed for DNA concentration following UV spectroscopy. The samples were diluted appropriately in TE buffer. Absorbance at 260 nm was determined on a UV spectrophotometer against a blank of TE buffer and the µg $DNA_{extracted}$/mg PLGA was calculated using the equation:

$$[DNA](\mu g/mg)=(Absorbance\lambda=_{260nm} X \text{ dilution factor})/(\varepsilon * w * b) \qquad \textbf{Equation 4. 1}$$

where $\varepsilon = 50^{-1}$ = extinction coefficient of DNA, *w* is the weight of microspheres in mg, *b* is the optical path length (1 cm) (Barman et al., 2000). The integrity of the released DNA was analysed following agarose gel electrophoresis (Capan et al., 1999).

4.3. 6 Spiking of DNA and empty microspheres

To ensure that 100 % of the DNA was recoverable from the method outlined above, spiking experiments were carried out. Approximately 1 mg of DNA in a total of 200 µl of TE buffer was added to 10 mg of empty microspheres. 500 µl of chloroform was added and the tubes were end-over-end rotated as a function of time. The samples were centrifuged for 5 minutes at 13,000 rpm. 100 µl of the supernatant was removed and assayed using UV. Agarose gel electrophoresis was carried out on the supernatant to characterise the DNA retrieved (Barman et al., 2000).

4.3. 7 Visualisation of ethidium bromide stained DNA

In order to characterise the microspheres, *renilla luciferase* was stained prior to encapsulation with ethidium bromide. 945 µg of *renilla luciferase* was incubated in 3 ml of ethidium bromide (100 µg/ml) for 30 minutes. Ethidium bromide is a dsDNA intercalator, which binds with little or no sequence preference of one dye per 4-5 base pairs of DNA. Excitation of bound ethidium bromide occurs at 518 nm and is 20 to 30-fold higher then non-bound ethidium bromide. The emission wavelength of ethidium bromide is 605 nm. The encapsulation protocol was identical to that described in Section 4.3.3 with the exception that due to the probability of quenching of the fluorescent dye the procedure was carried out in the dark.

4. 4 Characterisation of Microspheres

4.4. 1 Particle size analysis

The particle size of the protein-loaded microspheres was determined by laser diffraction with a Malvern 2600. A 63 mm lens was used which accommodates particles in the size range 0.5 μm - 118 μm. 10 mg of microspheres were blended with 5 ml of a 0.2% w/v solution of Tween 20 and sonicated for 10 minutes prior to measurement. The samples were measured in triplicate from different batches. The particles were plotted as cumulative (%) undersize versus size to give a histogram of the distribution and the span of particle distribution was recorded. The span is a measure of the width of the volume distribution relative to the median diameter.

4.4. 2 Structural analysis

Microsphere morphology and structure was determined by scanning electron microscopy (SEM). Samples microspheres were attached to a metal stub and gold coated using a sputter coater and viewed under a Hitachi S43000 scanning electron microscope.

4.4. 3 Differential Scanning Calorimetry (DSC)

Microspheres containing protein and DNA and empty microspheres were characterised by DSC in the range of 0°C to 250°C at a heating rate of 10°C per minute. Between 5 mg and 10 mg of microspheres were weighed out into 40 μl aluminium crucibles which were hermetically sealed and the top pierced.

4.4. 4 Gel Permeation Chromatography (GPC)

10 mg samples were suspended in 10 ml of PBS and were left shaking at 37°C over a specified time interval. The samples were filtered and stored under a vacuum until used. Approximately 5 mg of each sample was weighed out into 10 ml of tetrahydrafuran and allowed to stand overnight. The GPC consisted of a Waters column, a Waters 510 pump and a Waters Refractive index detector RI410. The mobile phase was THF and the flow rate was set at 1 ml/min. The internal and external temperatures were set at 30°C and 25°C

respectively. Millennium Chromatography software version 2.0 was employed to integrate the peaks and calculate the result. Samples were evaluated against a series of polystyrene standards with molecular weights of 2,500, 13,000, 90,000 (Aldrich) and 5,970, 37,900 (Tosoh, Japan). Samples were injected in triplicate.

4.4. 5 Confocal microscopy of DNA loaded microspheres

The microspheres containing ethidium bromide stained *renilla luciferase* were viewed under confocal microscopy. Approximately 0.5 mg of microspheres was suspended in 1 ml PBS. 1 µl of this suspension was added to 1 µl of antiquench (Appendix B) on a glass slide and the excess pipetted off. The coverslip was mounted and the edges sealed in. The microspheres were view under x 60 magnification.

4.4. 6 Determination of zeta potential

Approximately 10 mg of sample was dispersed in 10^{-3} M NaCl and phosphate buffer pH 7.4. A zeta standard DTS35050 (50mV $\pm$ 5mV) was used to analyse the performance of the Malvern Zetasizer prior to sample determination. Zeta potential readings were carried out five times and the average obtained.

4. 5 Cell culture studies

4.5. 1 Cell lines

Two cell lines were used. These were COS-7 cells and J774 macrophage cells. COS-7 cells are fibroblastic cells derived from kidney SV40-transformed African green monkey cells and were used in transfection experiments using DNA extracted from microspheres.

Macrophage cells are one of a group of differentiated cells in the immune system. They play an important role in host response against a variety of infectious agents and are the body's first line of defence. J774.1 macrophage cells (ATCC) were exposed to protein microspheres encapsulated in RG 752 and RG 502 and DNA microspheres encapsulated in RG 752.

4.5. 2 Maintenance of cells

Cells were grown in a CO_2 incubator at 37°C in supplemented Dulbecco's modified Eagles Medium (DMEM). The DMEM was supplemented with 2 mM L-glutamine, 10% v/v foetal calf serum (FCS) and 100 mg/ml gentamicin. They were grown in 75 cm^3 flasks in a 5% CO^2 incubator at 37°C. The cells were passaged approximately once a week to maintain cell viability and the media replaced twice weekly with fresh DMEM.

4.5. 3 Passaging of cells

The supplemented DMEM medium present was removed in order to remove the trypsin inhibitor present in the FCS and the cells washed in 10 ml of unsupplemented DMEM. The DMEM was poured off and 1 ml of trypsin added. The flasks were repeatedly disturbed to ensure removal of the cells from the flask walls. 10 ml of supplemented DMEM was added and the resulting solution was centrifuged at 1,000 rpm for 5 minutes. The supernatant was poured off and the pellet was resuspended in 6 ml of supplemented DMEM. Cell density was determined using a haemocytometer and the cells seeded at a density of 1-2 $x10^6$ cm^3 in an appropriate volume of media.

4.5. 4 Cell transfection

Prior to transfection the cells were seeded in 6-well plates at a density of 5 x 10^6 cm^2. 1 ml of DMEM was added to each well and the cells grown in a CO_2 incubator for 16-24 hours

2 μg of DNA was used to transfect the COS-7 cells and DOTAP was used as the transfection agent. A positive transfection mix was made up in two sterilin tubes. The first sterilin contained 2 μg DNA/500 μl Hanks Balanced Salt Solution (HBSS) and the second contained 35 μl DOTAP/800 μl HBSS. The two were mixed together and incubated for 15 minutes at 15-25°C. A negative control was also carried out using the same method as above with the exclusion of DOTAP. 500 μl of the positive transfection complex was added to three plates and 500 μl of the negative transfection complex was added to three plates. The plates were incubated for 4 hours at 37°C (transfection time) after which the transfection mix was removed and replaced with 1.5 mls of growth media. The plates were incubated for a further 24 hours at 37°C (expression time). The growth media was then removed, the cells washed twice with PBS and 250 μl of 1 x lysis buffer added to each well. The cells were then left at –80°C overnight.

The following day the cells were defrosted and scraped from the wells. They were centrifuged at 12,000 rpm at 4°C for 5 minutes. A 20 μl sample was used with 100 μl of luciferase assay and results read with a luminometer.

4.5. 5 Phagocytosis Assay

Macrophage cells were seeded at a density of 2 x 10^5 cells/well in 1 ml of DMEM media and incubated at 37°C in 5% CO_2. After 24 hours incubation 0.5 mg of microspheres were placed in 200 μl of DMEM media and added to each well (Walter et al., 2001). Wells were incubated at different time periods up to 24 hours. At each time point the cells were photographed using phase contrast microscopy and then washed thrice in PBS. They were then photographed again. At each time point 200 μl of enzyme free dissociation buffer was added to the washed cells and incubated for 5 minutes at 37°C. The cells were scraped out and were either spun down onto a slide or were processed for transmission microscopy. Cells that were spun onto a slide consisted of 100 μl of the dissociated cells attached by

gravity centrifugation onto glass slides. They were then stained with a cytoplasm stain and photographed. Dissociated cells were prepared for transmission microscopy by resuspension in 3 ml of 3% glutaraldehyde and spun down for 10 minutes at 3,000 rpm.

4.5. 6 Transmission microscopy

Macrophage cells were spun down in glutaraldehyde, the glutaraldehyde decanted and the cells were resuspended in approximately. 0.5 ml of agarose in a 2 ml eppendorf. This was allowed to solidify prior to cutting into small blocks (1 mm^3). The cubes of agarose were washed five times over an hour in 0.05M PBS (pH 6.8). 2% osmium tetroxide was then added to the cells and the samples rotated for half an hour. The samples were then dehydrated over a period of 110 minutes following incubation of the sample in increasing percentages of alcohol. The cells were then transferred from the alcohol solution to propylene oxide (a "transitional" fluid being miscible with both alcohol and resin) and then into resin where they were solidified and then cut ready for staining and examination by transmission microscopy. The samples were cut using a microtome with allows for the sectioning of thin samples using a glass knife (CMA, Trinity College).

Chapter 5

Microencapsulation of Human Serum Albumin

5. 1 Characterisation of RG 502 and RG 752 polymers

In recent years, there has been rapid growth in the area of drug discovery and the use of novel compounds as pharmaceutical products. In many cases these drugs are more potent and have poorer solubility then traditional agents. With the advent of novel drugs has come the development of novel techniques to deliver these drugs more effectively and efficiently. One such technique is the encapsulation of the active agent in a polymer matrix. The technology of polymeric delivery is in itself not a novel technique and has been well documented (O'Hagan et al., 1993; Uchida et al., 1994; McKeever et al., 2002).

PLGA polymers are available in a variety of co-polymer ratios and molecular weights both of which influence their physicochemical properties (Singh et al., 1998). PLGAs are generally amorphous compounds composed of a ratio of lactic and glycolic acids. Li et al. (1999) defined a PLA_xGA_Y copolymer as intrinsically amorphous if Y, the glycolic acid ratio is in the range of 10 to 70. Lactic acid is more hydrophobic than glycolic acid and as a result PLGAs with a higher lactic to glycolic ratio are more hydrophobic (Walter et al., 2001). Hence, PLGA polymers with higher lactic to glycolic ratios degrade more slowly and as a result have a longer half-life, than those with higher glycolic acid ratios. For example, it has been established that 50:50 lactide/glycolide co-polymers have a half-life of approximately 50-90 days in vivo. The 65:35, 75:25 and 85:15 polymers have progressively longer degradation half-lives with 85:15 lasting about 150 days (O'Hagan et al., 1998). Molecular weight is also an important factor in the degradation rates of PLGAs. Cohen et al. (1991) reported an inverse relationship between the burst release of an encapsulated drug and the molecular weight of the PLGA polymer. This observation was in agreement with Ogawa et al. (1988) who observed a more rapid rate of release with decreasing molecular weight. Hence not only is the copolymer ratio an important factor in the degradation of a polymer and hence release rate of the encapsulated agent, so too is the molecular weight, which is also a determining factor in both processes.

Commonly used polymers in the area of sustained delivery systems include polymers with lactide/glycolide ratios of 50:50 (Capan et al., 1999; Chen et al., 2001; Jiang et al., 2002) 65:35 (Yang et al., 2000), 75:25 (Mehta et al., 1996) and 85:15 (McGee et al., 1997). The choice of polymer is dependent on several factors including desired degradation rate,

polymer molecular weight, desired microsphere particle size and crystallinity. Therefore by careful selection of the appropriate microparticle characteristics and polymer composition it is possible to produce a drug delivery system with the desired release rate.

The PLGA copolymers used in this study were comprised of lactic and glycolic acids in the ratios of 50:50 (RG 502) and 75:25 (RG 752) (lactide/glycolide) and were chosen because of their degradation kinetics which allow them to be used over the period of the trial and their extensive use in the area of sustained delivery systems.

5.1. 1 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine the morphology of the two polymers. As can be seen from Figure 5-1 and 5-2 PLGA RG502 and RG752 are similar in appearance and morphology and are composed of rod-like structures.

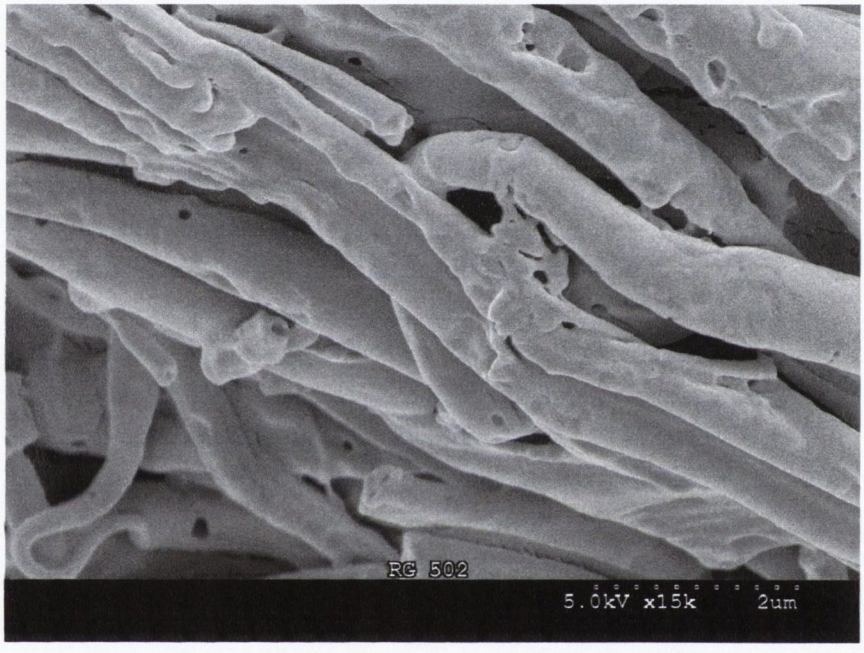


Figure 5- 1 Scanning electron micrograph of RG 502

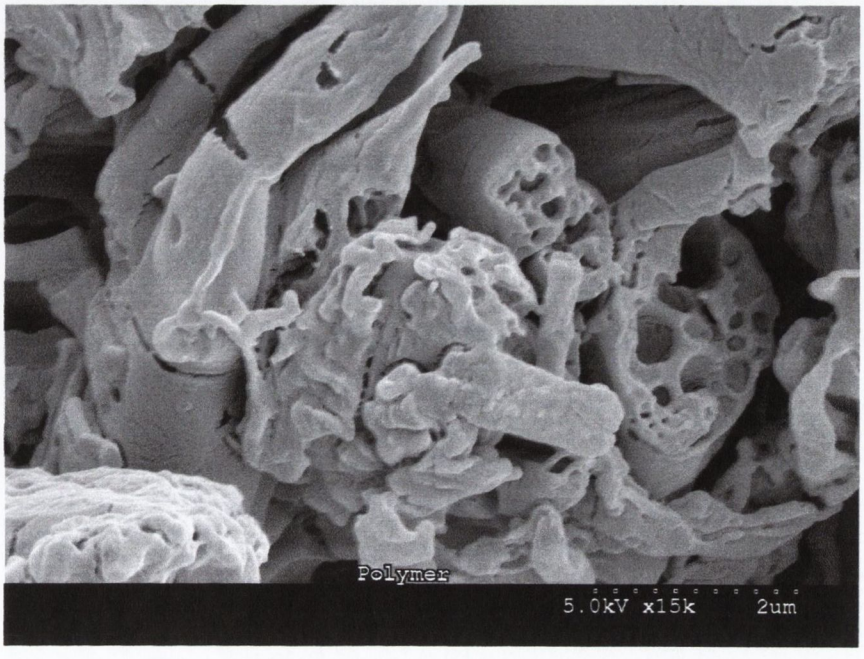


Figure 5- 2 Scanning electron micrograph of RG 752

5.1. 2 Differential Scanning Calorimetry (DSC)

DSC measures the heat capacity of the system as a function of temperature and allows for the detection of phase transitions of various orders. The glass transition temperature (T_g) corresponds to the temperature where the characteristics of a solid change from the glass state to the rubbery state. Below this temperature the atoms in the polymer can only undergo low vibrations however above it the polymer chains can undergo rotation and the polymer chains acquire enough mobility to crystallise.

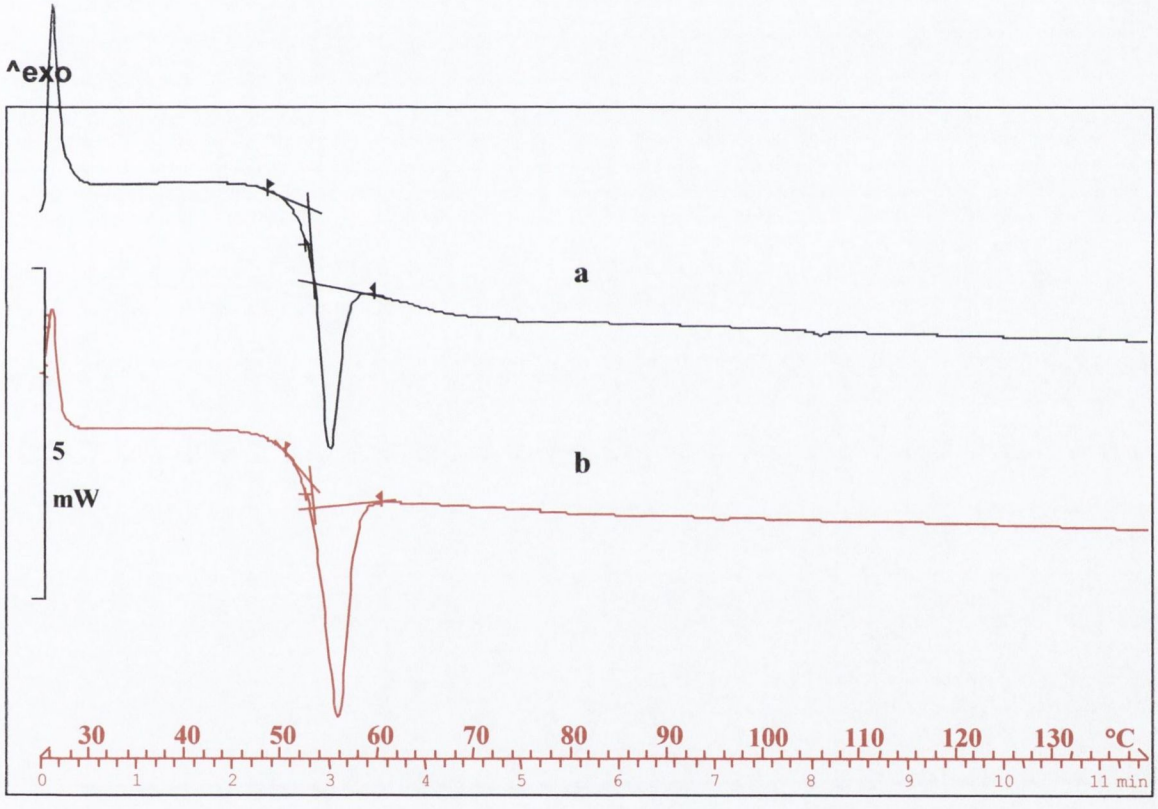


Figure 5- 3 DSC scans of (a) RG 502 and (b) RG 752

DSC scans of the two polymers showed glass transition temperatures of 52.68°C and 52.51°C for RG 502 (a) and RG 752 (b) respectively. Both polymers showed single sharp peaks. Ford and Timmins (1989) reported glass transition temperatures of approximately 67°C for poly-*d*-lactide and 36°C for polyglycolic acid. Both polymers used in this study,

PLGA 50:50 and PLGA 75:25, are based on copolymers of glycolic and lactic acid and are both amorphous compounds.

5.1. 3 Gel Permeation Chromatography

RG 502 and RG 752 were characterised by gel permeation chromatography (GPC). Table 5-2 shows the M_w, M_n, and the polydispersity (P) value obtained for each sample including the manufacturer's data (Boehringer Ingelheim Pharma) for both polymers. The methodology employed by the manufacturer in the determination of the molecular weight was not supplied. Both polymers were reported to have intrinsic viscosity values of 0.16 – 0.24 dl/g as determined by the manufacturer.

Polymer	**M_w (Daltons)**	**M_n (Daltons)**	**P**
RG 502	15,590.50 ± 78.49	6,359.00 ± 254.46	1.91 ± 0.32
Manufacturer Data (Batch 241846)	14,500	6,600	
RG 752	18,361.67 ± 74.63	6,539.00 ± 55.29	2.27 ± 0.02
Manufacturer Data (Batch 15056)	20,900	9,200	

Table 5- 1 Molecular weight characteristics of polymers studied
(samples represent the mean of three determinations ± standard deviation)

5. 2 Characterisation of protein-loaded microspheres prepared by high speed homogenisation

Due to the rapid proteolysis of proteins following parenteral administration, controlled protein and peptide drug delivery is essential for the optimisation of their therapeutic properties. Among the many available controlled drug delivery systems for parenteral administration, are those which involve the encapsulation of proteins or peptides in a polymer coating. These systems offer the potential for protein delivery due to their physicochemical properties, which allow the encapsulated agent to be released in a controlled way, and which at the same time protect the agent against degradation.

Encapsulation of proteins following a water/oil/water ($W_1/O/W_2$) double emulsion encapsulation technique is a widely utilised encapsulation method (Yang et al., 2000 a; Wang et al., 2002). The double emulsion is commonly made using a high-speed homogeniser to emulsify first the primary aqueous phase (W_1) into the oil phase (O) and subsequently to homogenise this single emulsion into a larger aqueous phase (W_2) producing a double emulsion (Li and Vert, 1995). The technique of double emulsion can lead to an entrapment of hydrophilic macromolecules in reasonable yields. It also offers the additional benefit of introducing aqueous soluble proteins into the process (Cho et al., 2001).

Previous work carried out by Uchida et al. (1994) examined the release of ovalbumin from RG 502 microspheres. In the present study, another albumin protein, human serum albumin (HSA) was investigated. Albumins are a group of proteins, which are found in nearly all body tissues (Groves, 1999). HSA is a 66 kDa protein, which is found in human blood where it accounts for about 60% of the total plasma protein. It is routinely used as a plasma volume expander and in the measurement of blood volume once separated from human blood. HSA is involved in many transport and regulatory processes in the body and has multifunctional binding properties, which range from various metals, calcium and copper, to fatty acids, hormones and therapeutic drugs (Mohamadi-Nejad et al., 2002). HSA is easily assayed by spectrophotometry and its integrity examined by the technique of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), which involves electrophoresis using a polyacrylamide gel containing sodium dodecyl sulphate. HSA has

not been studied as extensively as other members of the albumin group (Pean et al., 1998; Deng et al., 2001) hence our choice of it as a model protein.

5.2. 1 RG 502 microspheres

RG 502 microspheres were prepared by a modified solvent evaporation technique as has been described in detail in Chapter 4 (Yang et al., 2000 a; Chen et al., 2001). RG 502 microspheres were formulated following high-speed homogenisation (Ultra Turrax T25) for 180 and 240 seconds with the primary and secondary emulsion being homogenised for either 90 or 120 seconds each. The emulsification times chosen in this study were based on typical emulsification times employed for proteins, which range from 80 seconds to 30 minutes (Sah et al., 1994; Uchida et al., 1994; Coombes et al., 1998; Wang et al., 2002). Microspheres manufactured following 180 seconds emulsification showed what appeared to be flocculation during formation.

5.2.1. 1 Scanning Electron Microscopy

Scanning electron microscopy was used to characterise the morphology of empty microspheres (Figure 5-4) and protein-loaded microspheres (Figure 5-5 and 5-6).

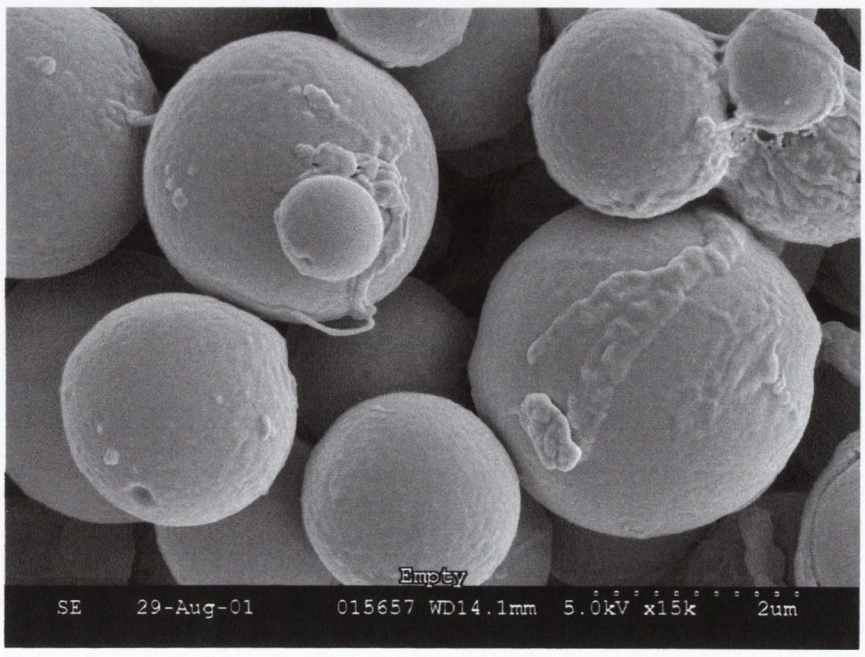


Figure 5- 4 RG 502 empty microspheres

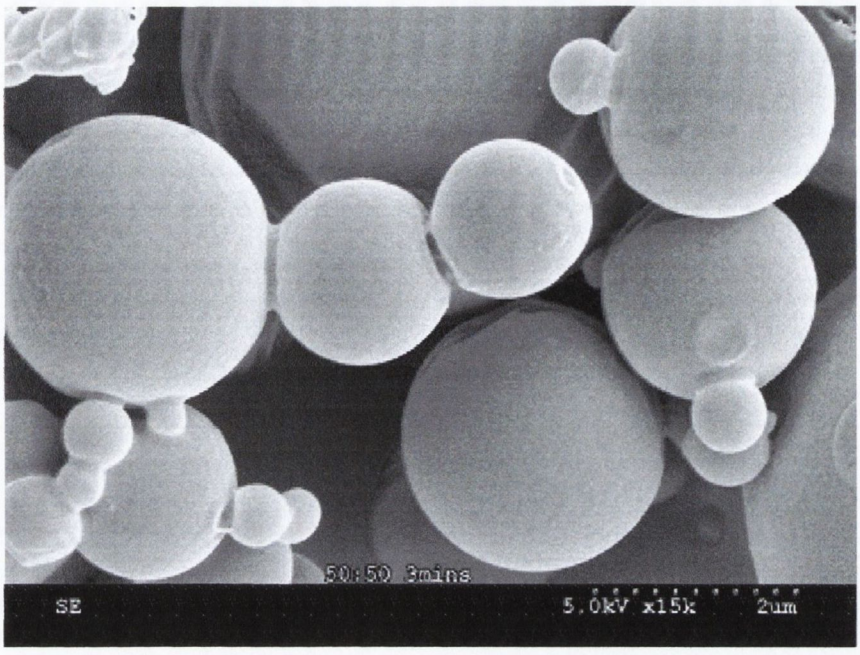


Figure 5-5 RG 502 HSA-loaded microspheres - 180 seconds emulsification

Micrographs revealed that protein-loaded microspheres were manufactured with relatively smooth surfaces while empty microspheres were observed to have rough surfaces.

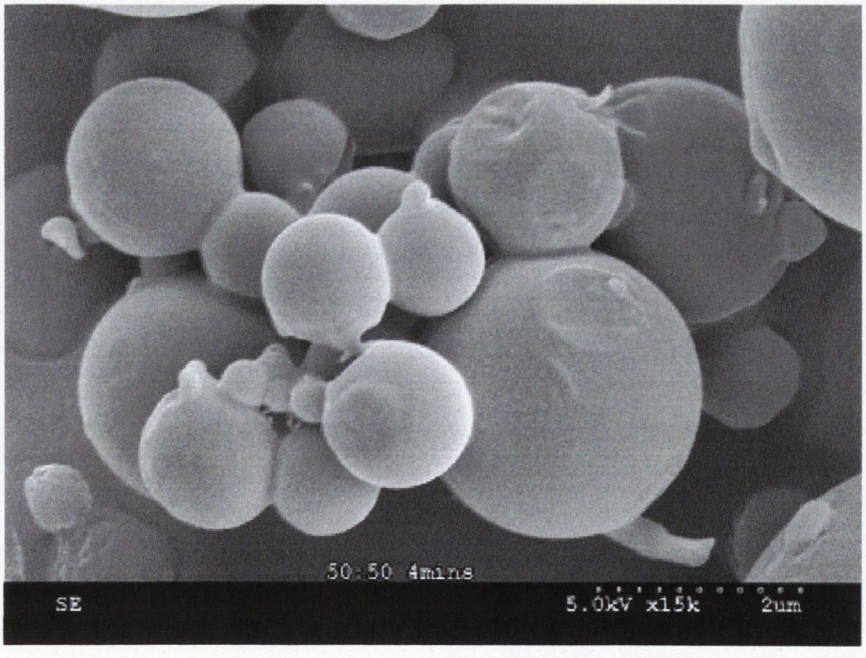


Figure 5-6 RG 502HSA-loaded microspheres - 240 seconds emulsification

Micrographs of microspheres manufactured following 240 seconds showed microspheres with smooth outer surfaces which were spherical in shape. Microspheres manufactured

following 180 seconds emulsification appeared to aggregate during formation. This phenomenon was still observed in microspheres manufactured following 240 seconds emulsification but was observed to occur in fewer microspheres. Empty microspheres on the other hand exhibited rough surfaces with irregular outer membrane formation giving rise to deposits of polymer on the outer surface.

5.2.1. 2 Particle Size Data

Particle size data obtained for RG 502 systems are illustrated in Table 5-2.

Emulsification time (seconds)	**D[ν,0.5] μm**	**SD**	**D[ν,0.9] μm**	**SD**	**Span**
Empty microspheres	4.56	± 0.21	8.79	± 0.96	1.58
180	6.33	± 0.41	19.19	± 2.19	2.55
240	4.92	± 0.15	10.46	± 0.35	2.44

Table 5- 2 Particle size *(samples represent the mean of three determinations)*

Particle size data for batches were observed to vary significantly ($p < 0.05$) at both the D[v,0.5] and D[v,0.9] levels as emulsification time was increased from 180 to 240 seconds.

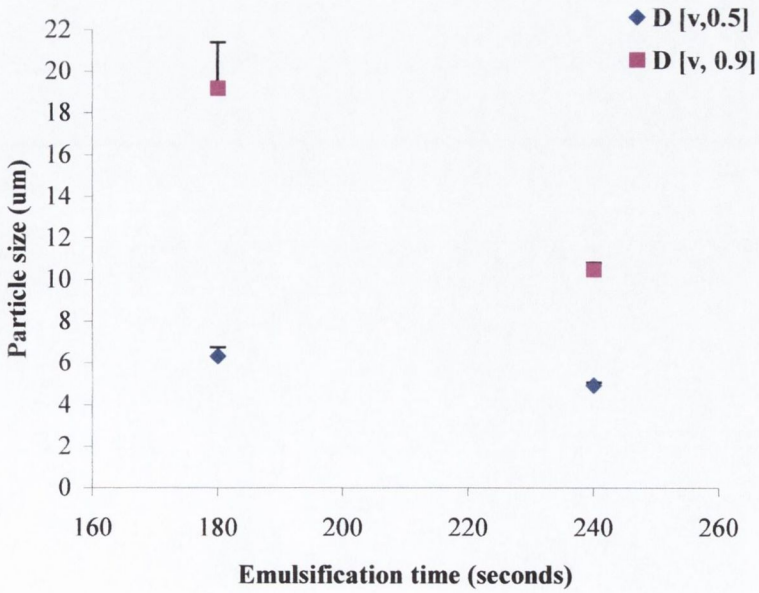


Figure 5- 7 Particle size for microspheres subjected to different emulsification times *(samples represent the mean of three determinations)*

In the current study mean particle size was observed to decrease as time of emulsification increased (Figure 5-7). McGee et al. (1997) studied the effect of processing parameters on the size of protein-loaded microspheres. It was reported that following emulsification for 390 seconds at 15,000 rpm all batches of microspheres had D[ν,0.5] values of less than 1.5 μm. A second emulsification time of 750 seconds was used with a speed of 5,700 rpm and 1,000 rpm, which resulted in microspheres of greater than 10 μm. Uchida et al. (1994) found that increasing the stirring rate during emulsification from 800 rpm to 3000 pm resulted in a decrease in mean average particle size diameter of PLGA 50:50 (mw 53,000) ovalbumin-loaded microspheres from 14.3 to 1.2 μm.

5.2.1. 3 Differential Scanning Calorimetry

Figure 5-8 illustrates the DSC scans of (a) RG 502, (b) protein polymer blend and (c) HSA.

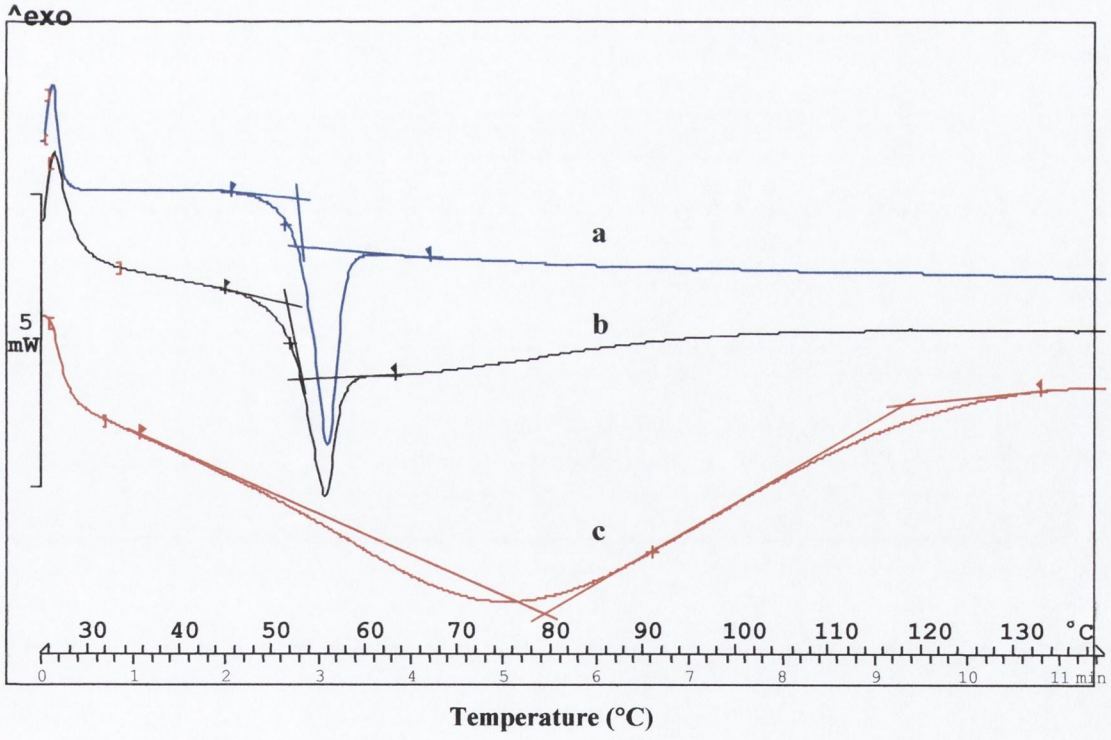


Figure 5- 8 DSC scans of (a) RG 502, (b) protein polymer blend and (c) human serum albumin

Figure 5-8 illustrates the thermal characteristics of (a) RG 752 (b) the protein polymer blend and (c) the protein alone. The blend was formulated using the same ratio of protein to polymer as was used in the manufacture of the protein-loaded microspheres. As can be seen from these traces the protein alone shows a broad endotherm and was not amenable to analysis for glass transition. Glass transition temperatures of between 40°C and 80°C have been reported for proteins belonging to the albumin family and the broad peak observed in this study is typical of the thermal characteristics of albumins. This occurs due to the gradual unfolding of the protein at higher temperatures (thermal unfolding) and eventual denaturation of the protein (Farruggia et al. 1999). Thermal denaturation of human serum albumin has been shown to be reversible up to 75°C but irreversible above 75°C (Mohamadi-Nejad et al., 2002).

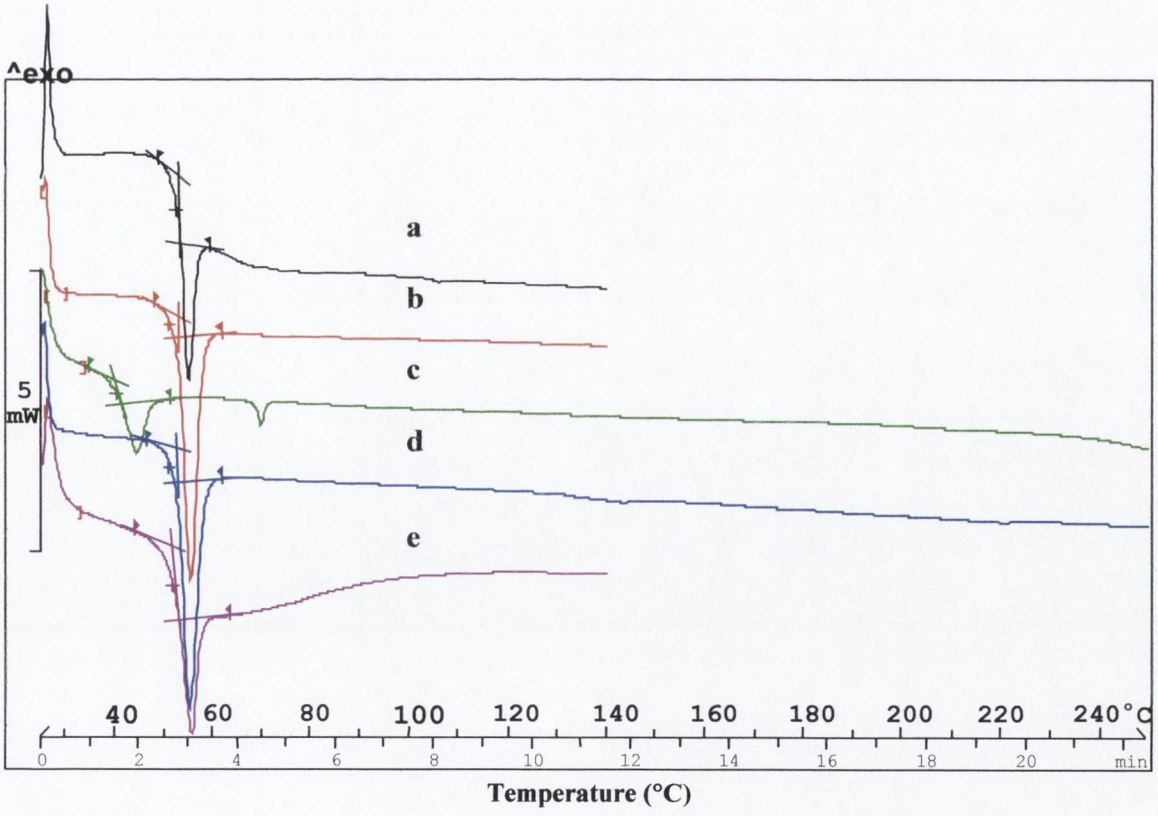


Figure 5- 9 DSC scan of (a) PLGA 5050, (b) empty microspheres and (e) a blend of protein and polymer. Scans of protein-loaded microspheres; (c) 180 seconds, (d) 240 seconds

Figure 5-9 illustrates the thermal characteristics of the RG 502 polymer, empty microspheres, RG 502 protein-loaded microspheres emulsified for 180 and 240 seconds and a blend of protein and polymer. DSC traces showed glass transition temperatures for RG 502, empty microspheres and protein-loaded microspheres manufactured for 180 and

240 seconds of 52.95°C, 52.79°C, 40.18°C and 52.53°C respectively. DSC was also carried out on a blend of 10% protein and RG 502 polymer to investigate the profile of the protein under the same experimental conditions as the loaded microspheres. This gave a glass transition temperature of 51.66°C and appeared to have a slightly different profile to either the protein-loaded microsphere or the polymer alone. The DSC did not exhibit a sharp peak as was evident in the previous scans rather it appeared to slope upwards thus increasing the width of the peak. From Figure 5-8 it was observed that the DSC of protein exhibited a broad endotherm. The DSC of the blend appears to exhibit a similar broad endotherm and the sharp peak seen with the polymer alone. This observation was not seen in the scans of the protein-loaded microspheres.

The glass transition temperatures of the polymer, the empty microspheres and drug loaded microspheres emulsified for 240 seconds confirm that, although there was a slight reduction in the T_g values of the microspheres when compared with the polymer, there was no major change in the glass transition temperatures (T_g) of the microspheres when compared with those of the polymer alone. This suggests that the protein was molecularly dispersed throughout the polymer. However there was a significant decrease in the glass transition of those microspheres emulsified for 180 seconds with two apparent endotherms evident. It has also been suggested that the slight reduction observed in the protein-loaded microspheres when compared with the polymer alone may be due to the plasticising affect of the encapsulated drug (Ford and Timmins, 1989). The slight reduction in T_g of the protein-loaded microspheres in this study could be attributed to this suggestion.

5.2.1. 4 Protein loading

Sharif et al. (1995) compared two methods for the determination of the levels of proteins entrapped in microspheres. The researchers compared protein loading values attained following extraction of the protein into dichloromethane (DCM) and following alkaline hydrolysis by sodium hydroxide (NaOH) and extraction into sodium dodecyl sulphate (SDS). Results showed that while extraction with DCM gave underestimation of protein levels, extraction via the alkaline hydrolysis method resulted in accurate extraction and determination of protein levels when compared with radiolabelled BSA protein. In the current study protein loading in PLGA microspheres was determined following alkaline

hydrolysis and extraction into Tris-EDTA buffer. The encapsulation efficiency (EE) was determined for protein-loaded microspheres and was expressed by relating the actual HSA entrapment to the theoretical HSA entrapment.

Emulsification time (seconds)	**Protein concentration μg/mg microsphere**	**SD**	**Encapsulation efficiency (%)**
180	12.86	± 3.03	12.61
240	11.38	± 6.23	10.95

Table 5- 3 Protein loading RG 502 microspheres
(samples represent the mean of nine determinations)

Table 5-3 illustrates the effect of emulsification time on protein loading. Statistical analysis indicated that loading did not vary significantly with increasing emulsification times ($p > 0.05$). Results show that encapsulation efficiency was highest for RG 502 microspheres manufactured following 180 seconds emulsification.

Uchida et al. (1994) encapsulated ovalbumin in PLGA 50:50 and reported encapsulation efficiencies of between 7 and 22% depending on the stirring rate used (3,000 rpm to 800 rpm). They reported higher encapsulation efficiencies with lower stirring rates. In the current study the stirring rate (8,000 rpm) was kept constant but time of emulsification was varied and it was observed that there was no statistical difference between encapsulation efficiencies ($p> 0.05$). The results observed in this section were in good agreement with results published by Uchida et al. (1993). The low encapsulation efficiency might be attributed to the low ratio of protein to polymer used. Jeffery et al. (1993) studied ovalbumin encapsulation in PLGA 50:50 (M.W. 22K). They examined the effect of the ratio of protein to polymer and reported values of 42.9% entrapment with a 1:10 protein:PLGA ratio. In this study the ratio of protein: PLGA was also 1:10 protein: PLGA (40mg: 400mg). However, these researchers do not report on manufacturing variables such as internal or external aqueous volume ratios, which may have an effect on the encapsulation efficiency of the protein.

5.2. 1. 5 Integrity of extracted protein

The integrity of protein extracted from RG 502 microspheres manufactured following 180 and 240 seconds emulsification was studied following SDS-PAGE of protein samples (Figure 5-10). Samples were run in lanes 2 and 3 with a standard HSA sample in lane 1 and a molecular weight marker in lane 4.

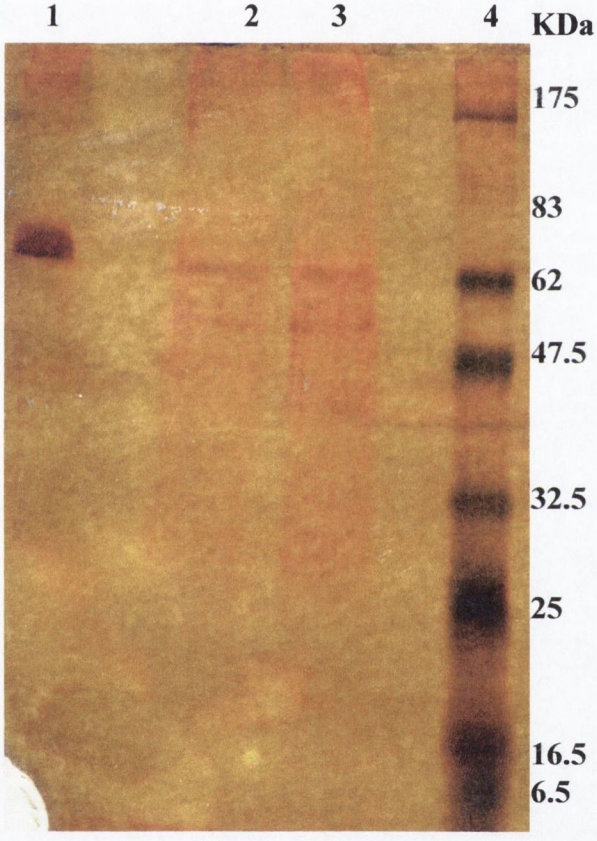


Figure 5- 10 SDS-PAGE of extracted protein

Lane 1: HSA protein standard
Lane 2: Extracted HSA from microspheres (RG 502 180 seconds)
Lane 3: Extracted HSA from microspheres (RG 502 240 seconds)
Lane 4: Molecular weight markers (MW range: 6.5 kDa – 175 kDa)

In the current study SDS-PAGE analysis was carried out on protein samples extracted from RG 502 microspheres. These were analysed for changes in protein structure by comparison against human serum albumin standard and a molecular weight marker. As can be seen from lanes 2 and 3 the relevant band corresponding to the HSA protein is clearly observed. Coomasie blue staining of the PAGE indicated that the structural integrity of the protein

was slightly affected by the encapsulation procedure as was evident from some smear further down the gel.

During microparticulate formation, the protein is exposed to potentially denaturing conditions such as mechanical agitation, high shear rate and organic solvents. These conditions may result in the irreversible denaturation of the encapsulated protein. Jeffery et al. (1993) used PAGE to investigate the integrity of ovalbumin protein extracted from PLGA 50:50 and 85:15 microspheres. Microspheres were manufactured following Silverson emulsification at 12,000 rpm. Park et al. (1995) also carried out PAGE on protein extracted from microspheres formulated following probe sonication. It was reported that protein extracted from release medium on day 1 of release tests showed no loss in structural integrity as a result of the encapsulation procedure.

5.2.1. 6 Protein release

Figure 5-11 illustrates the release of protein from microspheres formulated following 180 and 240 seconds emulsification.

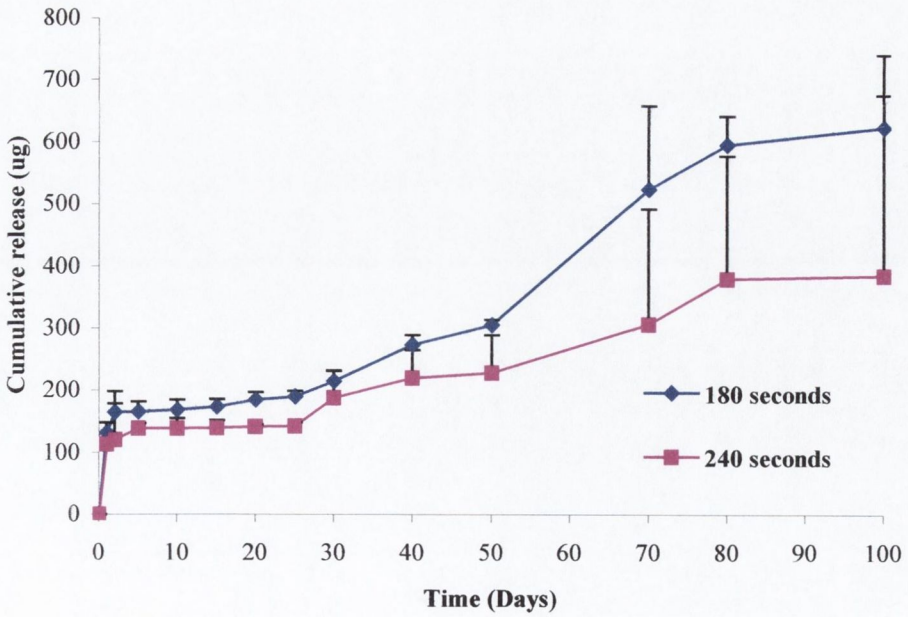


Figure 5- 11 Protein release from RG 502 microspheres
(samples represent the mean of three determinations)

Half-lives for typical PLGA 50:50 polymers were reported to be approximately between 3 and 10 weeks and typical release studies using similar molecular weight PLGA 50:50 (m.w. 18,000) have been reported to have been carried out over forty to one hundred days (McGee et al., 1997). Drug release studies in the current work were carried out over one hundred days.

From Figures 5-11 and 5-12 it can be seen that in both systems there is a triphasic release pattern over the 100 hundred days. This pattern appears to level off at about day 80 with between 70 and 95% of total encapsulated protein being released at this time. Microspheres formulated following 180 seconds homogenisation appeared to release a higher percentage of their content over one hundred days. These microspheres were also larger in size when compared with those formulated following 240 seconds emulsification time. McGee et al. (1997) showed an increasing rate of release with increasing particle size. The PLGA 50:50 polymer used in this study is a relatively low molecular weight PLGA polymer (m.w. 15.6 kDa) compared with other molecular weight grades of PLGA 50:50 used in the literature (Uchida et al. (1994) PLGA 50:50 m.w. 53,000; Coombes et al. (1998) PLGA 50:50 m.w. 34,000).

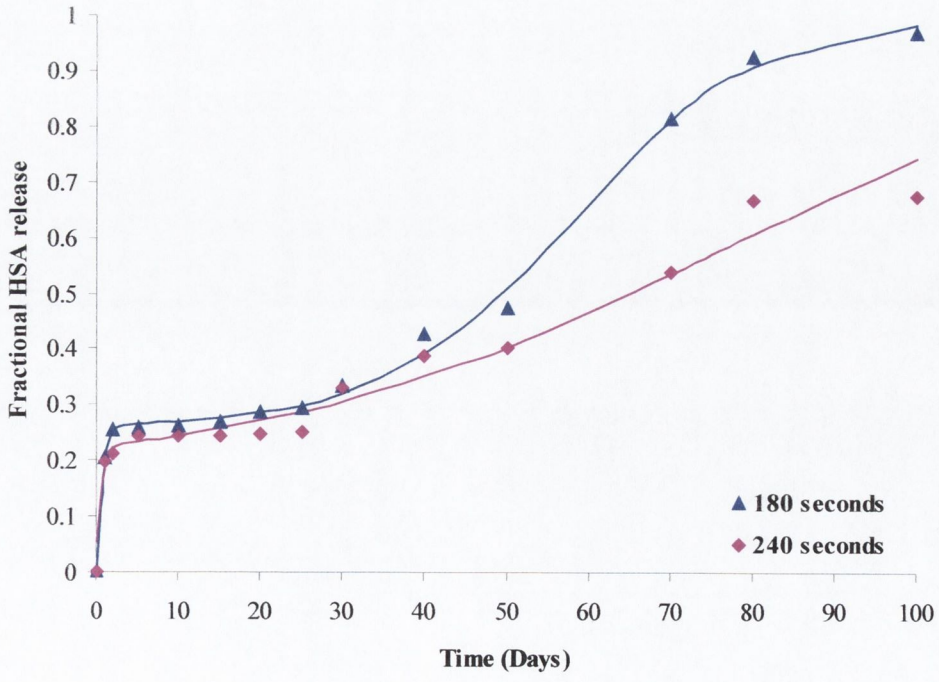


Figure 5- 12 Fractional protein release from RG 502 microspheres fitted to Equation 1-15
(samples represent the mean of three determinations)

The fractional release of protein from microspheres manufactured following 180 and 240 seconds was fitted to Equation 1-15 (Figure 5-12). Equation 1-15 was developed by Gallagher and Corrigan, (1996) and was initially used to describe the release of levamisole hydrochloride from PLGA 50:50 discs and combines the contribution from first order initial release phase and polymer degradation release phase.

Parameters obtained for the degradation controlled release rate and the burst release rate of the model were determined according to emulsification times and are shown in Table 5-4. The equation assumes two additive release processes: an exponential fast release phase with parameter F_B which can be defined as the fraction of drug available for direct surface release and K_B the release rate constant and the degradation controlled release phase with parameters k and $Tmax$.

Parameter	**180 seconds**	**240 seconds**
F_B	0.26	0.17
K_B	1.5	1.66
k	0.09	0.03
$Tmax$	57.68	77.34
CD	0.99	0.97
MSC	5.15	2.84

Table 5- 4 Parameters obtained from fractional release from RG 502 microspheres fitted to Equation 1-15

As can be seen from Table 5-4 the model selection criteria (MSC) for both graphs indicates a good fit of actual data to that generated by the model. Values for the coefficient of determination also indicated values close to 1, which is a further indication of a good fit between actual and predicted release data as generated by Equation 1-15.

Polymers which contain relatively high ratios of glycolic acid, an example of which is PLGA RG 502, are likely over a period of time to undergo bulk hydrolysis during which the permeability of the polymer to the drug increases. This increase in permeability leads to a release which is not predictable. This phenomenon leads to a situation whereby the release of the drug can be erratic and irregular thereby excluding such polymers for use in long term sustained release systems. Higher molecular weight and more hydrophobic

PLGAs have been reported to degrade more slowly than lower molecular weight species (Li and Vert, 1999). It was decided to investigate RG 752, a more hydrophobic polymer, in an attempt to develop longer and more predictable sustained release systems for HSA.

5.2. 2 Preparation of RG 752-loaded microspheres

RG 752 has a higher ratio of lactic to glycolic acid moieties relative to RG 502. Therefore it would be expected to degrade more slowly than a comparable weight RG 502 (Li and Vert, 1999). RG 752 microspheres were prepared as for the RG 502 microspheres discussed in the previous section.

5.2.2. 1 Scanning Electron Microscopy

Scanning electron microscopy was used to characterise the morphology of the microspheres. SEM micrographs of RG 752 microspheres emulsified for 180 and 240 seconds can be seen in Figures 5.13 and 5.14 respectively.

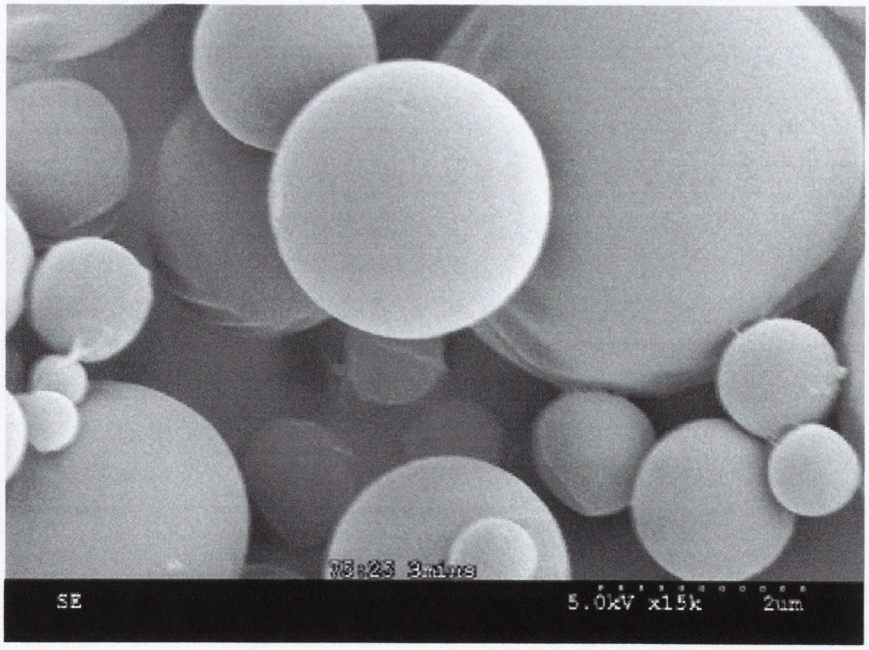


Figure 5- 13 RG 752 HSA-loaded microspheres - 180 seconds emulsification

The micrographs show typical microspheres from both systems with smooth outer surfaces. When compared with microspheres formed with RG 502 these microspheres are more spherical in shape and regular in size. Microspheres manufactured following 180 seconds homogenisation appeared to aggregate, as those manufactured with RG 502 did.

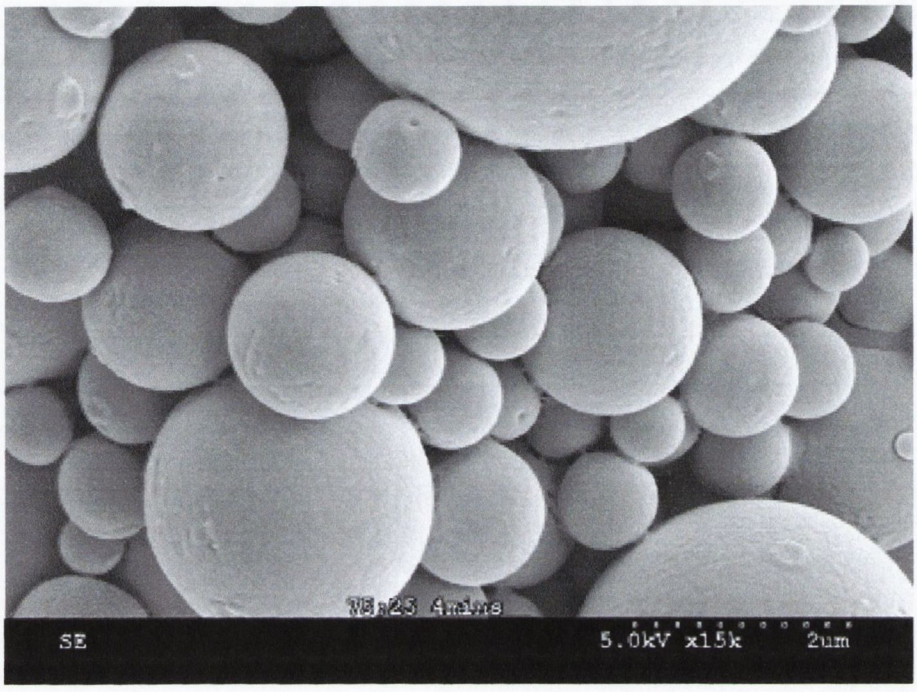


Figure 5- 14 SEM of HSA-loaded RG 752 microspheres – emulsified for 240 seconds

Microspheres manufactured following 240 seconds homogenisation did not appear to flocculate. Microspheres formed following longer homogenisation times show some surface pitting or slight irregularities in their surface layer.

5.2.2. 2 Particle size data

Particle size data obtained for RG 752 protein-loaded microspheres emulsified for 180 and 240 seconds are illustrated in Table 5-5.

Emulsification time (seconds)	**D[ν,0.5] μm**	**SD**	**D[ν0.9] μm**	**SD**	**Span**
180	4.79	± 0.20	12.08	± 2.03	2.65
240	4.55	± 0.02	9.16	± 1.10	2.37

Table 5- 5 Particle size data for RG 752 systems
(samples represent the mean of three determinations)

Mean particle sizes for batches subjected to different emulsification times did not exhibit any statistical differences ($p > 0.05$). Discrepancies in particle size between SEM images and Malvern laser diffraction results could be attributed to the aggregation exhibited by microspheres manufactured following 180 seconds homogenisation. RG 752 microspheres were significantly smaller in size when compared with those formulated with RG 502 (RG 502 microspheres - D[ν,0.5] values of 6.33 μm and 4.92 μm for emulsification of 180 and 240 seconds respectively) (Figure 5-15).

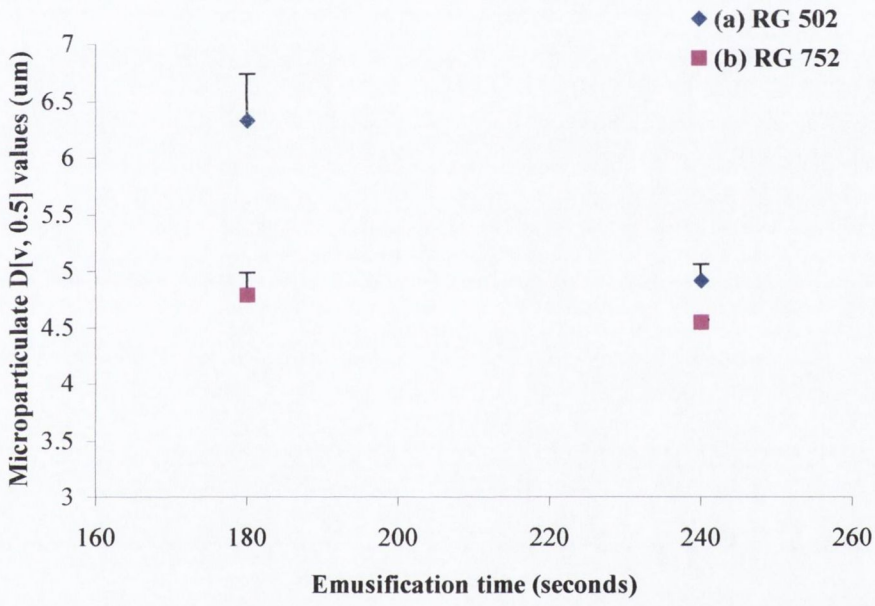


Figure 5- 15 Influence of polymer on particle size (a) RG 502 (b) RG 752
(samples represent the mean of three determinations)

According to Mehta et al. (1996) RG 752, with a molecular weight of 15,577, had a longer solidification time which resulted in smaller particle sizes for RG 752 microspheres when compared with RG 502 microspheres (molecular weight 10, 754). This observation was in good agreement with results found in the current work.

5.2.2. 3 Differential Scanning Calorimetry

Figure 5-16 shows traces obtained by DSC on heating from 25°C to 140°C at a rate of 10°C a minute of (a) RG 752 (b) empty microspheres and RG 752 protein-loaded microspheres emulsified for (c) 180 seconds and (d) 240 seconds and (e) polymer/protein blend.

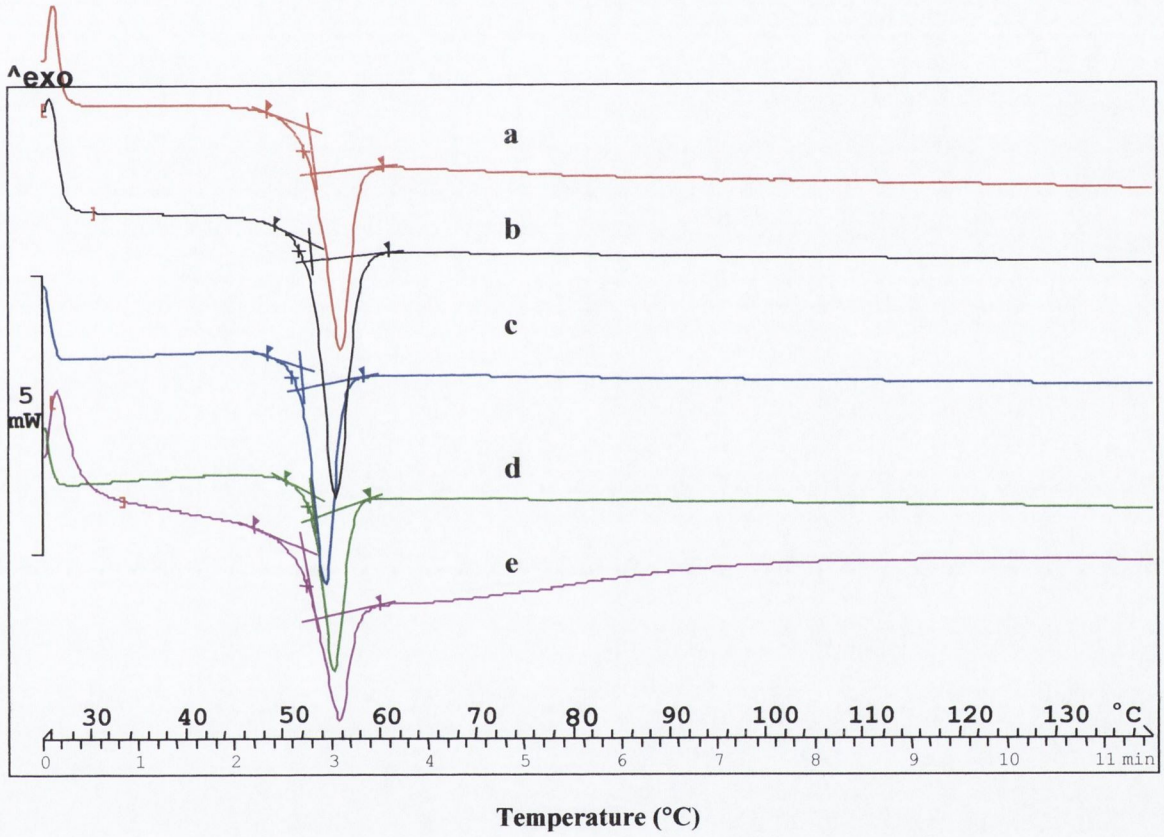


Figure 5- 16 DSC scans of (a) RG 752, (b) empty microspheres and protein-loaded microspheres; (c) 180 seconds and (d) 240 seconds and (e) polymer/protein blend

DSC scans showed glass transition temperatures of 52.78°C, 52.86°C, 51.80°C and 52.90°C for PLGA 7525, empty microspheres and protein-loaded microspheres emulsified for 180 and 240 seconds respectively. DSC carried out on blends of polymer and protein showed an endotherm with a glass transition temperature of 51.92°C. The resultant endotherm was similar in shape to that observed in Section 5.2.1.3. From these results it appears that protein-loaded microspheres do not exhibit similar endotherms to that of the blend. Rather they were similar in shape and temperature to empty microspheres and the

polymer powder. It can be concluded that the polymer maintained its thermal properties in the microspheres prepared following a w/o/w technique. No protein peak was observable which suggests the dispersion of the protein within the polymer matrix. The thermogram of the protein alone shows a typical wide broad peak with glass transition temperature of 78.53°C (Figure 5-8).

5.2.2. 4 Protein loading

Levels of protein encapsulated in RG 752 microspheres were determined by alkaline hydrolysis in a manner identical to extraction from RG 502 microspheres (Table 5-7). Encapsulation efficiency was determined by comparing actual loading levels to the theoretical loading (Table 5-6).

Microsphere Emulsification time	**Protein concentration μg/mg microsphere**	**SD**	**EE (%)**
180 seconds	8.54	± 2.73	8.22
240 seconds	9.20	± 3.15	8.81

Table 5- 6 Protein loading RG 752 microspheres
(samples represent the mean of nine determinations)

Protein encapsulation levels were not significantly different for batches subjected to different emulsification times ($p > 0.05$). There was no statistical difference in encapsulation levels of microspheres emulsified for 180 and 240 seconds. There was a slight increase in encapsulation efficiency observed in microspheres homogenised for 240 seconds when compared with those homogenised for 180 seconds.

Microspheres formulated with RG 502 showed higher loading values than those formulated with RG 752 at both emulsification time points. This observation might be ascribed to the hydrophilic nature of RG 502 and its rapid solidification of microsphere droplets when compared with more hydrophobic polymers (Mehta et al., 1996; Walter et al., 2001). This more rapid solidification would be expected to reduce the diffusion of protein into the continuous or outer aqueous phase of the double emulsion, resulting in increased entrapment and protein loading.

In the current study levels of protein encapsulation in RG 752 microspheres were significantly different from those observed in PLGA 50:50 ($p < 0.05$). The reported encapsulation efficiencies are in line with results of Uchida et al. (1994) who reported levels of between 7 and 22% encapsulation efficiencies for ovalbumin-loaded microspheres. Mehta et al. (1996) also reported higher levels of protein encapsulation in PLGA 50:50 when compared with PLGA 75:25. This was attributed to a lower solubility of PLGA 50:50 in the disperse phase which resulted in the rapid solidification of PLGA 50:50 microspheres thus reducing peptide diffusion during emulsification. This resulted in higher drug loads in PLGA 50:50 microspheres. In the current study this observation is supported by particle size results which showed smaller particle sizes for RG 752 microspheres which was attributed to a longer solidification time and higher shrinkage rate. The longer solidification time for PLGA 75:25 microspheres would allow for the diffusion of the protein into the outer aqueous phase and would result in a lower protein encapsulation when compared with PLGA 50:50 microspheres.

5.2.2. 5 Integrity of extracted protein

Protein samples extracted from RG 752 microspheres were analysed following SDS-PAGE and silver staining of the gel (Figure 5-17).

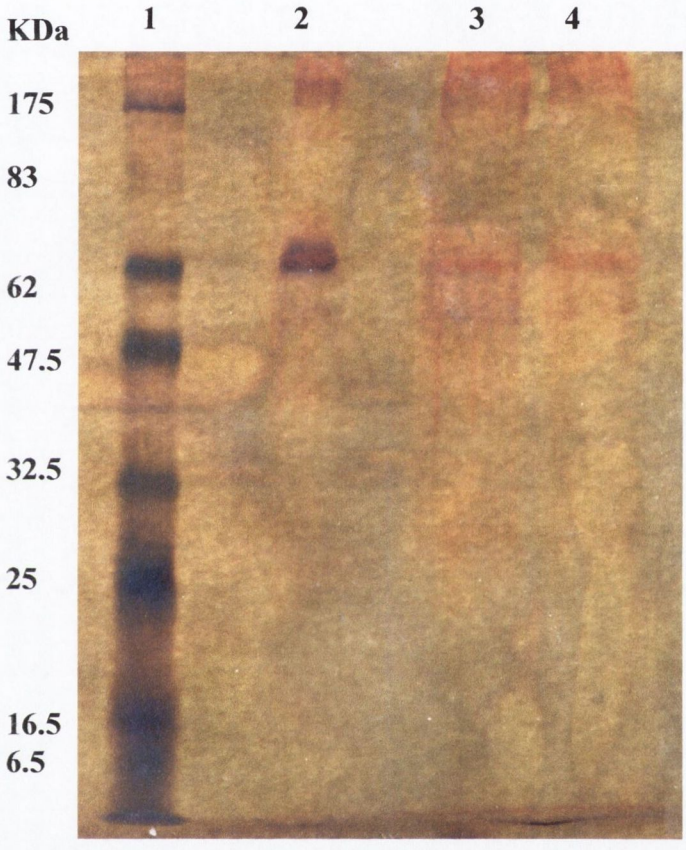


Figure 5-17 SDS-PAGE of protein extracted from RG 752 microspheres

Lane 1: Molecular weight markers (MW range: 6.5 kDa – 175 kDa)
Lane 2: HSA protein standard
Lane 3: Extracted HSA from microspheres (RG 752 180 seconds)
Lane 4: Extracted HSA from microspheres (RG 752 240 seconds)

In the current study SDS-PAGE analysis was carried out on protein samples extracted from RG 752 microspheres. These were analysed for changes in protein structure as a result of the microencapsulation procedure. As can be seen from lanes 3 and 4 the relevant band corresponding to the HSA protein is clearly observed at approximately 66 kDa indicating intact protein. However also visible on the gel is a small amount of smearing in the

extracted protein lane, which would indicate the presence of some denatured protein molecules.

5.2.2. 6 Protein release

Protein release studies were carried out on RG 752 microspheres containing protein over a one hundred day period. Figure 5-18 illustrates the release of protein from RG 752 microspheres formulated following 180 and 240 seconds emulsification.

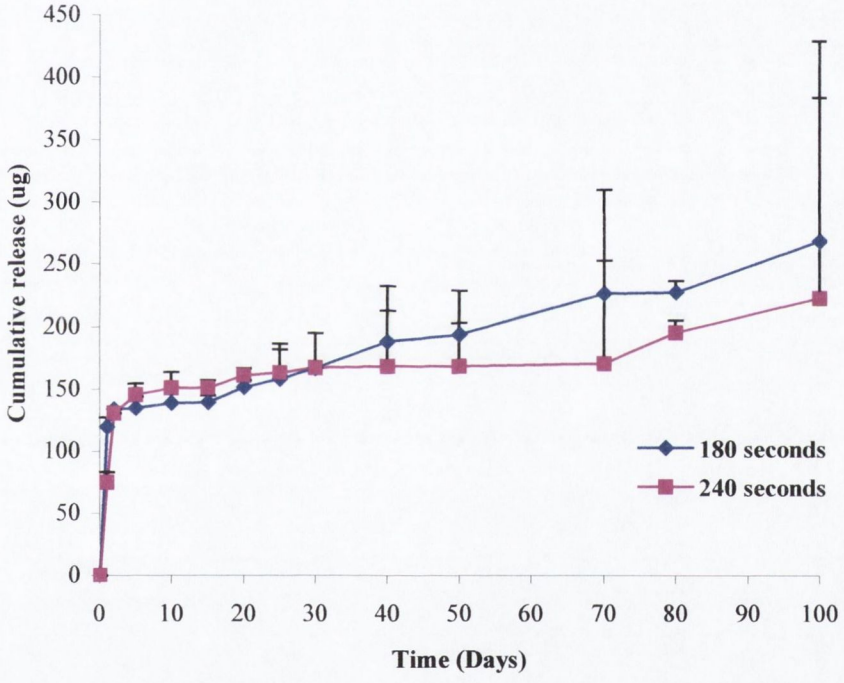


Figure 5- 18 Protein release from RG 752 microspheres

(samples represent the mean of three determinations)

Figure 5-18 shows the release of protein from protein-loaded RG 752 microspheres emulsified for 180 seconds and 240 seconds. As can be seen there is a more rapid protein release from microspheres formed following 180 seconds homogenisation when compared with that from microspheres formed following 240 seconds emulsification. The pattern of protein release appears to be an initial burst release from both systems, followed by a sustained release over the one hundred day trial. It is presumed that the release of protein would have continued until the microspheres had been depleted had the study not been terminated.

Figure 5-19 shows the plot of fractional release from RG 752 microspheres fitted to Equation 1-14. This set of release data was fitted to an equation initially developed by Higuchi to describe diffusion from a slab. This was then developed further by Peppas to describe diffusion from a sphere. The previous model used in relation to release from RG 502 microspheres was deemed not to be suitable for the release from RG 752 microspheres given the length of the trial and the lack of degradation release associated with RG 752 microspheres after one hundred days.

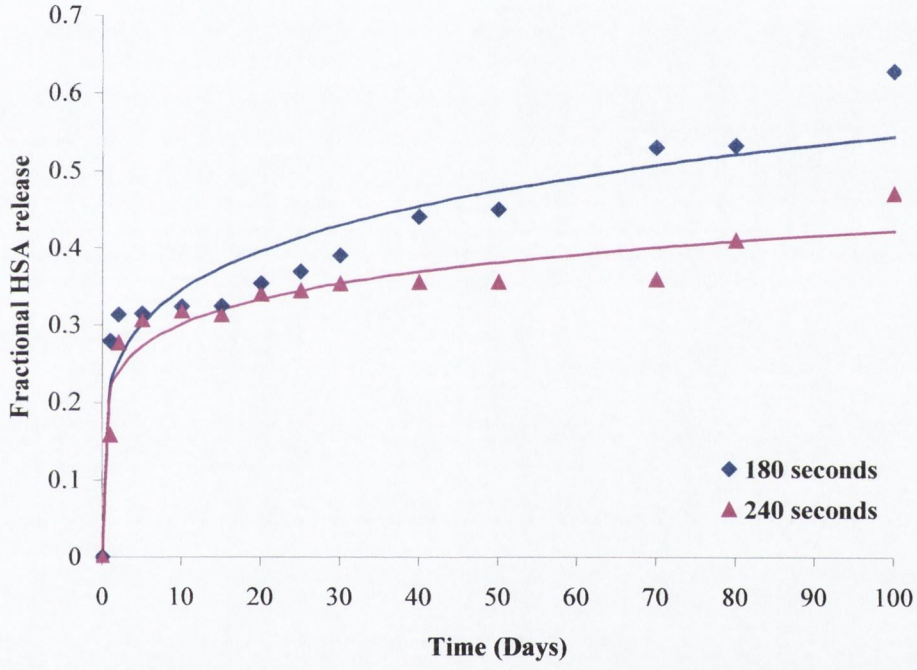


Figure 5- 19 Fractional release of HSA from RG 752 microspheres fitted to Equation 1-14

From Figure 5-19 it can be seen that there would appear to be a higher fraction of release from microspheres formed following 180 seconds emulsification when compared with the fraction released following 240 seconds emulsification.

Parameters obtained for the fitting of Equation 1-14 to release from RG 752 microspheres are shown in Table 5-7.

Parameter	180 seconds	240 seconds
K	0.219	0.215
n	0.197	0.146
CD	0.916	0.940
MSC	2.185	2.426

Table 5- 7 Parameters obtained following fitting of Equation 1-14 to release of HSA from RG 752 microspheres

From Table 5-7 it can be seen that there is a higher MSC value associated with RG 752 microspheres manufactured following 240 seconds when compared with that from microspheres manufactured following 180 seconds emulsification. This indicates a better fit to Equation 1-14 of release data from RG 752 240 seconds. Both batches showed good coefficients of determination with values close to 1. The variable n is the diffusional constant and can be set to 0.432 when the drug is released from a sphere. It has been reported that polydisperse systems will yield different n values for Fickian diffusion depending on the range of particle size used. However for such diffusion to occur n should fall in the range of 0.4 and 0.42. However n values in this case fell below 0.3 and are thus uninformative about the type of release which occurred.

5. 3 Characterisation of RG 752 delivery systems manufactured using alternative techniques

While protein microspheres manufactured via high-speed homogenisation have been well documented, this technique is not suitable for agents susceptible to the high shear rate of such a technique. Agents such as DNA and other nucleic acids have been shown to be susceptible to the high shear rate of high-speed homogenisation (Adami et al., 1998; Middaugh et al., 1998). It was for these reasons that the integrity and physiochemical properties of HSA encapsulated in RG 752 were studied following alternative encapsulation techniques. It was decided to investigate formulations using a Silverson mixer rather than the Ultra Turrax device, which was used in the previous sections. Emulsification using mixing devices with lower shearing rates (Silverson Laboratory mixer L4R) is a commonly used technique where the stability of the encapsulated agent is an issue. It is used in place of high-speed homogenisation and has been used with agents susceptible to denaturation or shearing (McKeever et al., 2002). The principle objective here was to employ HSA as a model compound to assist in the selection of optimal parameters for DNA encapsulation. It was decided to compare the effects of high-speed homogenisation (Ultra Turrax T25) and homogenisation with a Silverson mixer on protein encapsulation.

Spray-drying is used extensively in several industries including the chemical, biochemical, food and pharmaceutical industry (Broadhead et al., 1992; Johansen et al., 2000). Spray driers are designed to allow the passage of a solution through as a spray while at the same time removing the moisture from the solution by the passage of drying air either co-current (in the same direction) or counter-current (in the opposite direction) to the spray. The final step involves the separation of the final product from the air stream, which is usually accomplished using a cyclone separator. Spray-drying has been used in the encapsulation of a number of different agents including vitamin D_3 (Pavanetto et al., 1992) bovine serum albumin (Baras et al., 2000), 5-fluorouracil (Fu et al., 2002), insulin (Stahl et al., 2002) and DNA (Walter et al., 2001). Several of these agents are heat sensitive and it was decided to investigate the physicochemical properties of protein-loaded microspheres by spray-drying.

5.3. 1 Microspheres manufactured using a Silverson mixer (L4R)

Microspheres were manufactured as described in Chapter 4 Section 4.6.1.

5.3.1. 1 Scanning Electron Microscopy

Figures 5-20 and 5-21 illustrate scanning electron micrographs of microspheres manufactured following homogenisation with a Silverson mixer and an Ultra Turrax.

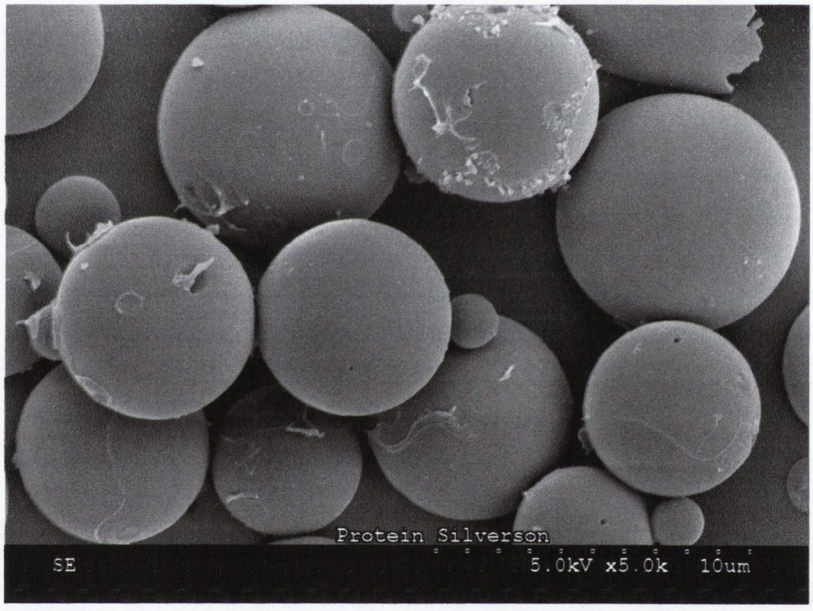


Figure 5- 20 RG 752 microspheres manufactured with a Silverson mixer

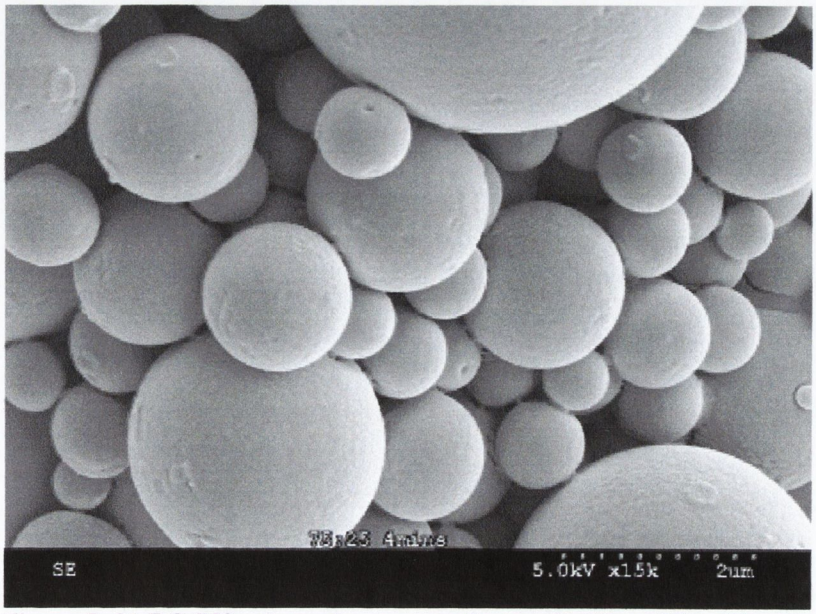


Figure 5- 21 RG 752 microspheres manufactured with an Ultra Turrax

Scanning electron micrographs show spherical microspheres with smooth surfaces with very few surface defects such as pores or channels. Microspheres formed following Silverson emulsification appear smoother and larger (Figure 5-20) when compared with microspheres formed following high-speed homogenisation (Ultra Turrax T25) (Figure 5-21). Another factor which contributes to particle morphology is the high stirring speeds associated with homogenisation using devices such as an Ultra Turrax (T25). This could promote the formation of irregular surfaces compared with those formed following other homogenisation techniques. This was reported by Pavanetto et al. (1992) who reported surface defects on microspheres which had been formed following homogenisation with an Ultra Turrax (T25).

5.3.1. 2 Particle size data

Particle size data for microspheres formulated using a Silverson mixer and an Ultra Turrax are illustrated in Table 5-8.

Technique (rpm)	**D[ν,0.5] μm**	**SD**	**D[ν,0.9] μm**	**SD**	**Span**
Silverson (L4R) (2,000)	6.65	± 0.01	10.03	± 0.12	3.08
Ultra Turrax (T25) (8,000)	4.55	± 0.02	9.16	± 1.10	2.37

Table 5- 8 Particle size data

(samples represent the mean of three determinations)

From Table 5-8 it can be seen that the increasing homogenisation speed from 2,000 rpm to 8,000 rpm results in a decrease in mean D[ν,0.5] particle size values. Statistical analysis indicated that the D[ν,0.5] values were statistically different between samples formed following emulsification with a Silverson mixer and a high-speed homogeniser (Ultra Turrax T25) ($p < 0.05$). Particle size data appears to support SEM findings that microspheres formulated following emulsification with a Silverson mixer resulted in larger particles when compared with those formulated following high-speed emulsification.

Several papers have been published on the effect of homogenisation speed on mean particle size including McGee et al. (1997) who reported that increasing homogenisation speeds from 5,700 rpm to 15,000 rpm leads to a decrease in mean D [v,0.5] particle size values from between 29.80 μm and 52.45 μm, depending on loading, to less than 1 μm. Jain (2000) also reported high homogenisation speed to be an important factor in the production of nano-sized particles. Uchida et al. (1994) reported that an increase in stirring rates from 800 rpm to 3,000 rpm led to a decrease in mean particle average diameter from 14.3 μm to 1.2 μm. In the current study it was observed that increasing the emulsification speed from 2,000 rpm to 8,000 rpm led to a decrease in median particle size from 6.65 μm to 4.55 μm.

5.3.1. 3 Differential Scanning Calorimetry

Figure 5-22 illustrates the thermal characteristics of RG 752, empty microspheres, protein loaded microspheres manufactured using an Ultra Turrax (T25) and a Silverson mixer (L4R) and human serum albumin/ polymer blend.

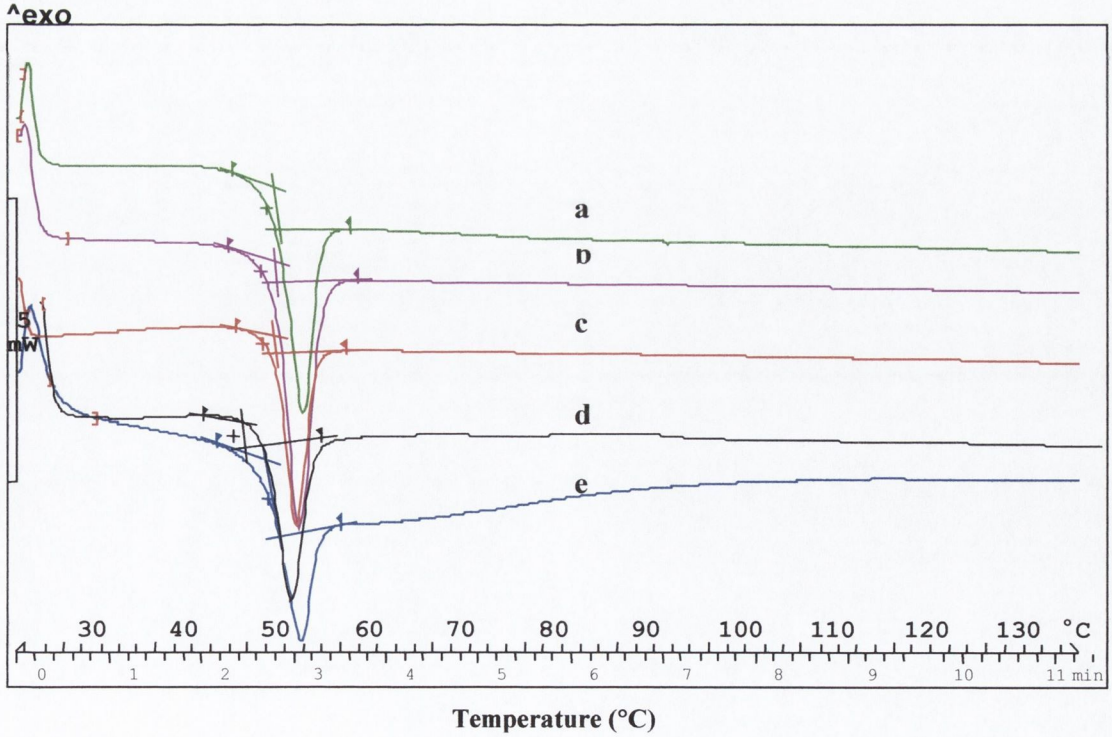


Figure 5- 22 DSC scan of (a) RG 752, (b) empty microspheres and (e) human serum albumin. Scans of protein loaded microspheres manufactured with (c) an Ultra Turrax (T25) and (d) a Silverson mixer (L4R)

DSC traces showed glass transition temperatures for RG 752, empty microspheres and protein loaded microspheres manufactured with an Ultra Turrax (T25) and a Silverson mixer (L4R) of 52.00°C, 51.19°C, 51.58°C and 49.43°C respectively. As can be seen from these results microspheres manufactured following Ultra Turrax homogenisation had similar T_g values to both RG 752 and empty microspheres and did not exhibit similar endotherms to that exhibited by the blend of protein and polymer with a transition temperature of 52.13°C. This suggested that the thermal characteristics of the polymer were not significantly altered following the encapsulation of protein manufactured in this manner. The glass transition temperature of microspheres manufactured following Silverson homogenisation is lower than that of the polymer alone or the empty microspheres which would suggest that microencapsulation using this method does affect the thermal characteristics of the polymer either through the plasticising effect of the encapsulated protein or through the incomplete dispersion of the protein in the polymer matrix. All thermal events were seen as sharp endothermic peaks with the exception of those for the protein and polymer blend, which showed a broad peak. Human serum albumin exhibited a broad endotherm with a glass transition of 78.6°C (Figure 5-5). As can be seen there was no evidence of a similar broad endotherm in any of the protein samples, which suggests the complete encapsulation of the protein within the polymer. This also suggests that the reduction in glass transition temperature associated with microspheres manufactured using a Silverson mixer is most likely due to the plasticising effect of an encapsulated agent as reported by Ford and Timmins (1989).

5.3.1. 4 Protein loading

Protein loading was determined following alkaline hydrolysis and extraction of the released protein into TE buffer. Table 5-9 illustrates the levels of protein loading according to emulsification technique and speed.

Emulsification technique (rpm)	**Protein concentration μg/mg microsphere**	**SD**	**EE (%)**
Silverson (2,000)	11.69	± 1.58	8.79
High-speed homogenisation (8,000)	9.20	± 3.15	6.92

Table 5- 9 Protein loading

(samples represent the mean of nine determinations)

Statistical analysis found there was no statistical difference between the two sets of loading data with respect to homogenisation technique ($p > 0.05$). Although there is no statistical difference in loading values, thermal characteristics of protein-loaded microspheres may reflect the trend in protein loading results. From Figure 5-22 it can be seen that the glass transition temperature of microspheres manufactured with an Ultra Turrax is higher than that for microspheres manufactured with a Silverson mixer. The decrease in T_g seen in microspheres manufactured with a Silverson mixer might be attributed to higher protein loading values.

Uchida et al. (1994) studied the effect of homogenisation speed on ovalbumin loading and reported higher loading values with decreasing homogenisation speeds. Although in the current work, there is no statistical difference between samples homogenised at different speeds there is a general increase in encapsulation efficiency with a decrease in homogenisation speed. This observation mirrors previous trends observed in the case of particles formulated with RG 502 following high-speed homogenisation and is also in good agreement with other researchers (Jeffery et al., 1993; Uchida et al., 1994).

5.3.1. 5 Integrity of encapsulated protein

The integrity of encapsulated protein was examined following SDS-PAGE of extracted protein samples (Figure 5-23).

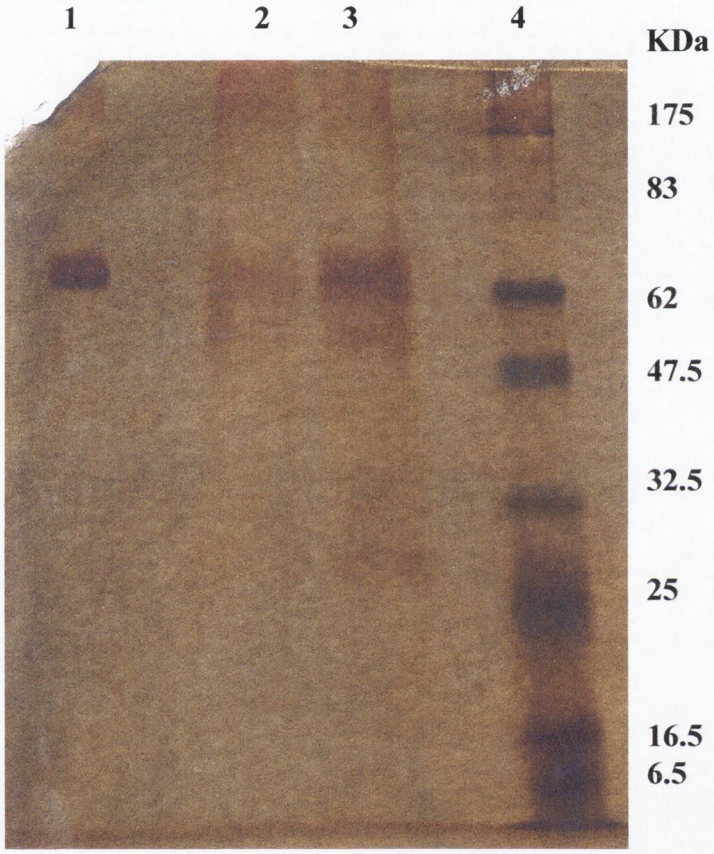


Figure 5- 23 SDS-PAGE of extracted protein

Lane 1: HSA standard (MW 66 kDa)
Lane 2: HSA extracted from microspheres (Ultra Turrax (T25))
Lane 3: HSA extracted from microspheres (Silverson mixer (L4R))
Lane 4: Broad range molecular weight marker (MW range: 6.5 kDa – 175 kDa)

In the current study, PAGE analysis was carried out on protein extracted from microspheres manufactured using an Ultra Turrax (T25) and a Silverson mixer (L4R) (Figure 5-23). The gel was stained with silver nitrate and revealed identical bands for the native and extracted protein (Lane 1, 2 and 3). HSA is a 66 kDa protein and from lanes 2 and 3 it can be seen that there are identical bands which when compared with the molecular weight marker correspond to the 66 kDa molecular weight marker (Lane 4). There were some additional bands in the case of the extracted protein from both batches of

microspheres however, these bands were very faint with the biggest band showing a molecular weight of 66 kDa. These additional smears are indications of the presence of additional fragments of protein, which could be attributed to the fragmentation or denaturation of the protein during the encapsulation procedure. Results indicated that the encapsulation processes used here, i.e. homogenisation with an Ultra Turrax (T25) and a Silverson mixer did not significantly affect the integrity of the encapsulated protein with most of the protein maintaining its integrity as determined by molecular weight comparison with protein standards.

5.3.1. 6 Protein release

Figure 5-24 illustrates the release from microspheres formed following homogenisation with a Silverson mixer and high-speed homogenisation.

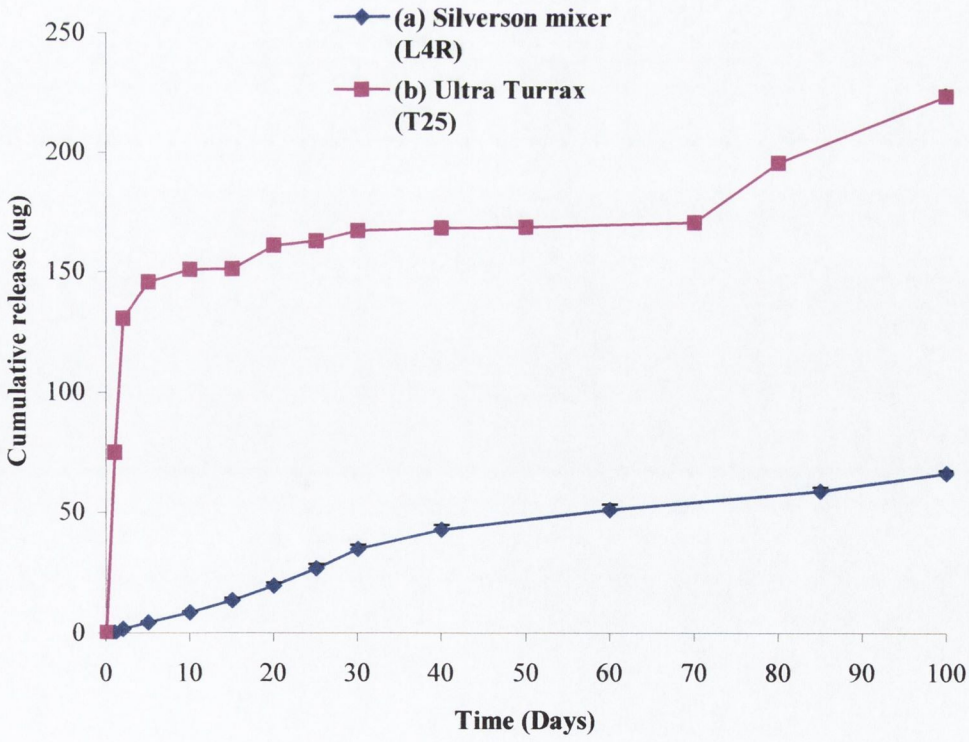


Figure 5- 24 Protein release from microspheres manufactured using (a) a Silverson mixer (b) Ultra Turrax (T25)
(samples represent the mean of three determinations)

As can be seen from Figure 5-24 there is a higher cumulative release from microspheres formed following emulsification at 8,000 rpm when compared with those formed following homogenisation at 2,000. From scanning electron micrographs of the two batches of microspheres it was observed that those manufactured using an Ultra Turrax (T25) exhibited a higher incidence of surface pores and defects when compared with those manufactured following homogenisation with a Silverson mixer (L4R). Microparticles, which have surface defects such as pores and channels, have been reported to release entrapped drug at a different rate to those, which do not exhibit such surface anomalies (McGee et al., 1997). Surface pores observed in microspheres following homogenisation with an Ultra Turrax (T25) would contribute to the increased burst release observed from these microspheres when compared with microspheres manufactured at lower emulsification speeds.

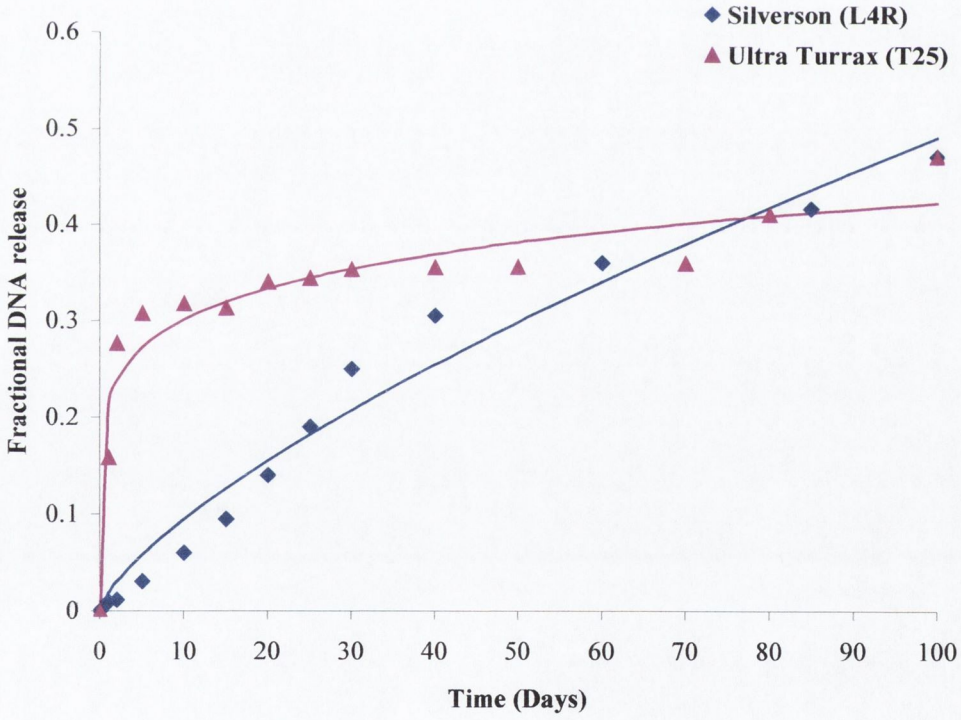


Figure 5- 25 Fractional protein release from microspheres manufactured using (a) a Silverson mixer (b) Ultra Turrax (T25) fitted to Equation 1-14

The main observation from both Figure 5-24 and Figure 5-25 is the absence of a burst effect from microspheres formed following Silverson homogenisation which is very evident following high-speed homogenisation (approximately 32% by day 10).

Parameters obtained from fitting Equation 1-14 to fractional release from microspheres manufactured using an Ultra Turrax (T25) and a Silverson mixer (L4R) are listed in Table 5-10.

Parameter	Silverson	Ultra Turrax
K	0.018	0.215
n	0.714	0.146
CD	0.972	0.940
MSC	3.264	2.426

Table 5- 10 Parameters obtained following fitting of Equation 1-14 to release of HSA from RG 752 microspheres

From Table 5-10 it can be seen that the model selection criteria is higher for release data from microspheres manufactured following homogenisation with a Silverson mixer (L4R) when compared with that from microspheres manufactured following homogenisation with an Ultra Turrax (T25). This indicates that there is a better fit of the model to the release data for these microspheres when compared with that observed with microspheres manufactured following homogenisation with an Ultra Turrax (T25). The coefficient of determination value is also closer to 1 in this batch when compared to microspheres manufactured using an Ultra Turrax (T25), which also reflects a good fit between actual data and the mathematical model.

5.3. 2 Microspheres manufactured using a Büchi spray drier

Emulsions were manufactured as described in Chapter 4 Section 4.6.1 and subsequently spray dried. Inlet temperatures of between 50°C and 80°C and a variety of air flow speeds were examined in this study and it was found that spray-drying at temperatures lower than 80°C resulted in the incomplete evaporation of the dichloromethane solvent. Hence 80°C was chosen as the inlet temperature and to examine the effect of these high temperatures on protein integrity. Typical inlet temperatures for spray dried agents such as insulin and protein antigens range from 40°C to 100°C (Baras et al., 2000; Ståhl et al., 2002).

5.3.2. 1 Scanning Electron Microscopy

Figure 5-26 and 5-27 show scanning electron micrographs of empty spray dried and protein loaded spray dried microspheres respectively.

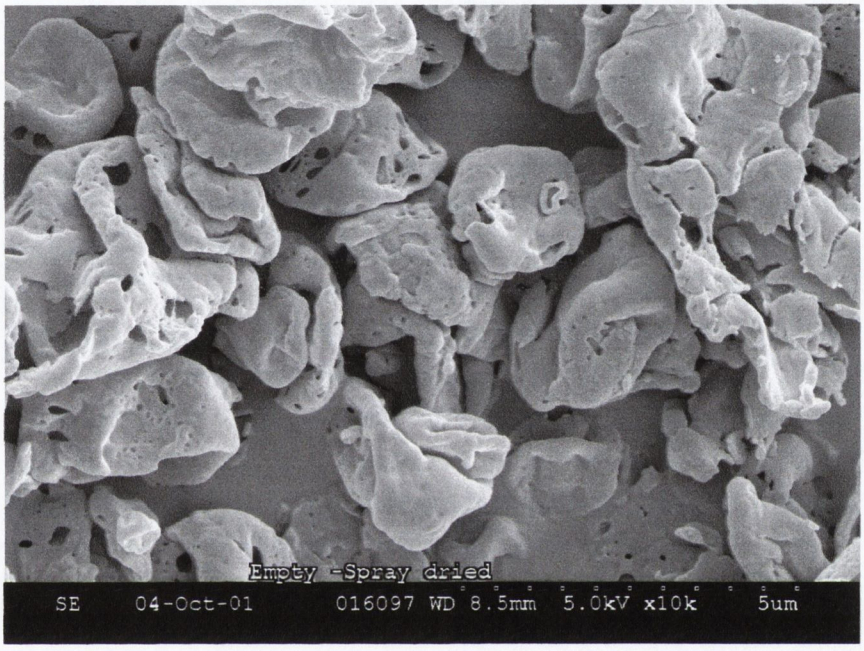


Figure 5- 26 Scanning electron micrograph of typical spray dried empty microspheres

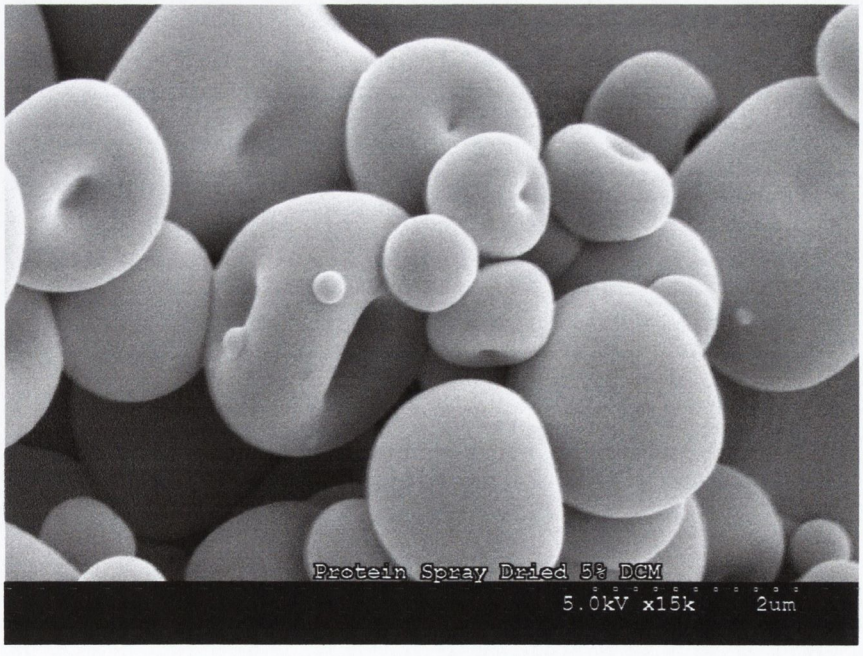


Figure 5- 27 Scanning electron micrograph of typical protein-loaded spray dried microspheres

Micrographs of protein-loaded spray dried microspheres (Figure 5-27) show doughnut shaped microspheres with a great diversity in shape and size compared with empty microspheres which appear fragmented (Figure 5-26). This is in contrast with solvent-evaporated microspheres where loaded and empty microspheres appear to have the same morphology.

Microspheres formed following spray-drying are usually spherical but have been reported to be hollow (Jain et al., 2000). Broadhead et al. (1992) also reported that spray dried microspheres were typically produced with hollow interiors. Hsu et al. (1996) presented a theory, which is based on the formation of a skin at high temperatures on the outer surface of the spray particles. They suggested that when the inner water phase is evaporated off the skin is destroyed and the outer surface collapses. The doughnut shape of protein-loaded microspheres observed here could be attributed to the collapse of the external microsphere wall at high temperatures.

5.3.2. 2 Particle size data

Particle size data for protein-loaded microspheres formed following emulsification with a Silverson mixer or Ultra Turrax (T25) emulsification followed by either spray-drying of the resultant emulsion or solvent evaporation are illustrated in Table 5-11 and Figure 5-28.

Manufacture technique		**D [ν, 0.5] μm**	**S.D.**	**D [ν, 0.9] μm**	**S.D.**	**Span**
Spray-drying	Silverson (L4R)	48.12	± 0.24	95.58	± 1.10	2.55
	Ultra Turrax (T25)	8.30	± 0.51	102.16	±1.05	3.08
Solvent evaporation	Silverson (L4R)	6.65	± 0.01	10.03	± 0.12	2.47
	Ultra Turrax (T25)	4.55	± 0.02	9.16	± 1.10	2.37

Table 5- 11 Particle size data for HSA-loaded microspheres manufactured following spray-drying or solvent evaporation

(samples represent the mean of three determinations)

Results show that particles formed following Silverson homogenisation and spray-drying were larger than those formed following any other type of manufacture procedure. Statistical analysis of particle size data indicates that spray-drying resulted in particles with significantly different dimensions relative to those manufactured with the Silverson mixer or with the Ultra Turrax ($p<0.05$).

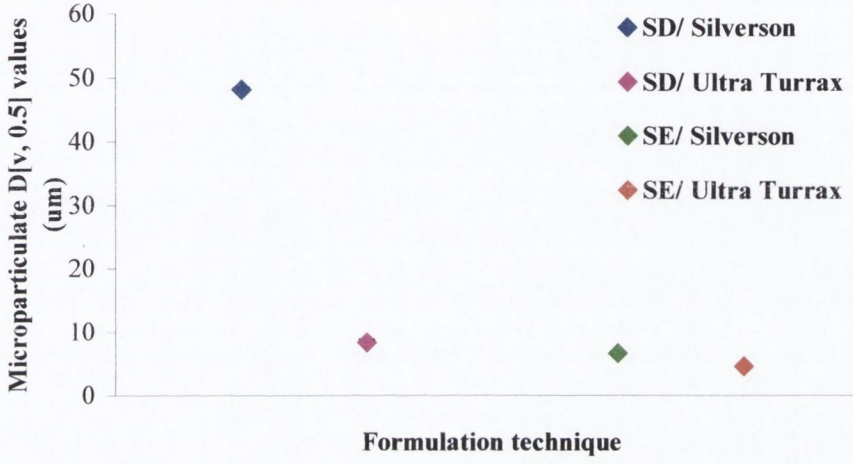


Figure 5- 28 Effect of formulation technique on particle size: Spray dried (a) Silverson (b) High-speed homogenisation: Solvent evaporation (c) Silverson (d) High-speed homogenisation

This difference in particle size between spray dried and solvent-evaporated particles could also be ascribed to the nature of solvent evaporation in which the particles were stirred constantly for a significant period of time (24 hours), allowing for the formation of smaller droplets.

Scanning electron micrographs and laser diffraction particle sizing of spray-dried microspheres indicate a difference in apparent particle size. This might be ascribed to the aggregation of particles following laser diffraction particle sizing which is not apparent following SEM analysis. Baras et al. (2000) reported finding a high occurrence of microparticulate aggregation following spray-drying when compared to solvent-evaporated microspheres, which could also explain the relatively high particle sizes found in this study. It was reported that this observation was probably due to the high atomisation air rates (between 400-500 l/h), which were used during spray-drying. These researchers overcame this problem by sonicating the resultant spray dried microspheres. In this current study the atomisation rate was set at 600 l/h. The aggregation of spray-dried particles was also reported by Jain (2000). The effect of inlet air temperature has also been cited as a

further cause of particle aggregation by several researchers (Broadhead et al., 1992; Ståhl et al., 2002).

Ståhl et al. (2002) investigated the production of spray-dried insulin particles using a factorial design. These researchers observed an increase in particle sizes with increasing inlet air temperature. Particle sizes reported in the current study were slightly higher than those reported by Ståhl et al. (2002). Broadhead et al. (1994) suggested that the increase in particle size might be the effect of increased agglomeration at the higher inlet air temperatures.

5.3.2. 3 Differential Scanning Calorimetry

The thermal characteristics of RG 752, empty microspheres, spray-dried protein loaded microspheres manufactured following homogenisation with an Ultra Turrax (T25) and a Silverson mixer (L4R) and human serum albumin were examined by DSC analysis (Figure 5-29).

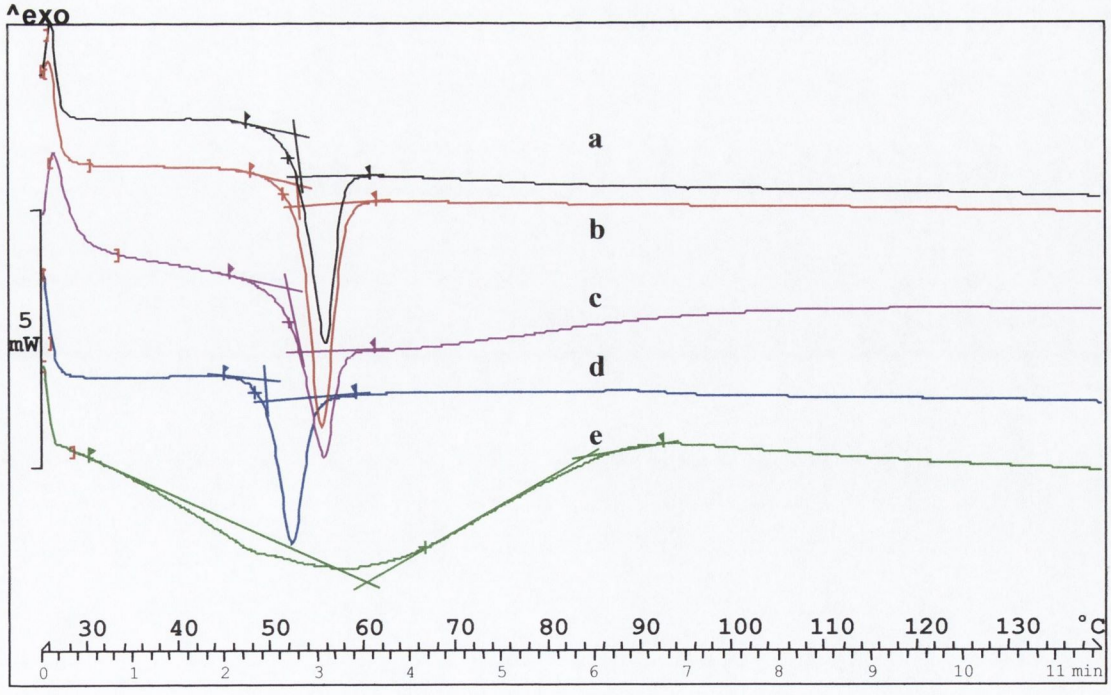


Figure 5- 29 DSC scan of (a) PLGA 7525, (b) empty microspheres (c) protein polymer blend, spray dried protein loaded microspheres manufactured with (d) Silverson mixer (L4R) and (e) Ultra Turrax (T25)

DSC analysis of PLGA 75:25, empty microspheres, and the protein polymer blend showed glass transition temperatures of 51.94°C, 52.31°C and 51.84°C respectively. Protein loaded microspheres manufactured following homogenisation with a Silverson mixer (L4R) and those manufactured following Ultra Turrax (T25) homogenisation showed endotherms of 48.25°C and 66.62°C respectively. As can be seen from the thermal traces for (a) PLGA 75:25 and (b) empty microspheres, narrow sharp peaks were observed. DSC carried out on a blend of polymer and protein showed an endotherm with a glass transition temperature of 51.84°C. The resultant endotherm was similar in shape to that observed in Section 5.2.1.3. In the case of the Silverson homogenised spray-dried protein loaded microspheres (d) there appears to be no significant differences in thermal characteristics when compared with the polymer, empty microspheres or the protein polymer blend. The glass transition temperature is slightly lower which can be attributed to the plasticising effect of the encapsulated agent. However microspheres formed following homogenisation with an Ultra Turrax (T25) (d) exhibited a broad peak with a glass transition temperature of 60.12°C and is similar in shape to that of the protein alone (Figure 5-8) with a glass transition temperature of 84.21°C. This suggested that in the case of these microspheres the protein was not completely encapsulated inside the polymer allowing the thermal characteristics of the protein to change those of the polymer microspheres.

5.3.2. 4 Protein loading

Levels of protein loading in spray-dried microspheres were determined following alkaline hydrolysis and TE buffer extraction according to Sharif et al. (1995).

Manufacturing technique		**Protein concentration μg/mg microsphere**	**SD**	**EE (%)**
Spray drying	Silverson	25.61	± 2.26	41.11
	High-speed homogenisation	3.66	± 0.37	6.13
Solvent evaporation	Silverson	11.69	± 1.58	8.79
	High-speed homogenisation	9.20	± 3.15	6.92

Table 5- 12 Effect of manufacturing process on loading
(samples represent the mean of nine determinations)

From Table 5-12 it can be seen that protein loading and encapsulation efficiency levels were highest for spray–dried microspheres manufactured using a Silverson mixer (L4R). These levels were significantly different from those observed following the other three manufacturing techniques. Protein loading in microspheres, which were subsequently spray-dried, was shown to be more dependent on emulsification speeds than microspheres formed following solvent evaporation. This was observed in the statistical differences between spray-dried microspheres and the lack of statistical difference between microspheres which had been solvent-evaporated. Pavanetto et al. (1992) investigated loadings of vitamin D_3 in polylactide (MW 109,000) microspheres produced by solvent evaporation or spray-drying and reported higher loadings in spray-dried microspheres when compared with batches formed following solvent evaporation.

In the current study the lowest loading values were found in spray dried microspheres emulsified using an Ultra Turrax (T25). Levels of encapsulation in these microspheres were significantly lower than levels attained from other batches ($p<0.05$). Loading values reflected the results observed in particle size studies. Batches with higher loadings also

exhibited higher average particle sizes relative to those batches with lower loadings ($p < 0.05$). This observation is in agreement with several other authors (Jeffery et al., 1993; Uchida et al., 1994).

5.3.2. 5 Integrity of extracted protein

The structural integrity of extracted protein was investigated via SDS-PAGE analysis (Figure 5-30).

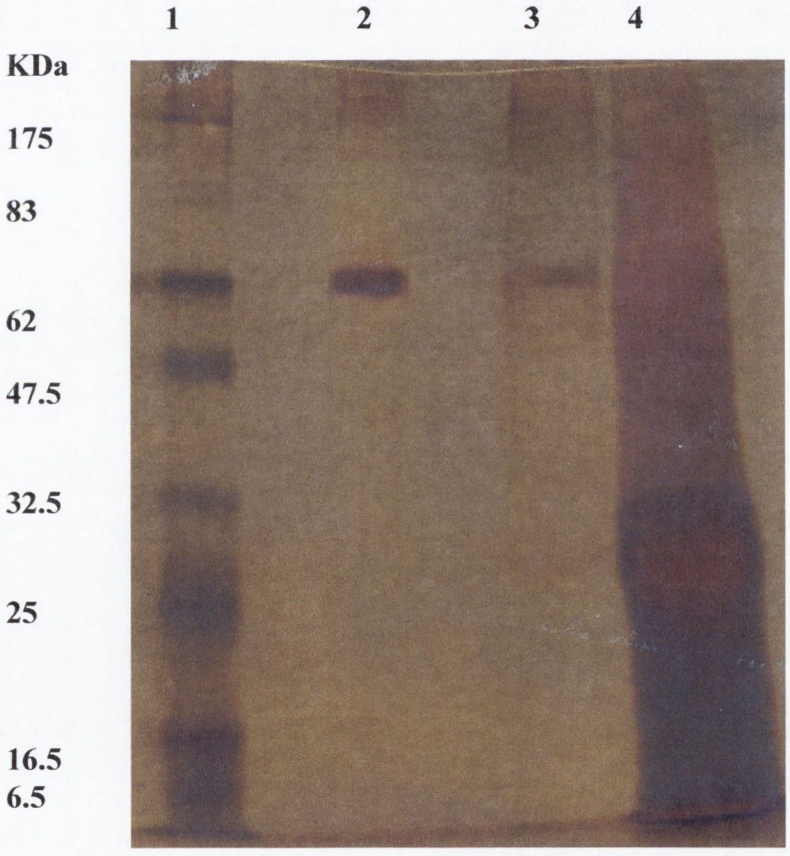


Figure 5- 30 SDS-PAGE of extracted protein

Lane 1: Molecular weight markers (M.W. range: 6.5 kDa – 175 kDa)
Lane 2: HSA protein standard (66 kDa)
Lane 3: Extracted HSA from microspheres (Silverson/ spray-drying)
Lane 4: Extracted HSA from microspheres (Ultra Turrax/ spray-drying)

Figure 5-30 illustrates a polyacrylamide gel of protein extracted from spray-dried microspheres manufactured following homogenisation with a Silverson mixer (L4R) (lane

3) and an Ultra Turrax (T25) (lane 4). When compared with the protein standard (lane 2) it can be seen that the protein sample extracted from microspheres homogenised with a Silverson mixer (L4R) (lane 3) retained its integrity and had a molecular weight of approximately 66 kDa as determined by comparison with molecular weight markers and the protein standard. This indicates that protein encapsulation via a method employing relatively high temperatures can be accomplished without compromising protein integrity unduly. However microspheres manufactured following homogenisation with an Ultra Turrax (T25) showed a large smear in lane 3. This would indicate the presence of denatured protein or protein fragments. This was attributed to the shear speed of homogenisation associated with Ultra Turrax (T25) homogenisation coupled with the high inlet temperature used during spray-drying. This is reflective of the thermal analysis results observed following DSC analysis, which indicated that there was incomplete encapsulation of the protein following manufacturing via this technique. The incomplete encapsulation would expose a higher percentage of protein to the high inlet temperature.

5.3.2. 6 Protein release

Figure 5-31 illustrates the release from spray dried microspheres formed following homogenisation with a Silverson mixer and an Ultra Turrax (T25).

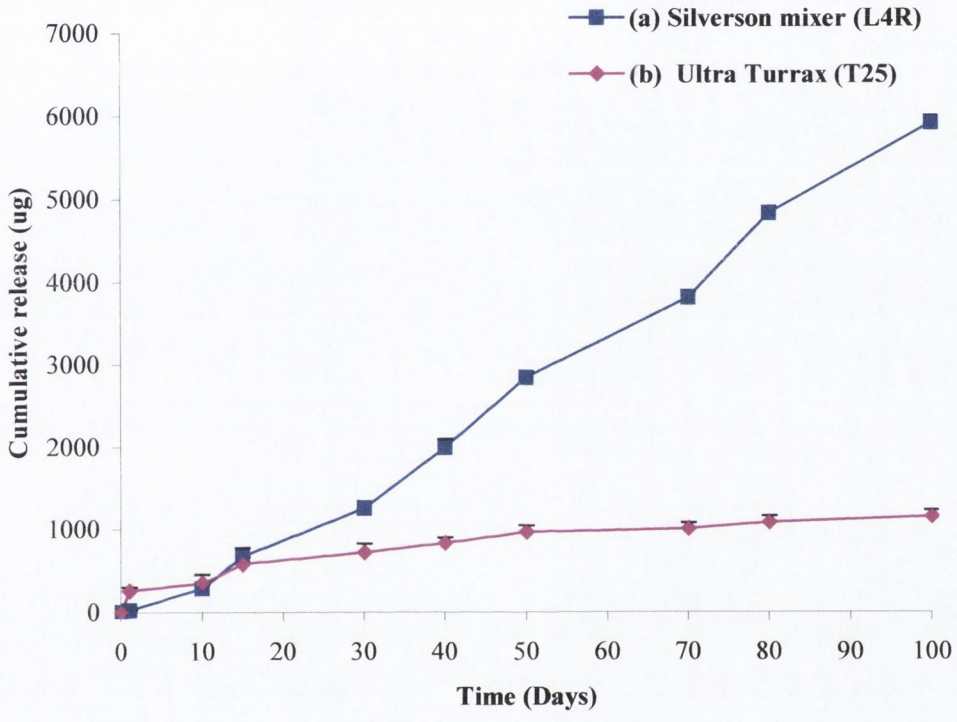


Figure 5- 31 Cumulative release from spray dried microspheres manufactured following emulsification with (a) a Silverson mixer (L4R) and (b) an Ultra Turrax (T 25).

(samples represent the mean of three determinations)

Figure 5-31 illustrates the cumulative release from spray-dried microspheres manufactured following homogenisation with a Silverson mixer (L4R) and an Ultra Turrax (T25). As can be seen there is a much greater amount of protein released over the one hundred days in the case of spray-dried microspheres manufactured with a Silverson mixer (L4R). In the case of both batches there appears to be a distinct lack of burst release when compared with microspheres manufactured following solvent evaporation (Figure 5-11 and 5-18). This result has been reported by Pavanetto et al. (1992) who observed a lack of burst release in microspheres manufactured following spray-drying. Figure 5-32 illustrates the fractional release from spray-dried microspheres fitted to Equation 1-14.

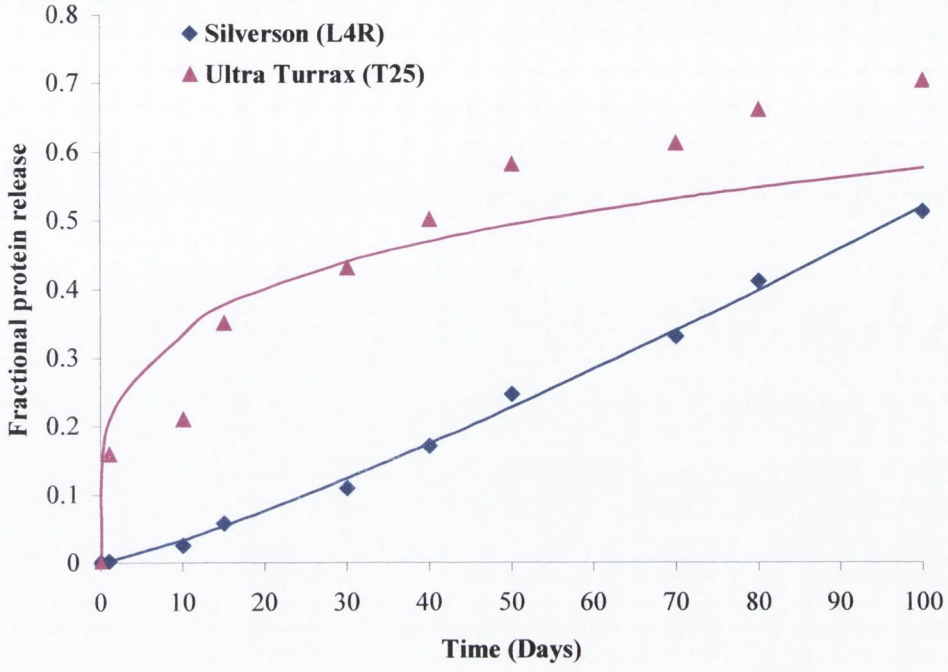


Figure 5-32 Fractional release of HSA fitted to Equation 1-14

As can be seen from Figure 5-32 release from microspheres homogenised with a Silverson mixer (L4R) fit the diffusion model better than those homogenised with an Ultra Turrax (T25). The difference in amount released over one hundred days between the two batches is reflective of their different loading values in which it was observed that there was a significantly higher loading and encapsulation efficiency in spray-dried microspheres manufactured following homogenisation with a Silverson mixer (L4R) (25.61 μg/mg and 41.11%) when compared with that in microspheres manufactured following homogenisation with an Ultra Turrax (T25) (3.66 μg/mg and 6.13%). Differences between these two systems have also been noted in the case of DSC analysis and protein integrity following SDS-PAGE analysis. Microspheres manufactured following Ultra Turrax (T25) homogenisation illustrated thermal characteristics similar to that of the native protein and showed a large amount of degradation following SDS-PAGE analysis. It is unclear whether this degradation was as a result of the high shear rate associated with homogenisation with an Ultra Turrax or with the high inlet temperatures used during spray-drying. In both cases however, other systems subjected to either technique did not display similar characteristics. It appears that the combination of both techniques (high

speed homogenisation and spray-drying) in this system was responsible for the characteristics of these microspheres.

Parameters generated from the model are listed in Table 5-13.

Parameter	Silverson (Spray dried)	Ultra Turrax (Spray Dried)
K	0.002	0.275
n	1.189	0.221
CD	0.997	0.639
MSC	5.319	0.621

Table 5-13 Parameters generated from fitting release from spray dried microspheres to Equation 1-14.

As can be seen from Table 5-13 release data from microspheres homogenised with a Silverson mixer (L4R) was described better by Equation 1-14 than that from microspheres homogenised with an Ultra Turrax (T25) with MSC and CD values of 5.319 and 0.997 compare with 0.621 and 0.639.

5. 4 Discussion

Release systems employing PLGA 50:50 and 75:25 in the encapsulation of proteins and nucleic acids have been well documented (Park et al., 1995; Mehta et al., 1996; Walter et al., 2001). However, the efficiency of these systems for optimal delivery of biologicals such as proteins and nucleotides has not yet been fully developed. In the current study factors such as polymer weight and composition, emulsification speed and microencapsulation technique were examined and their effect on characteristics such as protein release from PLGA microspheres, microparticulate size and protein integrity were studied.

Polymer characteristics such as composition and molecular weight were found to have an effect on several microparticulate characteristics. Polymer composition was found to influence protein loading. In this study it was observed that the lower molecular weight polymer resulted in increased protein loading efficiency. This finding might be ascribed to the hydrophilic nature of RG 502, which promotes faster solidification of microsphere droplets when compared with more hydrophobic polymers with higher glycolic acid ratios (Mehta et al., 1996; Walter et al., 2001). This more rapid solidification would be expected to reduce the diffusion of protein into the continuous or outer aqueous phase of the double emulsion, resulting in increased entrapment and protein loading.

Due to the insolubility of proteins in polymers and their relatively large molecular weight the rate of protein release from PLGA microspheres is believed to be largely dependent on the rate of polymer degradation (Hutchinson et al., 1985; O'Hagan et al., 1994). Protein release from biodegradable polymer matrices can occur by two main routes: by diffusion of the protein from the matrix surface or through pores and by polymer erosion or degradation (Baras et al., 2000). In this study an initial burst release was observed from both systems followed by a triphasic release profile in the case of RG 502 microspheres and a biphasic in the case of the RG 752 microspheres. The differences in the release patterns could be attributed to the differences in degradation profiles and to the fact that RG 752 has a longer half-life which would presumably have resulted in the continued release of protein until the microspheres had been depleted had the study not been terminated. The initial burst release, which in the case of RG 502 accounted for

approximately 30% of the total release and in the case of RG 752 was approximately 40%, has been reported in other studies and has been attributed to incomplete incorporation at the polymer surface (Shah et al., 1992; O'Hagan et al., 1994; Mehta et al., 1996). O'Hagan et al. (1994) reported that as the molecular weight of the polymer increased (from 33 kDa to 84 kDa) the burst effect decreased. This was not observed here perhaps due to the similarity in molecular weight of the two polymers (RG 502 15 kDa and RG 752 18 kDa) when compared with those employed by O'Hagan et al.

Polymer molecular weight was another important factor in the median particle size of microspheres. This was attributed to the slower solidification of the RG 752 polymer with a higher molecular weight, which resulted in a more rapid shrinkage of RG 752 microspheres when compared with RG 502 microspheres and has been observed in previous studies (Mehta et al., 1996). This slower solidification also allows for the diffusion of protein from the inner aqueous phase and resulted in decreased loading when compared with microspheres formed with RG 502.

Particles manufactured using different emulsification speeds followed by solvent evaporation were examined. It was observed that emulsification speed influenced particle size which has shown good agreement with other studies (Uchida et al., 1994; Jain, 2000). In this current work it was found that increasing homogenisation speed from 2,000 rpm to 8,000 rpm resulted in a decrease in median particle size from 6.65 μm to 4.45 μm. Protein loading was also influenced by homogenisation speed. As the homogenisation rate increased the levels of encapsulated protein decreased. This observation has also been reported by several other researchers (Uchida et al., 1994; McGee et al., 1997).

The glass transition or T_g temperature of a polymer has important implications for pharmaceutical devices based on PLGA. The release of the encapsulated agent is influenced by the T_g temperature. For example if the T_g of the polymer is above body temperature the polymer is in a glassy state. A rubbery polymer matrix has a higher permeability to water and loaded drugs than a glassy polymer, which results in faster polymer hydration, degradation and drug release. A glassy polymer will eventually become more rubbery due to the T_g decrease cause by hydration. The hydration for a glassy polymer results in a longer time lag for drug release and polymer degradation (Wang et al., 2000). Apart from determining the transition temperature, DSC is an important tool in the

evaluation of the form of the drug in the microsphere. The absence of an endotherm corresponding to drug fusion is indicative of the drug being dispersed in the polymer (Ford and Timmins, 1995). In the current study DSC analysis was used to evaluate the encapsulation of the protein in the polymer. In the majority of cases it was found that there were no endotherms corresponding to the protein, which indicated the complete encapsulation of the protein within the polymeric matrix. However one batch of microspheres, those which had been manufactured with an Ultra Turrax (T25) homogeniser and spray-dried showed some thermal characteristics of the unencapsulated protein indicating incomplete encapsulation using this technique. These microspheres also showed a large amount of protein denaturation when samples were analysed using SDS-PAGE.

Of major concern in microencapsulation is the effect of the actual encapsulation procedure on the integrity of the encapsulated agent. The integrity of HSA following encapsulation was examined following SDS-PAGE and silver staining of the gel. It was observed that following emulsification at 8,000 rpm and at 2,000 rpm there was no denaturation of the protein when compared with control protein. However following homogenisation with an Ultra Turrax (T25) and spray dried at an inlet temperature of 80°C extracted protein showed some evidence of denaturation with the presence of extra bands in the sample of protein extracted from spray-dried microspheres. This was not observed in microspheres which were homogenised with a Silverson mixer (L4R) and spray-dried at the same temperature. These results suggest that even at high inlet temperatures there are manufacturing techniques which allow for the spray-drying of heat sensitive agents.

High inlet temperatures during spray-drying have also been shown to promote particle aggregation (Ståhl et al., 2002). This led to higher mean D[v, 0.5] particle sizes in this study when compared with microspheres manufactured following solvent evaporation. Particles sizes following Silverson homogenisation (2,000 rpm) and spray-drying were also significantly higher than those formed following high-speed homogenisation (8,000 rpm) and spray-drying. This mirrored results for solvent-evaporated particles and is in good agreement with other researchers (Uchida et al., 1994; Jain, 2000).

Chapter 6

Microencapsulation of plasmid DNA

6. Introduction

It has been over a decade since naked DNA was first injected intramuscularly and was shown to lead to the in situ expression of proteins (Wolff et al., 1990). Since then other methods and routes of DNA vaccination have been developed including viral and non-viral mediated delivery via intramuscular, intradermal and subcutaneous routes. In many cases however, mucosal immunisation and in particular, oral delivery continues to be the most attractive route for the delivery of all medicinal agents (Singh et al., 1998). This is due to the fact that most invading pathogens enter the body through these routes and the induction of mucosal immunity offers the most effective line of defence against such pathogens. Particulate DNA delivery systems such as microspheres and liposomes offer protection against degradation and the promise of a more effective mucosal DNA delivery system (Wang et al., 1999).

The first papers in the field do not comment on the manufacturing process and the factors critical for successful DNA encapsulation and vaccine formulation. The previous chapter described the microencapsulation of human serum albumin (HSA). This protein was employed as a model compound which would assist in the selection of appropriate processing conditions for encapsulation of a bioactive material such as DNA. From the protein study it was observed that microspheres homogenised for 240 seconds with the Silverson mixer (L4R) displayed favourable surface morphologies, particle sizes and release characteristics. These microspheres did not appear to flocculate and were less than 10 μm in size. Importantly this manufacturing technique did not appear to denature the protein as seen from SDS-PAGE of protein extracted from microspheres manufactured with the Silverson mixer (L4R). All DNA batches were manufactured following homogenisation with a Silverson mixer (L4R) for 240 seconds followed by solvent evaporation overnight unless otherwise stated. This study investigates the effect of several processing parameters and their effect on the physicochemical properties of encapsulated DNA

The physicochemical properties of plasmid DNA present a number of supplementary formulation challenges relative to the encapsulation of peptides and proteins (Huang et al., 1999). Plasmid DNA molecules are extremely large, negatively charged macromolecules

with typical molecular weights in the millions of daltons; at least a magnitude higher than proteins. Plasmid DNA molecules can exist in three different conformational forms; supercoiled, open circular and linear, each with different hydrodynamic characteristics. Gene transfer is most effective with the supercoiled form and thus delivery systems must optimise the encapsulation and release of this particular fraction. Plasmids are extremely sensitive to physical manipulation such as sonication and high-speed homogenisation both of which are common techniques in the encapsulation process. In the present work preliminary studies with plasmid DNA focused on the pGL3 luciferase vector.

6. 1 Influence of manufacturing temperature

Studies have been carried out examining the effect of processing temperature on the physicochemical properties of protein-loaded microspheres (Yang et al., 2000 a). Further studies by Yang et al. (2000 b) suggested that preparation temperature significantly affected protein-loaded microsphere formation leading to differences in their structural and protein release profiles. It was reported that preparation temperature affects the mass transfer and solubility of the active agent the higher the temperature the more soluble the agent and the higher the mass transfer. Hence preparation temperature should have a significant effect on particle characteristics. To our knowledge no studies have to date, reported on the effects of temperature on DNA-loaded microspheres. A pGL3 plasmid encoding for firefly luciferase was used as a model plasmid and was encapsulated at three different temperatures (4°C, 20°C and 37°C) to examine the effect of processing temperature on the physicochemical properties of DNA-loaded microspheres.

6.1. 1 Microsphere morphology

Figures 6-1 to 6-3 are scanning electron micrographs of microspheres formulated at 4°C, 20°C and 37°C respectively. Pores were observed on the surface of microspheres manufactured at all three temperatures. All three systems displayed microspheres with irregular surfaces and a variety of sizes. The surfaces of the microspheres were wrinkled

irrespective of the temperature and this was observed to increase with an increase in temperature.

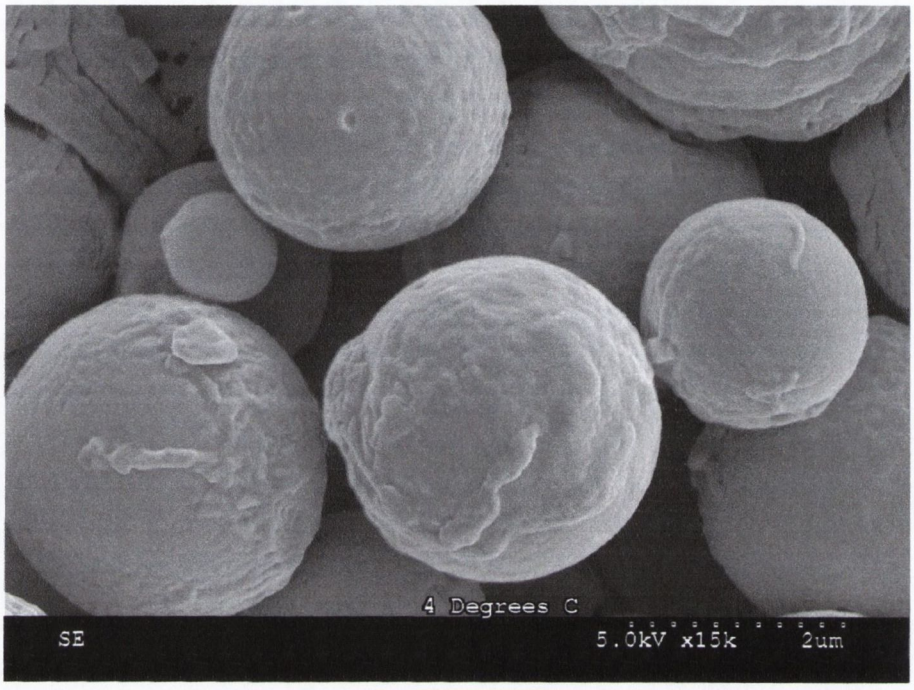


Figure 6- 1 DNA-loaded microspheres manufactured at 4°C

Figure 6-2 DNA-loaded microspheres manufactured at 20°C

In the current study there was an increase in frequency of surface pores as the manufacturing temperature was increased. It has been reported by Jeyanthi et al. (1996)

that the rate and method of solvent removal influences the matrix porosity and the surface area of the microspheres. The solvent used in this study was dichloromethane which is highly soluble in aqueous media at low temperatures such as 4°C but whose solubility decreases with increasing temperatures (Yang et al., 2000 a).

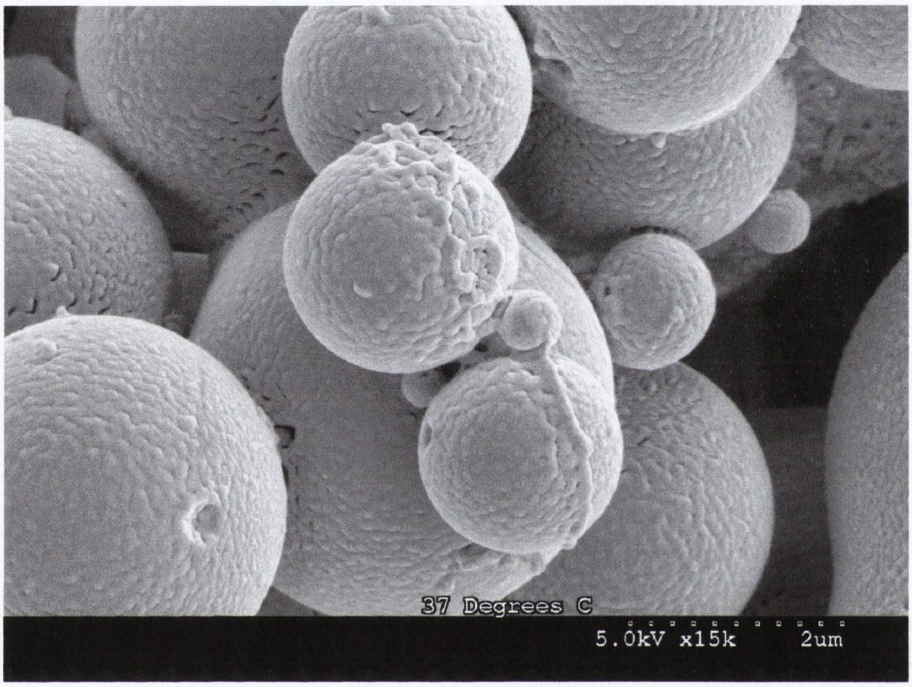


Figure 6-3 DNA-loaded microspheres manufactured at 37°C

Figure 6-3 displays scanning electron micrographs of DNA-loaded microspheres manufactured at 37°C. As can be seen from the micrograph at this high temperature surface wrinkling is more pronounced than at the previous two temperatures.

According to Li et al. (1995) the removal of dichloromethane is initially faster at lower temperatures due to its high solubility but then slows down when compared with higher temperatures due to the low evaporation capabilities. This affects the solidification rate of the polymer and the formation of microparticles. At higher temperatures polymer solidification occurs at a faster rate when compared with rates at lower temperatures due to this rapid evaporation of the solvent resulting in microspheres with thin and wrinkled walls (Jeyanthi et al., 1996). In the current study further examination would be required to determine the thickness of the microsphere wall however the increase in wrinkling at the outer surface as the temperature was increased was observed. At lower processing temperatures, the solvent is removed slowly thus increasing the time of microsphere

formation resulting in the formation of thicker outer walls. Jeyanthi et al. (1996) carried out work on the effect of solvent removal technique on the matrix characteristics of protein-loaded microspheres. This was carried out either by a temperature gradient technique or a dilution technique. Solvent removal was achieved in the first technique following solvent evaporation at high temperatures (40°C) and in the second, solvent removal was due to dilutions of the continuous phase or solvent extraction at 25°C. It was reported that, at these high temperatures pores could be seen on the microsphere surface irrespective of the solvent removal technique involved.

6.1. 2 Particle size analysis

Table 6-1 illustrates the size of microspheres manufactured at different temperatures.

Processing temperature (°C)	**D[ν,0.5] μm**	**SD**	**D[ν,0.9] μm**	**SD**	**Span**
4	6.92	± 0.63	7.83	± 0.63	2.59
20	4.48	± 0.51	5.77	± 0.65	2.52
37	3.32	± 0.14	4.29	± 0.68	2.35

Table 6- 1 Particle size data for different temperatures

(samples represent the mean of three determinations)

From Table 6-1 it can be seen that the size of the microspheres decreases with increasing temperature. Statistical analysis showed that the particle sizes, at 95% confidence level, for microspheres formulated at different temperatures were statistically different ($p < 0.05$).

Yang et al. (2000 a) suggested that increasing temperature affected particle size, through the removal rates of the organic solvent employed during the microencapsulation procedure. At higher temperatures the solvent, in this case dichloromethane undergoes rapid evaporation. This gives rise to smaller particles with thinner walls when compared with those formulated at lower temperatures where solvent removal is slower. It was postulated that increasing temperatures leads W/O/W double emulsions to become more viscous thus allowing the emulsion to be broken up into smaller droplets. In the present study increasing the temperature over the range of 4°C to 37°C resulted in increasingly smaller particles.

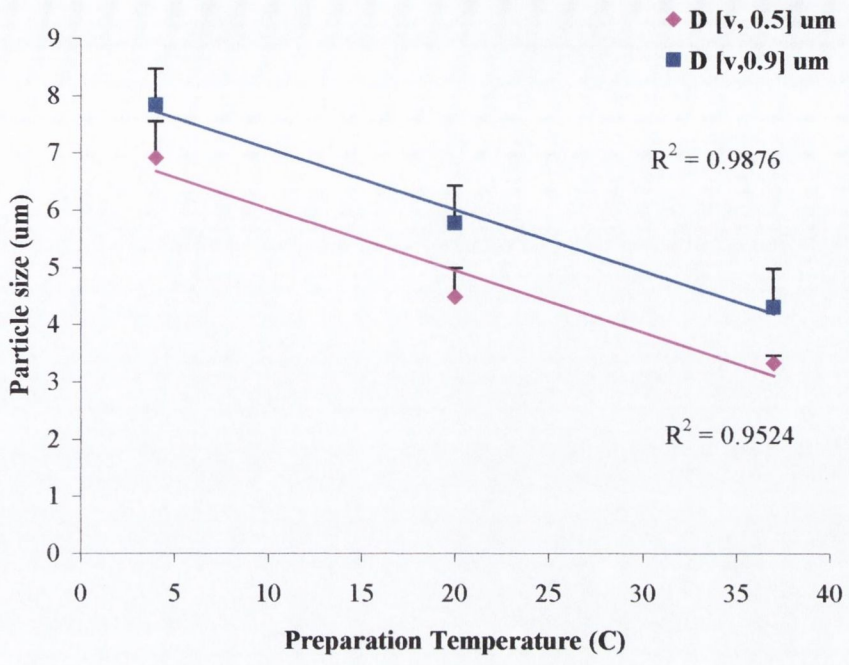


Figure 6- 4 Relationship of preparation temperature to particle size

(samples represent the mean of three determinations)

From Figure 6-4 there appears to be an inverse relationship between the values of the D [v, 0.5] and D [v, 0.9] particle sizes and manufacturing temperature. Linear regression analysis gave a relationship of the D [v, 0.5] particle sizes with an equation of y = -0.1087x + 7.1162 with a coefficient of determination of 0.9524 and the D [v, 0.9] particle sizes with an equation of y = -0.1071x + 8.1403 with a coefficient of determination of 0.9876.

6.1. 3 DNA loading

Table 6-2 outlines DNA loading in microspheres manufactured at different temperatures.

Microsphere	Theoretical DNA load μg DNA/mg microsphere	Actual DNA load μg DNA/mg microsphere	Encapsulation efficiency
4°C	3.09	1.34 ± 0.02	43.513%
20°C	2.86	0.82 ± 0.05	28.513 %
37°C	2.78	0.82 ± 0.032	29.344 %

Table 6- 2 Loading data

(samples represent the mean of three determinations ± standard deviation)

As can be seen from Table 6-2 there does not appear to be a direct relationship between encapsulation efficiency and manufacture temperature. Microspheres manufactured at different temperatures displayed different theoretical loading. Loading values were 3.088, 2.86 and 2.78 μg DNA/mg microsphere for microspheres manufactured at 4°C, 20°C and 37°C respectively. Encapsulation efficiency indicated that DNA loading was greatest for microspheres manufactured at 4°C while those manufactured at 20°C exhibited the lowest loading values. Wang et al. reported similar encapsulation efficiencies following encapsulation of plasmid DNA in PLGA 50:50 (1999). They reported values of between 22.5% and 53.3% encapsulation efficiencies depending on the molecular weight of the PLGA used.

With regard to the effect on encapsulation efficiency of manufacturing temperatures Yang et al. (2000 a) found that microspheres formulated at high and low temperatures had similar protein encapsulation efficiencies (59.6% at 5°C, 52.7% at 20°C and 54.1% at 38°C). They hypothesised that at high temperatures the solubility of the encapsulated agent and the mass transfer rate might cause the loss of protein from the dispersed phase. Encapsulation efficiencies reported here are in line with other researchers (Liu et al. 2002; Wang et al. 1999).

6.1. 4 Differential Scanning Calorimetry

Figure 6-5 illustrates the thermal characteristics of the polymer powder, empty microspheres and DNA-loaded microspheres manufactured at different temperatures.

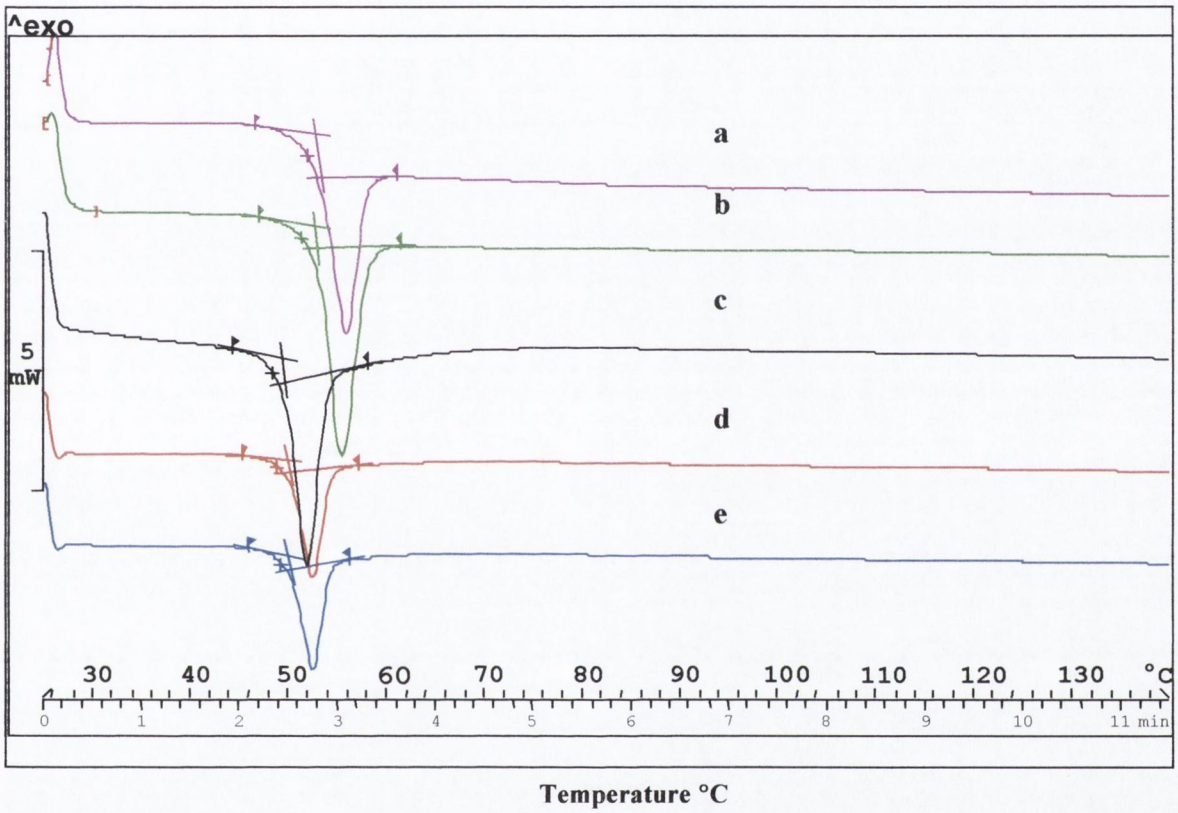


Figure 6- 5 DSC scans of (a) RG 752 polymer powder, (b) empty microspheres, and DNA–loaded microspheres manufactured at (c) 4°C, (d) 20°C and (e) 37°C.

The thermal characteristics of DNA-loaded microspheres manufactured at 4°C, 20°C and 37°C were analysed using differential scanning calorimerty. DSC scans showed glass transition temperatures of 48.30°C, 49.22°C and 48.84°C for microspheres manufactured at 4°C, 20°C and 37°C respectively. RG 752 polymer and empty microspheres were also analysed and were shown to have transition temperatures of 51.82°C and 51.15°C respectively. Analysis indicated that the thermal characteristics of the polymer alone were not significantly different from that of the empty microspheres indicating that the polymer does not undergo any significant changes, which would affect its glass transition temperature when formed into microspheres. The glass transition temperatures of the

DNA-loaded microspheres were also not significantly different from that of the polymer powder or the empty microspheres.

Interestingly the glass transition temperatures mirrored the loading values of the microspheres with microspheres encapsulating the highest concentration of DNA (4°C) showing the lowest glass transition value. The slight decrease in the glass transition temperatures observed for DNA–loaded microspheres compared with the polymer powder and the empty microspheres could be attributed to the plasticising effect of an encapsulated agent which would be more pronounced as microsphere loading increased (Ford and Timmins, 1989).

6.1. 5 DNA release

Figure 6-6 illustrates the effect of temperature on the release characteristics of DNA from PLGA microspheres.

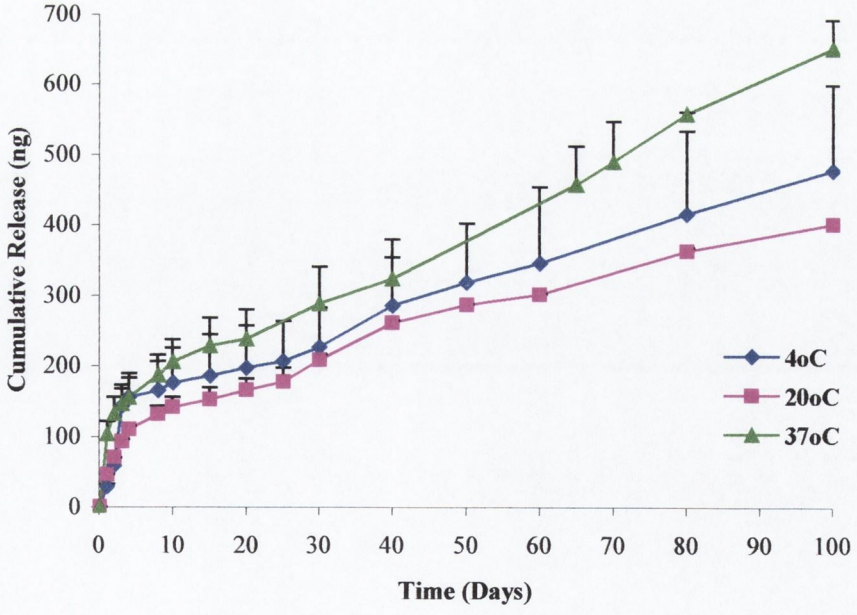


Figure 6- 6 Effect of processing temperature on release
(samples represent the mean of three determinations)

From Figure 6-6 it can be seen that microspheres formed at 37°C had the fastest rate of release followed by those formed at 4°C. It can be seen that there is an initial burst release of DNA from the microspheres irrespective of manufacturing temperature followed by a

sustained release. Fractional release from microspheres manufactured at different temperatures was fitted to Equation 1-14 which describes diffusion of an active agent from a polymer matrix (Figure 6-7). This model was described in Chapter 1 Section 1-6.

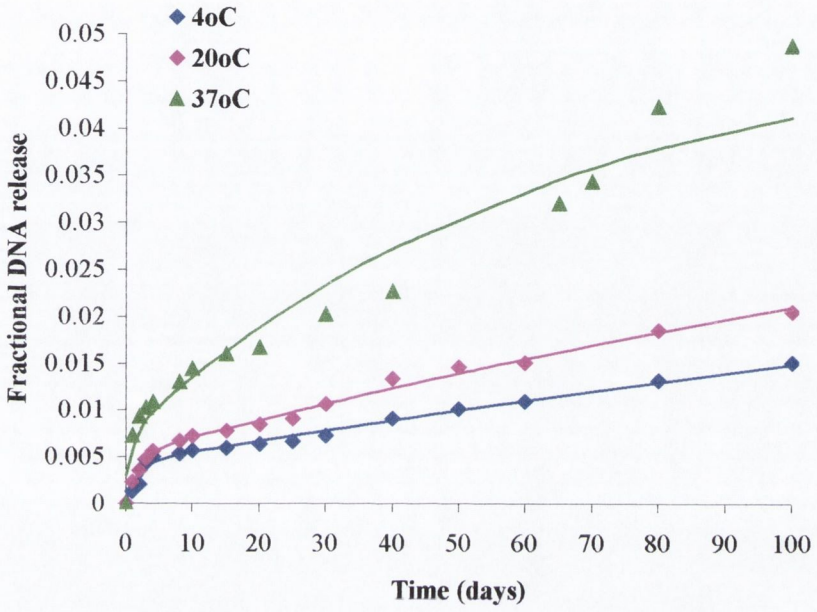


Figure 6- 7 Fractional release of DNA fitted to Equation 1-14

As can be seen from Figure 6-7 all systems exhibited a typical diffusion based release pattern. As was the case in previous protein studies RG 752 has a relatively long half-life which would presumably have resulted in the continued release of plasmid DNA until the microspheres had been depleted had the study not been terminated. Fractional release appears to be dependent on manufacture temperature with the fastest rate of release observed from microspheres manufactured at the highest temperature followed by that released from microspheres manufactured at 20°C. Levels of cumulative percentage release, similar to that observed in the current work has previously been reported by Wang et al. (1999) and Walter et al. (2001) Both researchers used a PLGA 50:50 polymer which has been shown to degrade faster than PLGA 75:25. In Chapter 1 it was reported that the half-life of PLGA 75:25 is 110 weeks. This trial was carried out for 12 weeks due to the possible denaturation of DNA after this point. However this led to a large proportion of the DNA remaining in the microspheres, which would presumably have been released, had the

study not been terminated. Table 6-3 lists the parameters obtained following fitting of fractional release to Equation 1-14.

Parameter	4°C	20°C	37°C
K	0.0023	0.0028	0.0036
n	0.434	0.467	0.515
CD	0.958	0.980	0.980
MSC	2.931	3.990	2.765

Table 6- 3 Parameters obtained from fitting fractional DNA release to Equation 1-14

The coefficient of determination (CD) and model selection criertion (MSC) were used as measures of goodness of fit. Table 6-3 summarises the results obtained when actual fractional release was fitted to the data generated by the model. The model was developed to model diffusion of a drug from a range of devices including microspheres.

As can be seen from Table 6-3 release from microspheres manufactured at 20°C exhibited the highest MSC value of 3.99, which indicated a good fit between points generated by the model and actual release data results. All batches showed good coefficient of determinations with values close to 1. The variable n is the diffusional constant and can be set to 0.432 when the drug is released from a sphere. It has been reported that polydisperse systems will yield different n values for Fickian diffusion depending on the range of particle size used. However for such diffusion to occur n should fall in the range of 0.4 and 0.42. For monodisperse systems the value of n should equal 0.42 for Fickian diffusion. Values of n higher than this indicate anomalous diffusion with values approaching 1 indicating zero order release. As can be seen from the parameters in Table 6-3 the n values obtained from these microspheres fall between 0.434 and 0.515. This would suggest that release from microspheres manufactured at lower temperatures might be based on Fickian diffusion. However as the temperature of manufacture increased the n value also increased indicating that anomalous diffusion might be the release mechanism.

The rate of formation of protein-loaded microspheres has been reported to be dependent on the manufacturing temperature (Yang et al., 2000 a). This in turn has been shown to influence the initial burst and release behaviour of an encapsulated drug. In relation to microsphere structure lower temperatures were reported to produce microspheres with a honeycomb internal structure when compared with the smaller core of microspheres produced at higher temperatures (Jeyanthi et al., 1996). Li et al. (1995) reported that microspheres formed at higher temperatures had thin walls and a network of interconnection between internal pores, which facilitates a more rapid release of the encapsulated agent.

6.1. 6 Degradation

Typical microspheres were examined for degradation by scanning electron microscopy and gel permeation chromatography. Figures 6-8 (a) to 6-10 (d) illustrate polymer degradation in microspheres formulated at 4°C, 20°C and 37°C respectively over 5, 55, 75 and 100 days.

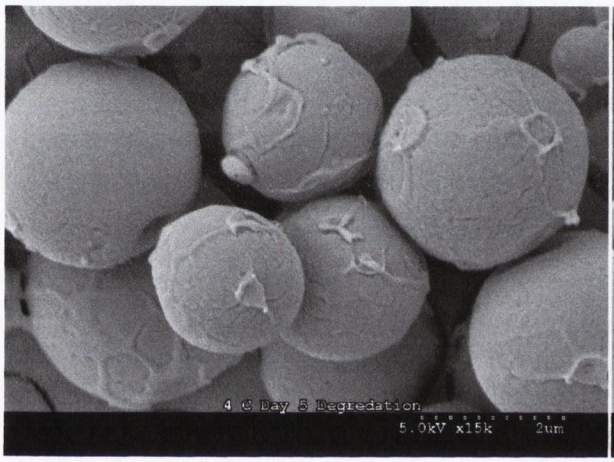


Figure 6-8 a Day 5 degradation

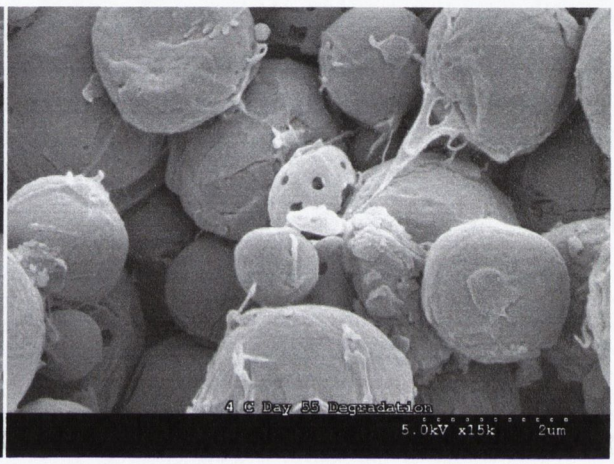


Figure 6-8 b Day 55

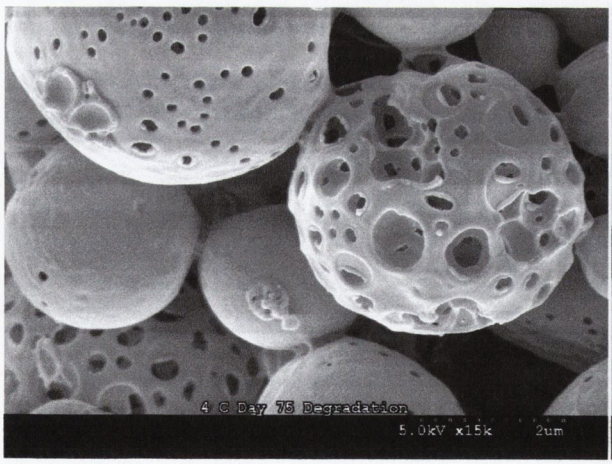


Figure 6-8 c Day 75

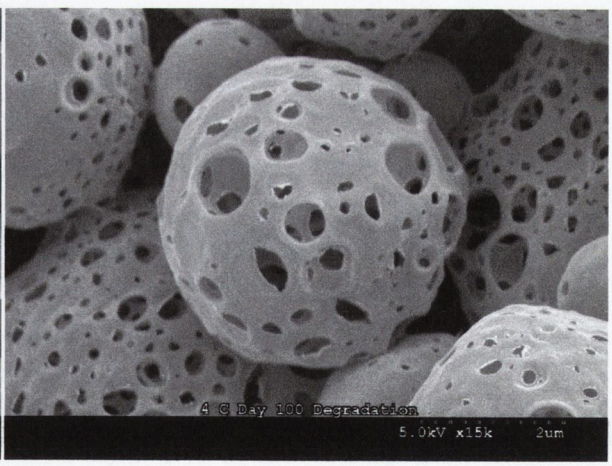


Figure 6-8 d Day 100

Figures 6-8 a to d illustrate the degradation of microspheres manufactured at 4°C. It can be seen that at days 5 and 55 a pronounced degradation of the polymer is not yet apparent. However at day 75 small regularly shaped pores can be seen on the outer surface. At 100 days degradation, much larger pores are seen in a proportion of the microspheres with hollow or extensively branched matrix filled interiors.

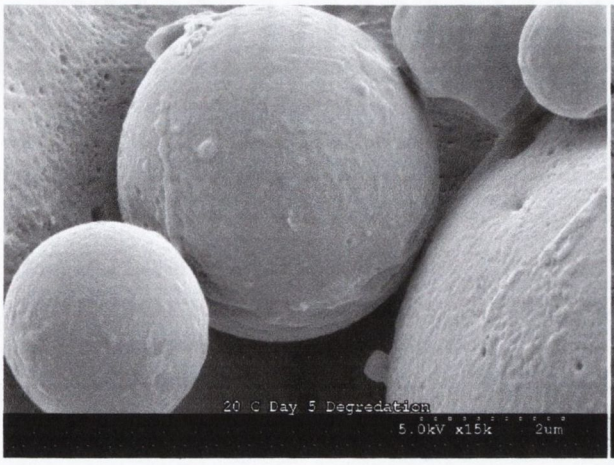


Figure 6-9 a Day 5 degradation

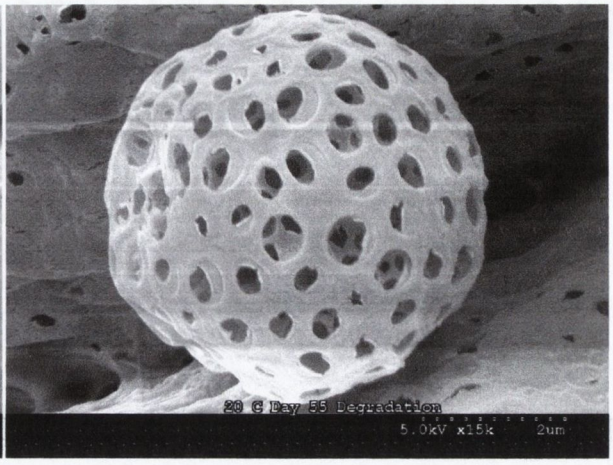


Figure 6-9 b Day 55

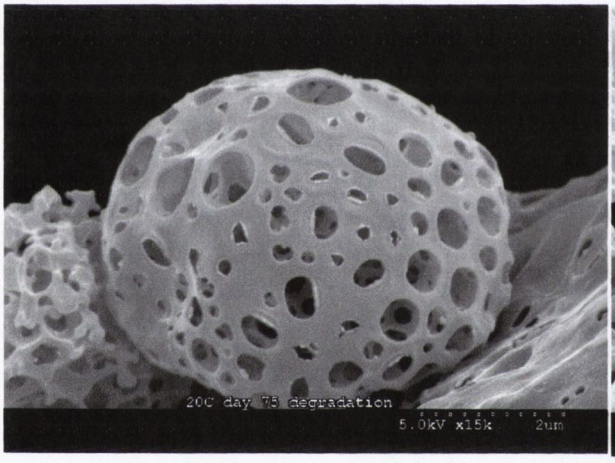


Figure 6-9 c Day 75

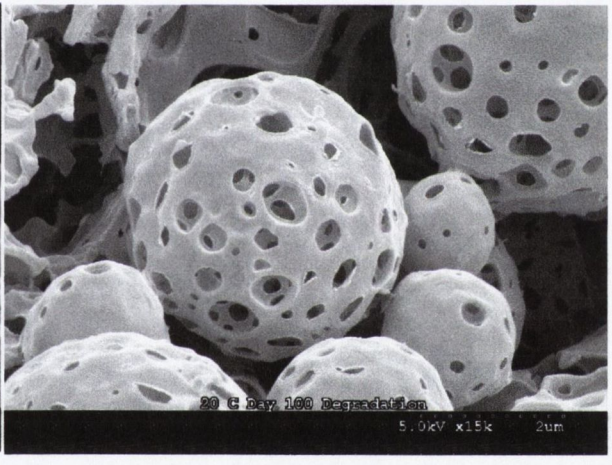


Figure 6-9 d Day 100

Figures 6-9 a to d illustrate the degradation of microspheres manufactured at 20°C. After 5 days of incubation small pores are formed on the outer layer of the microspheres followed by a rapid degradation up to day 55. By 75 days pores were visible in the outer surface layer some of the microspheres and by 100 days a proportion of microspheres appeared to have extensive pores on their surface.

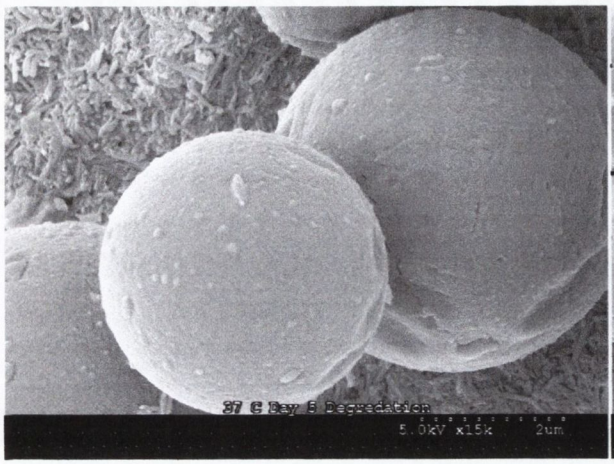


Figure 6-10 a Day 5 Degradation

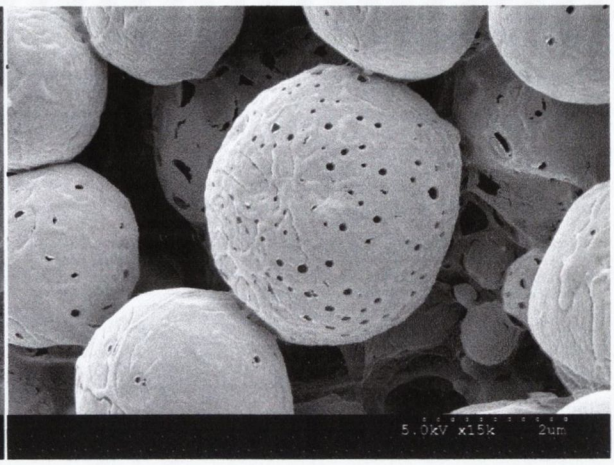


Figure 6-10 b Day 55

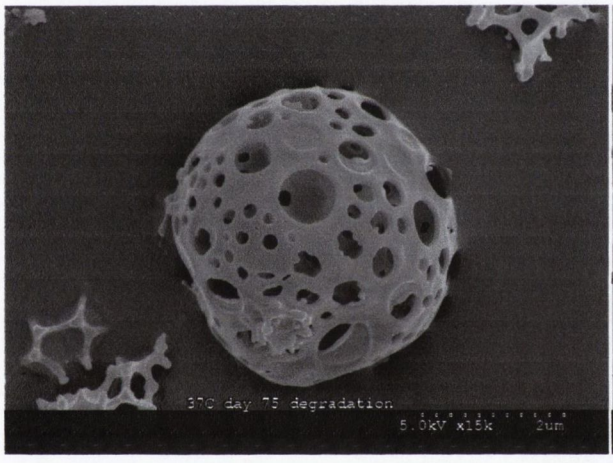


Figure 6-10 c Day 75

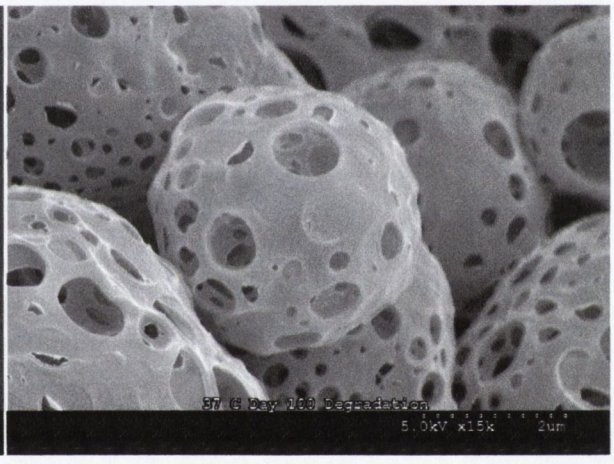


Figure 6-10 d Day 100

Figures 6-10 a to d illustrate the degradation of microspheres formulated at 37°C. Day 5 of the incubation time shows microspheres with wrinkled pock-marked surfaces. Fifty-five days into the incubation trial the microspheres appear to have small pores on their outer surface which then develop into larger more frequent pores. Some of the microspheres examined, which were incubated for 100 days, show extensive pores on their outer surface and a matrix of what appear to be interconnecting channels. When the structures of the microspheres are compared at 55 days degradation it can be seen that microspheres manufactured at 4°C remained generally intact until this time point.

Gel permeation chromatography was used to examine the fractional degradation of the RG 752 polymer and empty microspheres (Figure 6-11) and the effect of manufacturing temperature on the degradation of DNA-loaded microspheres (Figure 6-12). Linear regression analysis of the points was calculated as an estimation of the rate of degradation.

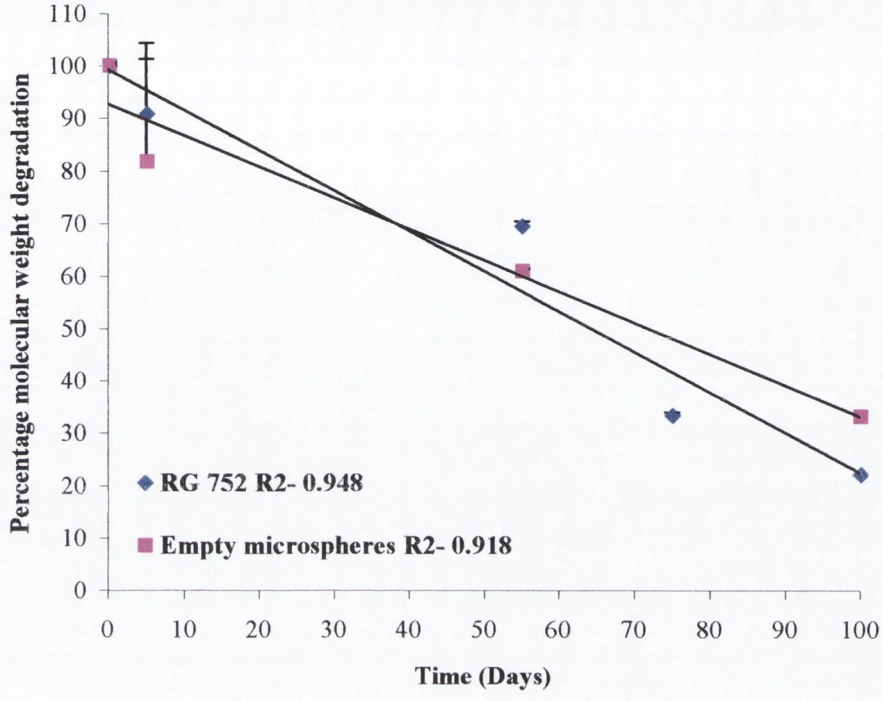


Figure 6- 11 Percentage degradation of RG 752 and empty microspheres

(samples represent the mean of three determinations)

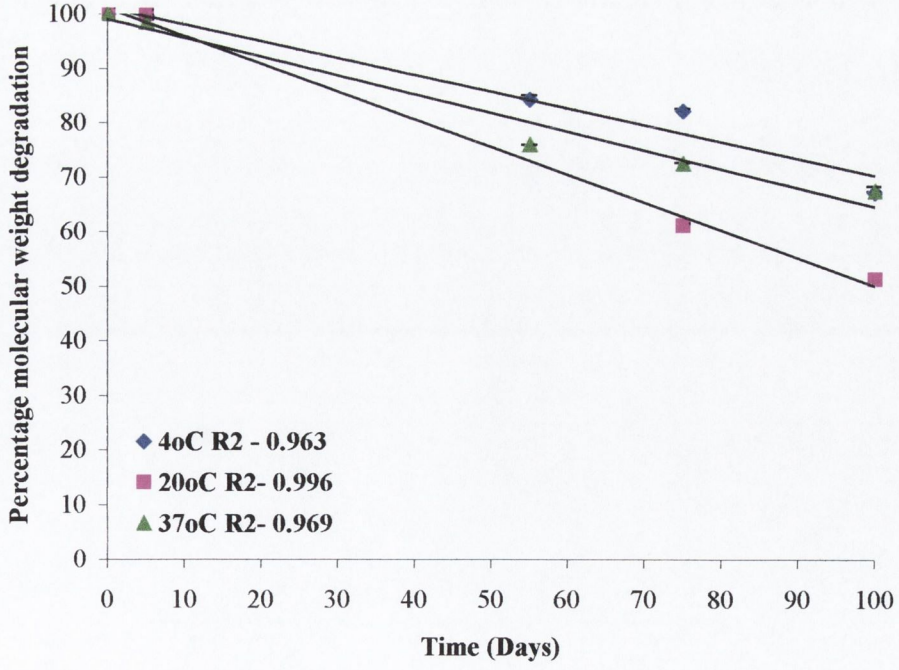


Figure 6- 12 Percentage molecular weight degradation of DNA-loaded microspheres manufactured at different temperatures following incubation over time

(samples represent the mean of three determinations)

From the calculated data it appears that the rate of degradation was constant and similar for the three DNA- loaded batches and for the polymer powder and the empty microspheres. However, as the manufacturing temperature increases so too does the rate of degradation with the increasing slope of the line from –51.423 in the case of 4°C to –107.510 in the case of those manufactured at 37°C. The relative rate of degradation of the empty microspheres and the polymer powder increases from – 0.595 for empty microspheres to – 0.767 in the case of RG 752. In the case of microspheres manufactured at 4°C the slope of the line generated from the degradation of the microspheres, indicates that degradation in these microspheres appears to be slightly slower than for those produced at higher temperatures. This phenomenon might suggest that microspheres formulated at lower temperatures have a thicker more hydrophobic outer layer. This could be due to the slower polymeric solidification and the slower rate of solvent evaporation, which occurs at low temperatures. Microspheres formulated at higher temperatures have been reported to form thin dense outer layers (Yang et al., 2000 b). In the present study a thin outer layer would allow the formation of pores more rapidly and hence would increase the rate of degradation.

6.1. 7 pH

Fractional changes in the pH of the phosphate buffer used in the release trials were analysed over a period of one hundred days. At specific time points, buffer was removed for analysis of released DNA and was replaced with fresh buffer. This extracted buffer was also analysed for changes in pH. As can be seen from Figure 6-13 the pH of the medium decreases with increasing time for all batches.

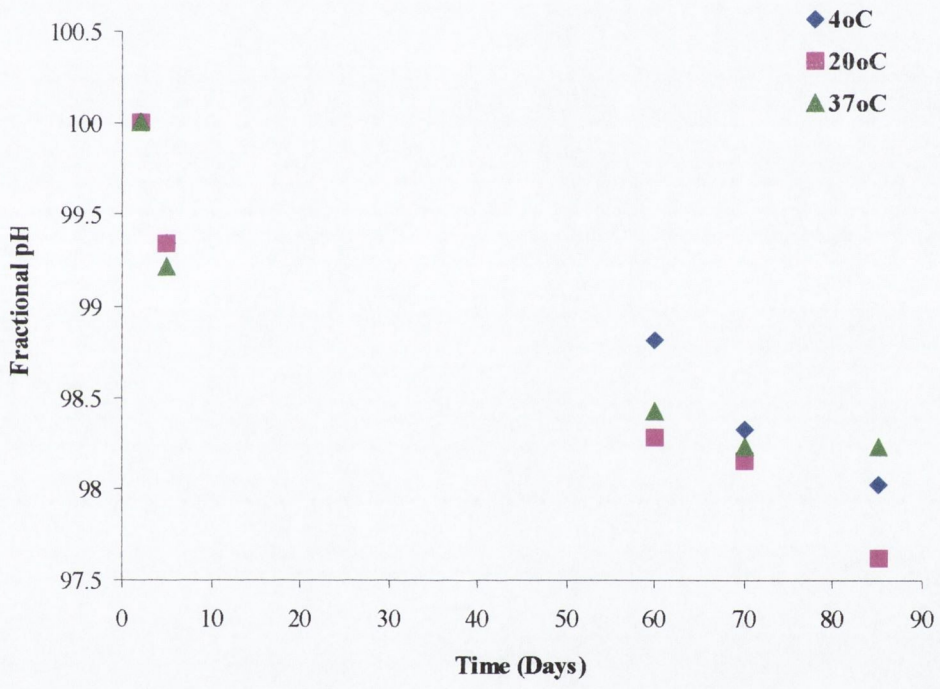


Figure 6- 13 pH profiles over eighty-five days
(samples represent the mean of three determinations)

The sampling technique used was chosen in an attempt to minimise pH changes during the trial. It was hypothesised that by replacing 100% of the sample buffer any variation in pH would be kept to a minimum. Figure 6-13 illustrates that the pH did fall significantly during the time trial despite the sampling method. GPC analysis showed a progressive weight loss in the degrading microspheres consistent with the bulk hydrolysis of the polyester arising from hydrolytic scission of ester groups (Li and Vert, 1999). The decrease in pH is attributed to this hydrolysis and to the formation of acidic species within the microspheres. Sah et al. (1994) examined the release from PLGA 75:25 microspheres and the accompanying change in pH over 24 days. They replaced 1/8 of the sample buffer at each time point. They reported a decrease in the pH of the buffer from pH 7.4 to

approximately pH 7.2. In the current study the pH of the buffer was seen to fall from approximately pH 7.6 to pH 7.4 over eighty-five days which is of the same magnitude as that seen by Sah et al. over a period of twenty four days.

6.1. 8 Discussion

As can be seen from the results presented microspheres manufactured at different temperatures result in batches of microspheres being formed with different physicochemical characteristics. Scanning electron microscopy illustrated that microspheres manufactured at high temperatures displayed a high degree of surface anomalies such as wrinkling when compared with microspheres manufactured at 4°C and 20°C. Particle size analysis via laser diffraction indicated that the size of the particles decreased significantly with increasing temperatures. Both observations were attributed to the rate of solvent removal which has been reported to be faster at higher temperatures than lower temperatures leading to a difference in the rate of microsphere solidification. Temperature was also observed to influence the encapsulation efficiency of DNA with microspheres manufactured at lower temperatures encapsulating a higher percentage of DNA. This might also be attributed to the rate of microsphere formation during which at low temperatures the rate of solidification is slower than at higher temperatures allowing for the increased encapsulation of DNA into the microsphere when compared with microspheres where solidification is faster thereby excluding a large percentage of the aqueous DNA. Fickian diffusion was seen as the method of DNA release in all cases with the fastest rate of release observed with microspheres manufactured at 37°C. Degradation of the polymer was observed to follow a temperature dependent path with increasing manufacturing temperatures resulting in an increase in the slope or relative rate of degradation from –51.423 in the case of 4°C to –107.510 in the case of those manufactured at 37°C. In summary we have shown that the physicochemical characteristics of microspheres are dependent on manufacturing temperature which influences the particle size, morphology, encapsulation efficiency, release characteristics and degradation of these microspheres. In this section only one plasmid was examined, the pGL3 DNA plasmid. In the next section three different molecular weight plasmids were studied in an attempt to assess the influence of molecular weight on the characteristics of DNA-loaded RG 752 microspheres.

6. 2 Influence of plasmid molecular weight

Previous researchers have studied the encapsulation of various proteins encapsulated in PLGA copolymer systems (Cohen et al., 1991). These studies have shown that molecular weight plays a significant role in the release of proteins, with lower molecular weight proteins being released at a faster rate than higher molecular weight proteins. Small microspheres have traditionally been shown to release drugs more quickly than larger microspheres, rods or discs due in part to their large surface area to volume ratio. In addition, proteins in these small microspheres can not only diffuse from the interior to the surface more quickly, but experience degradation-based protein-release more rapidly as well. Plasmid DNA is a large and negatively charged macromolecule with a molecular weight of kilos of Daltons or greater. Despite the fact that in recent years controlled release systems have been successfully employed to deliver proteins, polymer-based DNA controlled systems have not yet been fully explored. Studies examining the effect of DNA size and other limitations associated with the use of DNA as the active agent are detailed in a number of papers (Luo et al., 1999; Jong et al., 1997; Wang et al., 1999).

The present work investigates the effect of plasmid size on the physicochemical properties of PLGA based DNA delivery systems. Three plasmid vectors were encapsulated in PLGA 75:25 using a modified solvent evaporation technique. The size of a plasmid vector is generally described in terms of base pairs and can be converted to daltons since 1 kilobase of plasmid DNA corresponds to 6.5 x 10^5 daltons of duplex DNA (Mathowitz, 1999). The lowest molecular weight plasmid vector used belongs to a family of wildtype *Renilla* luciferase (R*luc*) control reporter vectors, which provide expression of a luciferase enzyme. These vectors contain the DNA encoding *Renilla* luciferase (R*luc*) cloned from the anthozoan coelenterate *Renilla reniformis* (sea pansy). The pRL–CMV vector is comprised of 4079 base pairs corresponding to a molecular weight of 2.65 x 10^3 kDa. The pGL3 vector consists of 5010 base pairs in size corresponding to 3.26 x 10^3 kDa. The largest molecular weight vector used is the pSV-β-Galactosidase (pSV-β-Gal) and is comprised of 6821 base pairs corresponding to 4.43 x 10^3 kDa.

6.2. 1 Microsphere morphology

DNA-loaded microspheres representative of each of the three plasmid DNA entities studied were examined using scanning electron microscopy for differences in their surface morphology (Figure 6-14 to 6-16).

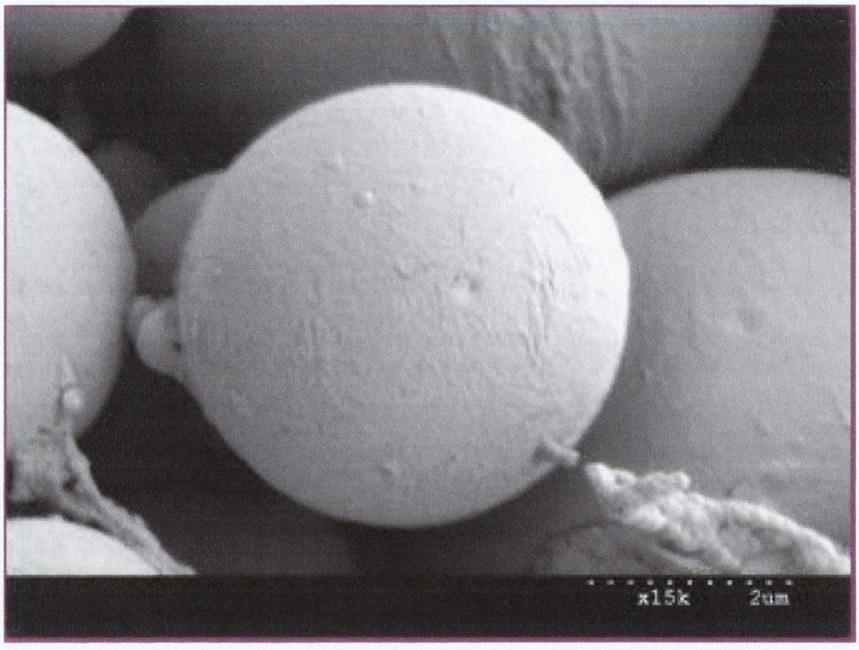


Figure 6- 14 pRL-CMV loaded microspheres

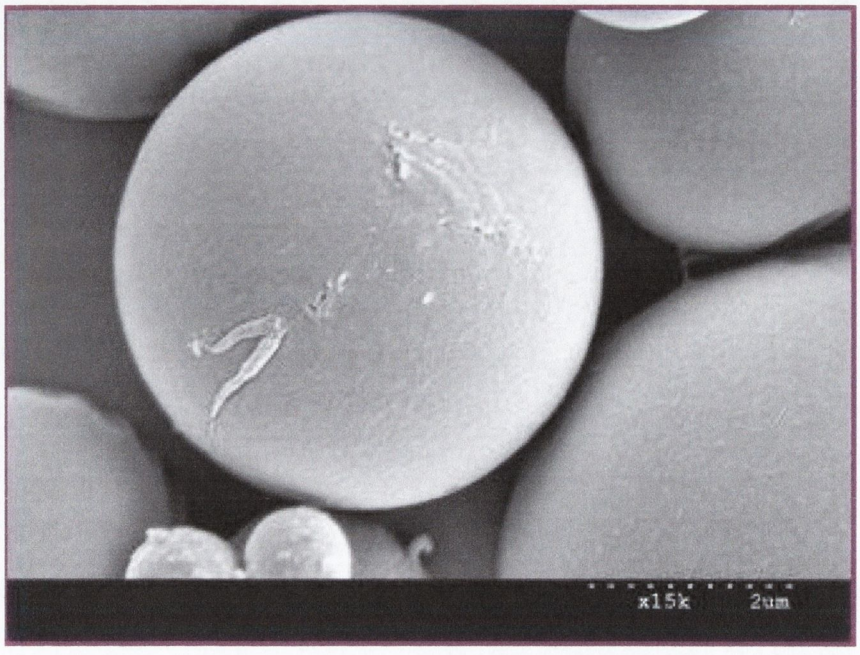


Figure 6-15 pGL3 loaded microspheres

Figures 6- 14 to 6-16 illustrate scanning electron micrographs of typical microspheres from batches produced with plasmids of different sizes. pRL-CMV and pGL3-loaded microspheres display smooth outer surfaces and are similar in size. However microspheres loaded with pSV-β-Gal appear to have a wrinkled outer surface. All batches of microspheres were manufactured at room temperature (20°C). Differences in the surface morphology observed should be largely independent of the processing conditions.

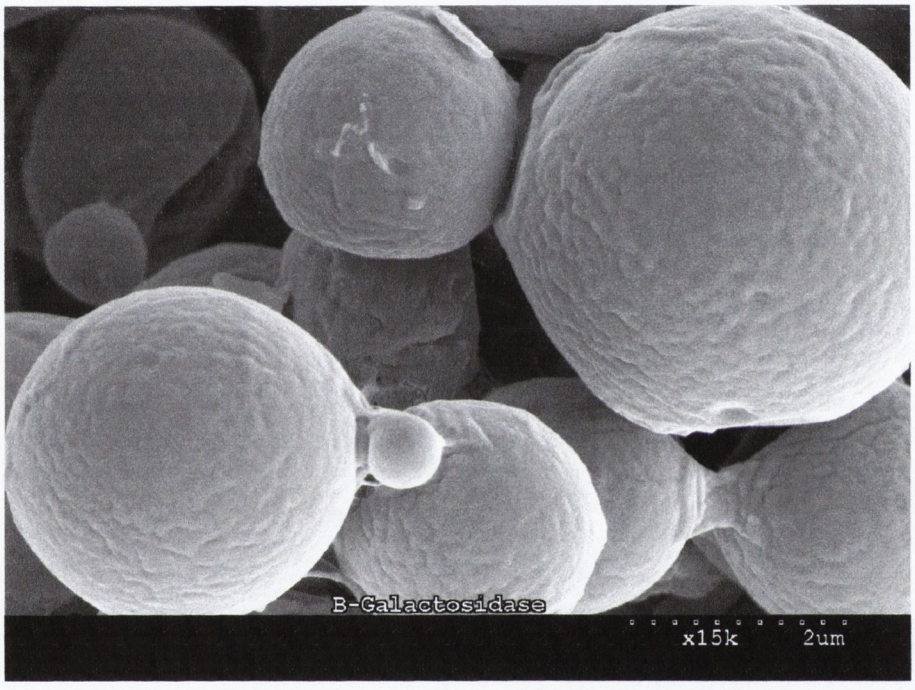


Figure 6- 16 pSV-β-Gal loaded microspheres

In this study pores can be seen on the outer surface of pSV-β-Gal loaded microspheres while they appear to be absent from microspheres containing the other two plasmids. Upon closer examination of the surface of the pSV-β-Gal loaded microspheres, pits could be seen in the microsphere surface suggesting that this is due to the presence of surface pores. This high degree of pitting on the surface skin is absent from microspheres encapsulating pRL-CMV, pGL3 or control microspheres.

6.2. 2 Particle size

Table 6-4 illustrates the influence of plasmid molecular weight on the size of the resultant microspheres.

Plasmid	Mw (x 10^3 kilo daltons)	D [ν, 0.5] μm	SD	D [ν, 0.9] μm	SD	Span
pRL-CMV	2.65	4.49	± 0.10	11.46	± 1.65	2.49
pGL3	3.26	4.07	± 0.28	9.99	± 0.37	2.37
pSV-β-Gal	4.43	2.89	± 0.00	5.10	± 0.03	2.25

Table 6- 4 Particle size data

(samples represent the mean of three determinations)

The mean D [ν,0.5] particle size for microspheres encapsulated with different molecular weight plasmids are statistically different at the 95% Confidence Interval ($p < 0.05$).

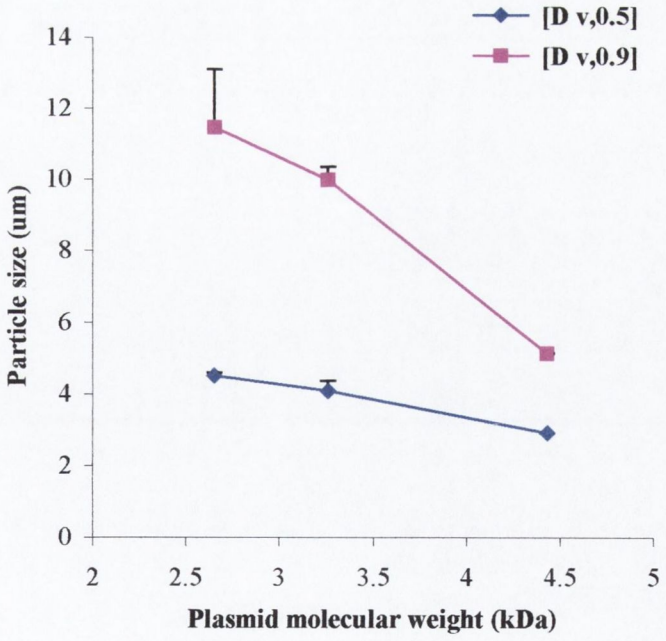


Figure 6- 17 Particle size for microencapsulated plasmid of varying molecular weights

(samples represent the mean of three determinations)

Figure 6-17 illustrates the effect of plasmid molecular weight on mean particle size at the D [v, 0.5] and D [v, 0.9] interval. There appears to be an inverse relationship between plasmid molecular weight and particle size. Wang et al. (1999) encapsulated plasmid DNA

(8131 base pairs or 5.28 x 10^3 kDa) which encoded for β-galactosidase in RG 502 microspheres. Mean particles sizes of between 0.4 and 2 μm in diameter were reported. Using a novel approach to the preparation of DNA microspheres Singh et al. (2000) adsorbed DNA plasmids onto the outer surface of RG 502 microparticles. Published data reported mean sizes of between 0.98 and 2.22 μm for the resultant particles. As can be seen from these studies, the particle sizes reported in the current work is in good agreement with other researchers.

6.2. 3 DNA loading

DNA loading in microspheres was determined following incubation in dichloromethane and extraction into TE buffer. Samples were analysed using UV absorbance at 260 nm (Barman et al., 2000).

Plasmid	**Mw (x 10^3 kilo daltons)**	**Theoretical DNA load μg DNA/mg**	**Actual DNA load μg DNA/mg microsphere**	**Encapsulation efficiency**
pRL	2.65	3.258	0.960 ± 0.020	29.465 %
pGL3	3.26	2.861	0.817 ± 0.036	28.513 %
pSV-β-Gal	4.43	3.030	0.469 ± 0.020	15.491 %

Table 6- 5 Influence of plasmid type on loading

(samples represent the mean of three determinations ± standard deviation)

From Table 6-5 loading appears to be dependent on plasmid molecular weight. The smaller the plasmid, the higher the loading and encapsulation efficiency. In the current study there is an inverse relationship observed between plasmid molecular weight and loading (Figure 6-15).

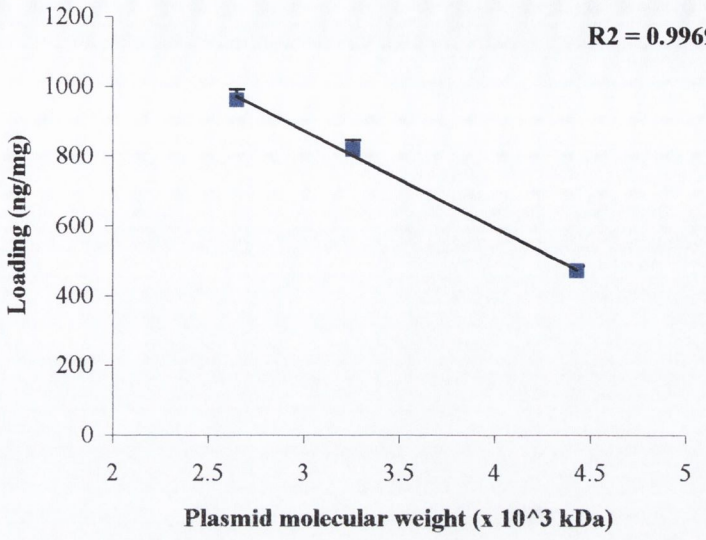


Figure 6- 18 Effect of plasmid molecular weight on microsphere loading

(samples represent the mean of three determinations)

The line with best fit had an equation of y = -27.863x + 170.89 with a coefficient of determination of 0.997. Walter et al. (1999) compared the encapsulation efficiency of two different molecular weight plasmids. They reported values of 48.3% encapsulation efficiencies for the higher molecular weight plasmid DNA (5 kilo base pairs) when compared with 68.5% encapsulation efficiency for low molecular weight calf thymus DNA (0.65 kilo base pairs). The observation that encapsulation efficiency increases as particle size increases was also reported by Capan et al. (1999). These researchers encapsulated plasmid DNA in PLGA 50:50 and reported particle size ranges of 3.1 μm to 3.5 μm with increases in encapsulation efficiency of 29.4% to 35.1%. Liu et al. (2002) also reported increasing particle size values with increasing loading efficiencies.

6.2. 4 Differential scanning calorimetry (DSC)

Figure 6-19 illustrates the thermal characteristics of the polymer powder, empty microspheres and DNA-loaded microspheres manufactured with varying molecular weight plasmids.

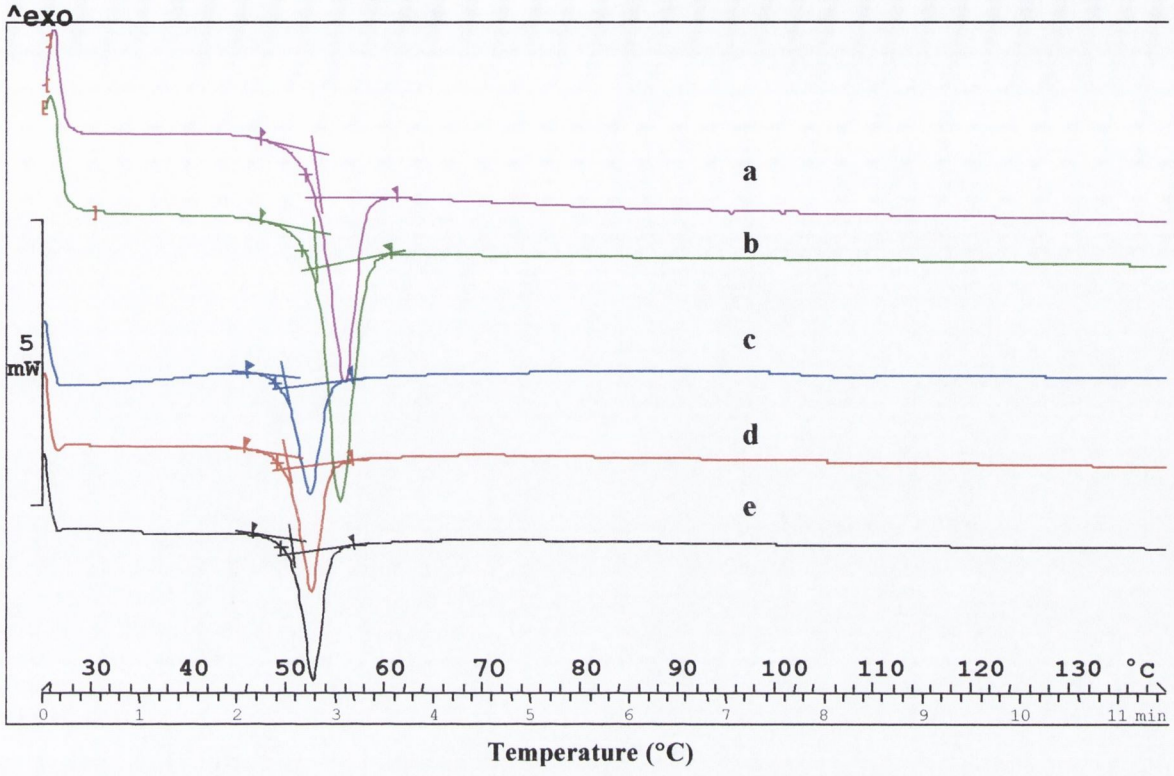


Figure 6- 19 DSC scan of (a) RG752 polymer powder (b) empty microspheres and DNA-loaded microspheres containing (c) pRL-CMV plasmid (d) pGL3 plasmid and (e) pSV-β-Gal

DSC scans of RG 752 polymer and empty microspheres show glass transition temperatures of 51.05°C and 51.57°C respectively. DNA-loaded microspheres showed glass transition temperatures of (c) 48.80°C, (d) 48.92°C and (e) 49.37°C for microspheres loaded with pRL-CMV, pGL3 and pSV-β-Gal respectively. When compared with thermograms of the RG 752 polymer alone and the empty microspheres it can be seen that the presence of the DNA plasmids does not cause a major change in the thermal characteristics of the polymer. Interestingly, as was observed in Section 6-1-4, glass transition temperature increases with decreased encapsulation efficiencies with the plasmid with the highest encapsulation efficiencies (pRL-CMV, 29.47% encapsulation efficiency) showing the lowest glass transition temperature (48.80°C). DSC thermograms show that only the endothermic peaks similar to that of the polymer were detectable, an observation which would suggest the dispersion of the plasmid within the polymer matrix.

6.2. 5 DNA release

Figure 6-20 illustrates the release pattern of pRL-CMV and pGL3 and pSV-β-Gal from RG 752 microspheres. pRL-CMV-loaded microspheres show the highest initial burst release followed by pSV-β-Gal–loaded microspheres.

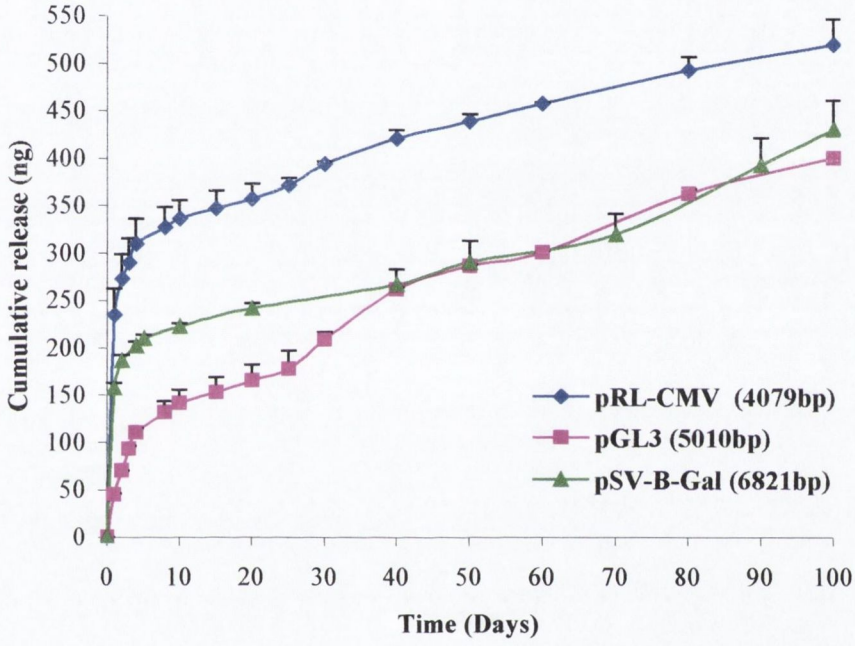


Figure 6- 20 Cumulative release for plasmids with varying molecular weights

(samples represent the mean of three determinations)

As can be seen from Figure 6-20 the release from plasmid loaded microspheres appears to be biphasic with an initial burst release of plasmid (until approximately day 4 or 5) followed by a lag phase during which time there is a slow rate of release for all three plasmid loaded microspheres systems. This pattern of release from a hydrophobic polymer such as RG 752 has been previously reported by Walter et al. (2001). As was the case in previous protein studies RG 752 has a relatively long half-life which would presumably have resulted in the continued release of plasmid DNA until the microspheres had been depleted had the study not been terminated.

The amount of DNA released appears greatest for the smallest molecular weight plasmid, the pRL-CMV plasmid. This mirrored studies by Sandor et al. (2001) who reported that the rate of release of protein from PLGA 50:50 microspheres was fastest for smaller molecular

weight proteins. Statistical analysis, at the 95% confidence level, indicated that the release from pGL3 and pSV-β-Gal- loaded microspheres was not statistically significant ($p > 0.05$). However the release of pRL-CMV was statistically different when compared with the release of the pGL3 and pSV-β-Gal ($p < 0.05$).

It is believed that lower molecular weight molecules tend to be encapsulated in the outer layer of the microsphere causing the observed initial release (Mathowitz et al., 1997). In this study it was found that the lowest molecular weight plasmids exhibit a faster release over the first ten days however with regards to the remaining plasmids this does not appear to be dependent on molecular weight. This may be due to the pores, which were visible on the outer surfaces of pSV-β-Gal–loaded microspheres and which were absent in the other two. If this is the case then the burst release appears to be dependent on the encapsulation of small molecules in the outer layer and their branched internal structure which facilitates rapid release and in the case of larger molecules, on the morphology of the outer layer which is highly pitted and porous also facilitating a rapid burst release. This is supported by Sandor et al. (2001) who reported that microspheres encapsulating small molecules had an open internal structure and that microspheres encapsulating larger molecules, had more pores on their outer surface with a denser internal structure. Hence in both cases a burst release would be expected for different reasons. Fractional release from DNA microspheres manufactured with different molecular weight plasmids was fitted to Equation 1-14 (Figure 6-21).

Figure 6-21 illustrates the actual release data fitted to those generated by the model. Two phases can be distinguished for the release pattern of the DNA: the initial release and the second release phase. In this study the burst effect appears to be more significant in the case of the largest molecular weight plasmid studied, the pSV-β-Gal plasmid. In the case of this plasmid there also appears to be the beginning of a third release phase after 70 days of incubation. In the case of the remaining plasmids the release pattern appears to be biphasic. The coefficient of determination (CD) and model selection criterion (MSC) were used as measures of goodness of fit. Table 6-6 summarises the results obtained when fractional release was fitted to the model (Equation 1-14).

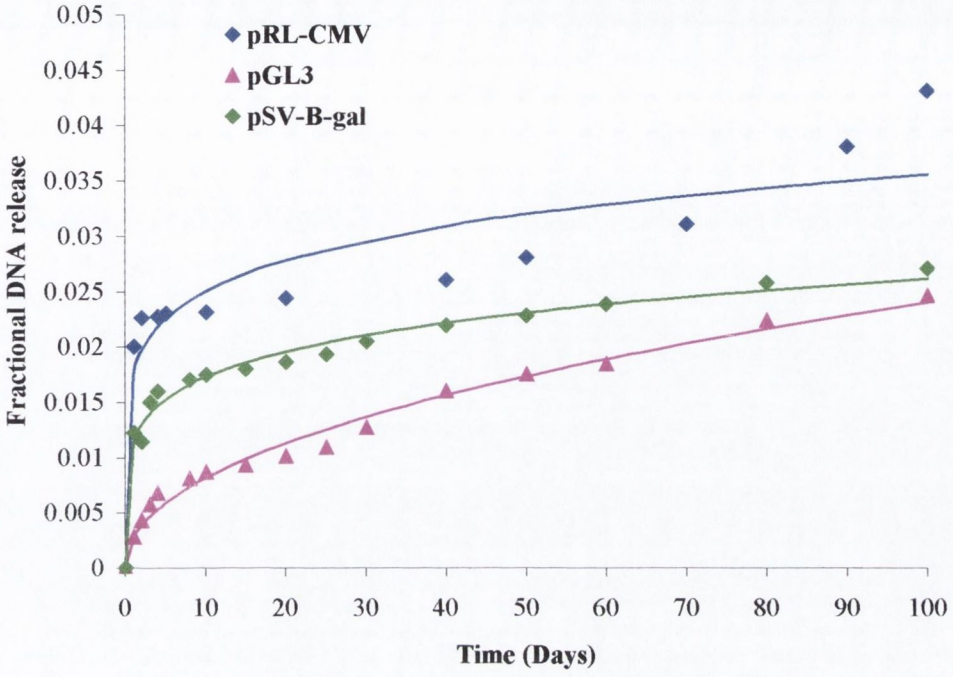


Figure 6- 21 Fractional release of DNA from microspheres manufactured with different molecular weight plasmids fitted to Equation 1-14

Parameter	PRL-CMV	pGL3	pSV-β-Gal
	2.65 x 10^3 kDa	3.26 x 10^3kDa	4.43 x 10^3kDa
K	0.0012	0.0028	0.0018
n	0.178	0.467	0.151
CD	0.984	0.980	0.886
MSC	3.880	3.990	1.811

Table 6- 6 Parameters obtained for the fitting of release of different molecular weight plasmids to Equation 1-14

From Table 6-6 it can be seen that both the pRL-CMV and pGL3 plasmids were released at a rate which was in good agreement with the diffusion model (Equation 1-14) resulting in MSC values of over 3 and coefficient of determination values close to 1. The n values associated with release of varying molecular weight plasmids varied between 0.151 and

0.467. Values close to 0.432 indicated that the release might be based on Fickian diffusion such as is the case in the release of pGL3 from these microspheres.

6.2. 6 Degradation

Samples of microspheres were examined for degradation following scanning electron microscopy and gel permeation chromatography analysis (Figure 6-22).

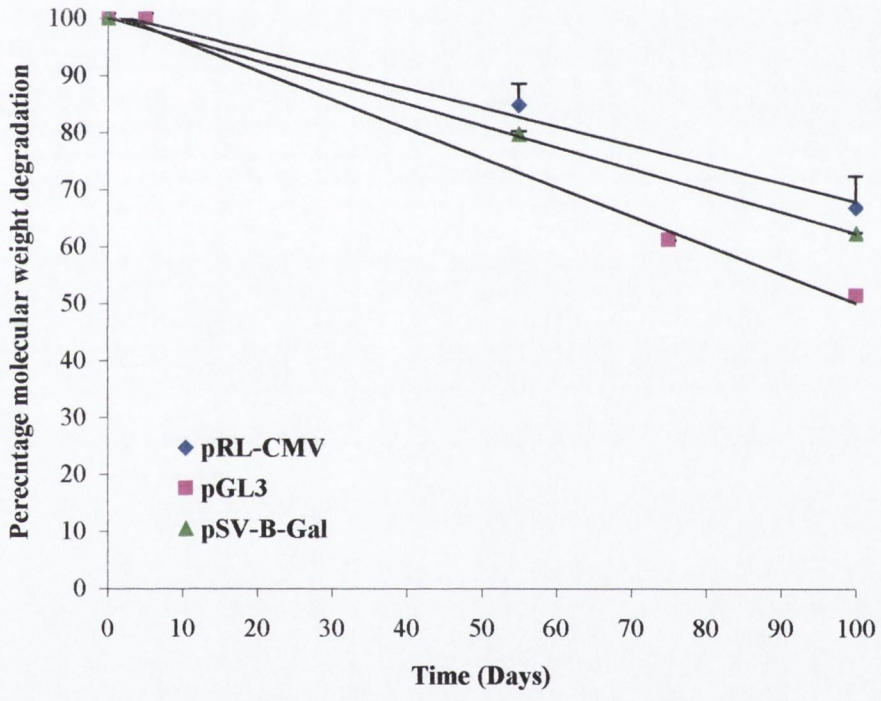


Figure 6- 22 Degradation of microspheres and polymer

(samples represent the mean of three determinations)

From Figure 6-22 it can be seen that the presence of an encapsulated agent decreases the slope or relative rate of degradation of the microspheres. The slope of the line and corresponding R^2 value through the points are as follows: *pRL-CMV* - y = -0.330x + 100.920, R^2 = 0.988, *pGL3* - y = -0.509x + 102.340, R^2 = 0.996, *pSV-β-Gal* y = -0.379x + 100.140, R^2 = .998, The slope of the line was taken as an approximation of the relative rate of degradation of the sample in that degradation rates in decreasing order can be ranked as follows pGL3 < pSV-β-Gal< pRL-CMV. Figure 6-8 illustrated the rate of degradation of RG 752 and empty microspheres. The rate of degradation of the empty microspheres and

the polymer powder increases from – 0.595 for empty microspheres to – 0.767 in the case of RG 752. This indicated that the rate of degradation of microspheres loaded with different molecular weight plasmids was less than that of empty microspheres or of the polymer alone.

6.2. 7 Discussion

In the current section the influence of the molecular weight of the encapsulated agent on the characteristics of RG 752 microspheres was investigated. A high degree of pitting in the surface of pSV-β-Gal encapsulated microspheres was observed following scanning electron microscopy which was absent from microspheres encapsulating pRL-CMV, pGL3 or control microspheres.

Following particle size analysis with laser diffraction there appeared to be an inverse relationship between plasmid molecular weight and particle size. This phenomenon was also present in the encapsulation efficiency results where the levels of encapsulation was highest in the case of the smallest plasmid, pRL-CMV. Levels of encapsulation reported in the current study were similar to those reported by other researchers.

The release of the different molecular weight plasmids followed different release patterns with the two smallest plasmids, pRL-CMV and pGL3 following a diffusion based pattern Fractional release studies indicated that the release of pSV-β-Gal was greater than for the remaining two plasmid however the amount released was greater for the smaller plasmid pRL-CMV. This might be attributed to the higher loading associated with the smaller plasmid, pRL-CMV compared with the remaining two plasmids.

In terms of microspheres' degradation the slope of the line following linear regression analysis was taken as the approximation of the rate of degradation of the microspheres. It was observed that degradation was quickest for microspheres loaded with pGL3 followed by pRL-CMV. Studies also indicated that that the rate of degradation of microspheres loaded with different molecular weight plasmids was less than that of empty microspheres or of the polymer alone.

In conclusion we have shown in this section that the molecular weight of the encapsulated agent is an important factor in the characteristics of encapsulated systems. Loading and particle size have both been shown to be significantly influenced by the molecular weight of the encapsulated agent with microspheres encapsulating the smallest plasmid exhibiting both the largest particle size and the highest encapsulation efficiency. In the next section the influence of the external aqueous phase was examined with respect to pGL3 encapsulated microspheres.

6. 3 Influence of external aqueous volume

Microspheres were manufactured following a modified solvent evaporation technique in which an aqueous solution containing the active ingredient was added to an organic phase containing the polymer and then to a larger aqueous phase containing a stabiliser such as polyvinyl alcohol (PVA). This technique is termed a water/oil/water ($W_1/O/W_2$) double emulsion and is widely used in the field of microencapsulation (Coombes et al., 1998; Li et al., 2000). An emulsion is achieved by applying a mechanical energy which deforms the interface between the water and oil layer to such an extent that droplets form. In order to aid emulsification a surfactant such as polyvinyl alcohol (PVA) can be dissolved in the second water phase before it is added. The PVA acts as a surfactant in the outer aqueous layer and associates with the surface of the drug containing droplets produced in the primary emulsion and prevents their coalescence. Polymer precipitation and gradual diffusion of the solvent from the polymer solution into the outer aqueous phase eventually leads to microparticulate formation and hardening. Several investigators have studied the effect of PVA concentration (Jeffery et al., 1993; Coombes et al., 1998) and the effect of inner water volume on the encapsulation of proteins (Cohen et al. 1991; Jeffery et al. 1993; Chen et al., 2001) however the influence of external aqueous volume has not been extensively reported in the literature (Jeffery et al. 1993).

Although o/w emulsion solvent evaporation has been used successfully by several groups to entrap hydrophobic materials into PLGA microparticles, poor levels of entrapment have normally been observed which results from the partitioning of the water soluble material from the inner oil phase to the outer aqueous phase (Jeffery et al., 1993). Ogawa et al (1988) developed a water-in-oil-in-water solvent evaporation to entrap water-soluble molecules into PLGA microparticles. In this section the influence of the ratio of W_1 to W_2 in the encapsulation of plasmid DNA was investigated. The aqueous phase containing the DNA was kept constant while the outer phase containing the PVA stabiliser was varied with the PVA concentration maintained at 0.27%. However in doing so the ratio of polymer to PVA varied. The ratios of outer to inner aqueous volumes used were 20:1, 15:1 and 10:1.

6.3. 1 Microsphere morphology

Figures 6-23 to 6-25 illustrate scanning electron micrographs of microspheres formulated with a ratio of external to internal aqueous phases of (a) 10:1, (b) 15:1 and (c) 20:1.

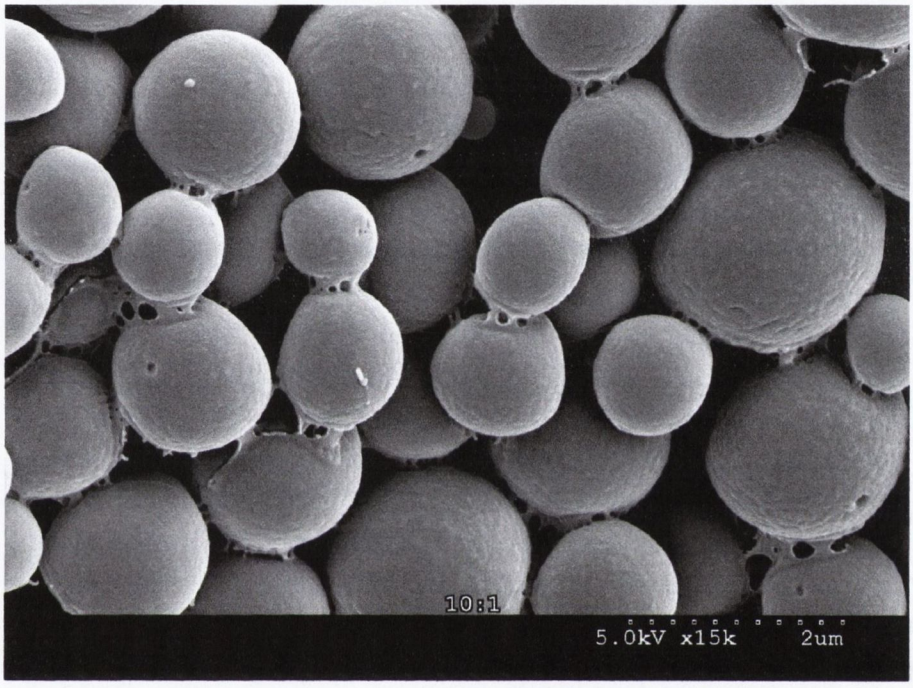


Figure 6- 23 Microspheres formulated with 10:1 outer: inner aqueous volume

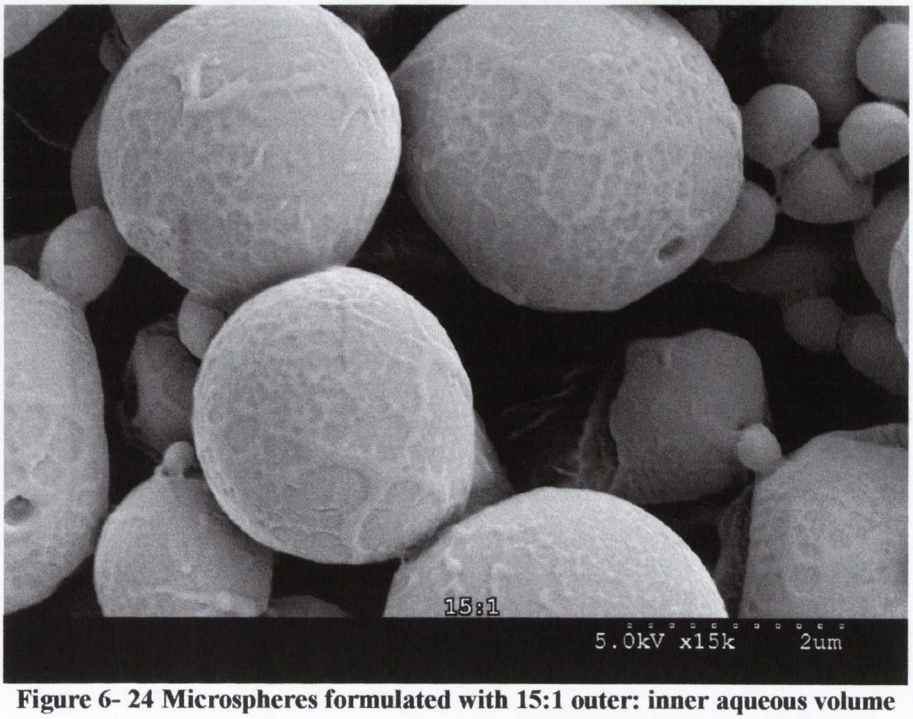


Figure 6- 24 Microspheres formulated with 15:1 outer: inner aqueous volume

From these micrographs it can be seen that as the aqueous volume increases the surface of the microspheres becomes smoother, larger and their shape becomes more spherical

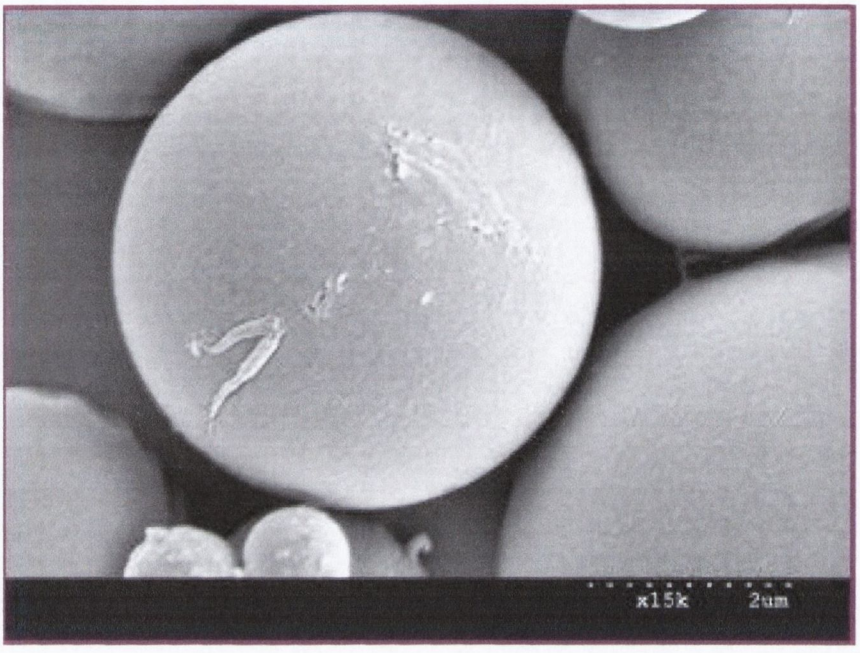


Figure 6- 25 Microspheres formulated with 20:1 outer: inner aqueous volume

Microspheres formed with 10:1 outer:inner aqueous volume ratios can be seen to aggregate and are not free flowing (Figure 6-23). They appear to have a rough outer surface and are not regular in shape or size. This might be attributed to the decrease in polymer: PVA concentration which occurs as the aqueous volume is reduced thus reducing the stabilising effect of the PVA. This would allow for the flocculation of droplets during the emulsification procedure. Microspheres made with a 15:1 outer:inner aqueous volume ratio, appear to be more regular in shape but have a rough wrinkled surface. (Figure 6-24). They also appear to coalesce but not to the same degree as microspheres formulated at the lower ratio. Those formulated with a 20:1 outer:inner aqueous volume ratio have very smooth outer layers and are regular in shape (Figure 6-25). Also as the inner aqueous volume increases the size of the microspheres is seen to increase.

6.3. 2 Particle size

Table 6-8 illustrates the influence of aqueous volume on particle size. Increasing the outer aqueous volume from 10:1 to 20:1 would appear to result in an increase in particle size. This was also seen in the scanning electron micrographs of the particles.

Outer:inner aqueous phase ratio	D[v,0.5] μm	SD	D[v,0.9] μm	SD	Span
10:1	3.49	± 0.20	3.88	± 0.17	1.24
15:1	3.50	± 0.05	5.69	± 1.60	2.47
20:1	4.48	± 0.51	5.77	± 0.65	2.52

Table 6- 8 Effect of aqueous volume on particle size
(samples represent the mean of three determinations)

Particle sizes (D [v, 0.5]) of systems manufactured with a 10:1 aqueous phase ratio did not differ significantly from systems with a 15:1 aqueous phase ratio ($p > 0.05$). However the particle sizes of these microspheres are significantly different from those particles formed with a 20:1 aqueous phase ratio ($p < 0.05$).

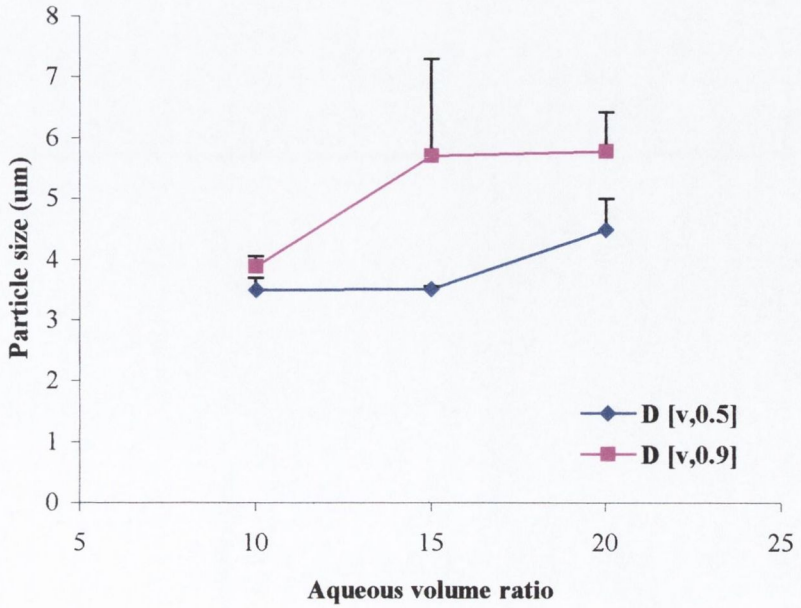


Figure 6- 26 Particle size data for microspheres manufactured with varying outer aqueous volumes
(samples represent the mean of three determinations)

Particle sizes (D [v, 0.9]) of systems manufactured with a 10:1 aqueous phase ratio were significantly different from systems with a 15:1 and a 20:1 aqueous phase ratio ($p < 0.05$). However the (D [v, 0.9]) particle sizes of microspheres manufactured with 15:1 and 20:1 aqueous phase ratios were not significantly different ($p > 0.05$).

Jeffery et al. (1993) investigated the effect of external aqueous phase ratio on the particle size of PLGA 50:50 and 85:15 microparticles. These researchers reported that adjustment of the outer aqueous volume from a ratio of 2:1 to a ratio of 20:1 resulted in an increase in particle size from 2.8 μm to 6.5 μm. This was attributed to the reduction in agitation, which resulted from the decrease in mixing efficiency associated with larger volumes. This would in turn promote the formation of increased droplet sizes in the double emulsion, which would have resulted in larger microspheres. The results reported in the current study (Figure 6-26) are in good agreement with Jeffery et al. (1993).

6.3. 3 DNA loading

Loading in microspheres manufactured with different aqueous volumes is illustrated in Table 6-9.

Outer: inner aqueous volume ratio	**Theoretical DNA load μg DNA/mg microsphere**	**Actual DNA load μg DNA/mg microsphere**	**Encapsulation efficiency**
10:1	3.058	0.427 ± 0.027	13.954 %
15:1	3.539	0.357 ± 0.058	10.094 %
20:1	2.86	0.817 ± 0.0492	28.513%

Table 6- 9 DNA loadings in different aqueous volumes

(samples represent the mean of three determinations)

As can be seen from Figure 6-27 there appears to be no relationship between outer aqueous volume and DNA loading. In the current study the effect of increasing aqueous volume on particle size and on DNA loading appear to be similar. With regard to particle size there is no statistical difference between particles formed with 10:1 and 15:1 outer aqueous phase ratios while there is a significant increase in particle size once the outer:inner aqueous phase ratio is increased to 20:1. In the case of DNA loading there is a similar observation.

DNA loading in microspheres prepared with a 10:1 and 15:1 outer:inner phase ratio appear to be very similar while loading in microspheres prepared with an outer: inner aqueous phase ratio of 20:1 is significantly higher. The highest encapsulation efficiency is observed in the case of microspheres manufactured with 20:1 outer to inner volume ratio which are also the microspheres which displayed the largest particle size.

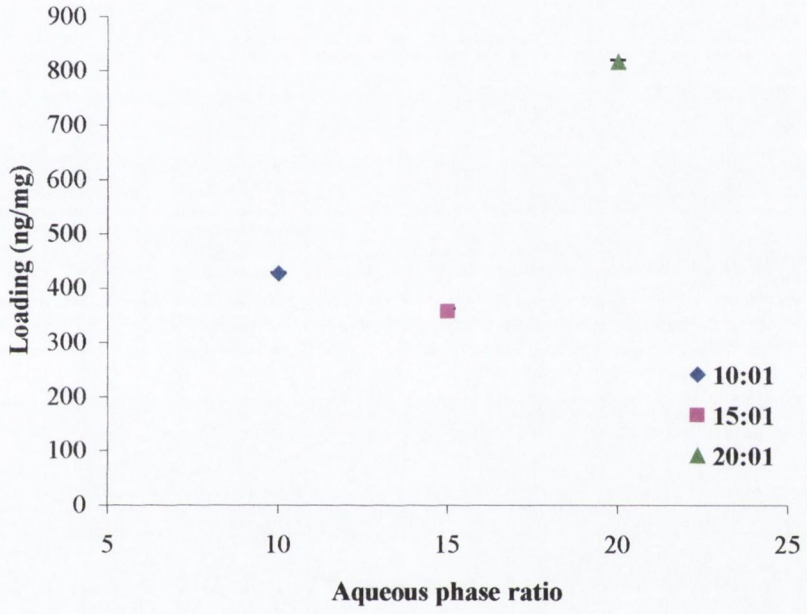


Figure 6-27 Influence of outer aqueous volume on loading of DNA

Jeffery et al. (1993) studied the effect of outer aqueous volume phase on the loading of ovalbumin in PLGA 50:50 and 85:15 microspheres and reported that there was a linear relationship between the volume of external aqueous phase and the amount of protein entrapped. This was also associated with the increase in particle size and was attributed to the resultant increase in particle volume, which would enable the entrapment of a higher concentration of protein.

6.3. 4 Differential Scanning Calorimetry (DSC)

Figure 6- 28 illustrates the thermal properties of RG 752 polymer powder, empty microspheres and DNA-loaded PLGA microspheres.

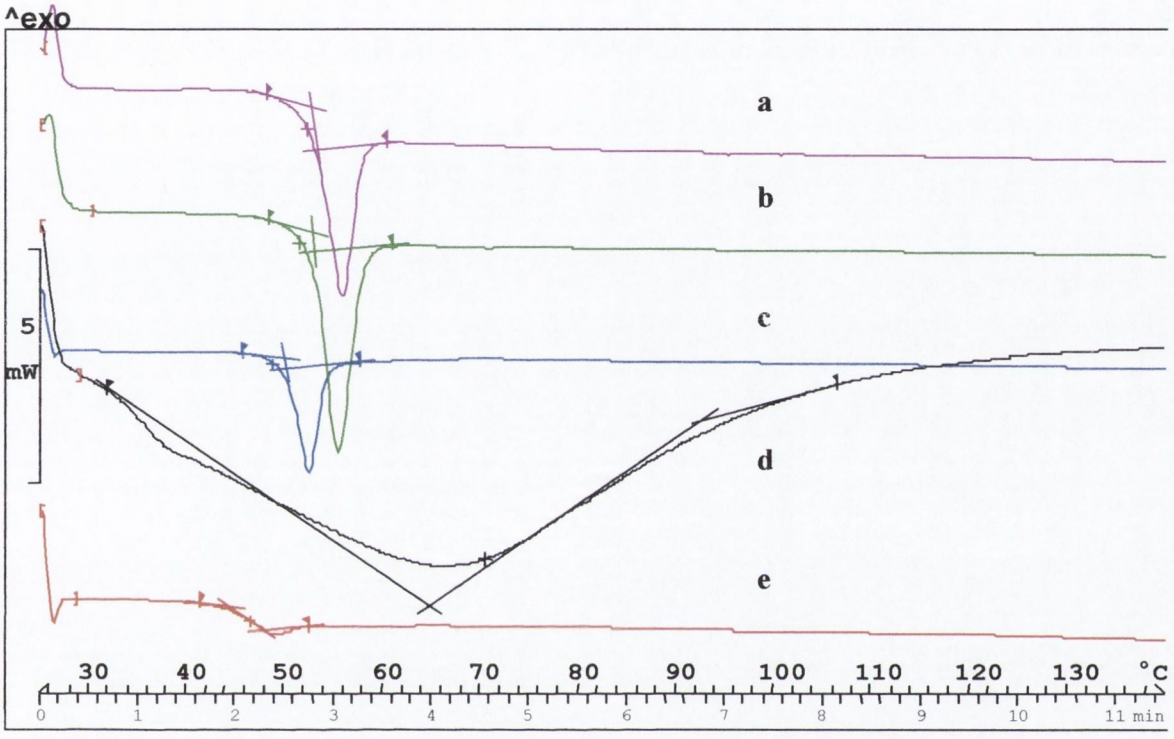


Figure 6- 28 DSC scan of (a) RG752 polymer powder (b) empty microspheres and DNA-loaded microspheres manufactured with an aqueous phase ratio of (c) 20:1 (d) 15:1 (e) 10:1

Glass transition onset temperatures were 48.78°C, 70.33°C and 46.32°C for 20:1, 15:1 and 10:1 aqueous volumes respectively. The scan for those particles formulated with 15:1 aqueous volume (d) is most likely to be as a result of residual liquid, which is masking the thermal analysis of the microsphere itself. As can be seen from the remaining thermograms the presence of the DNA plasmid causes a slight decrease in the glass transition value of DNA-loaded microspheres when compared with the PLGA polymer (a) and empty microspheres (b) with glass transition temperatures of 52.21°C and 51.50°C respectively. This might be attributed to the plasticising effect of the encapsulated agent (Ford and Timmins, 1989).

6.3. 5 DNA release

Figure 6-29 illustrates the effect of aqueous volume on the release from DNA-loaded microspheres.

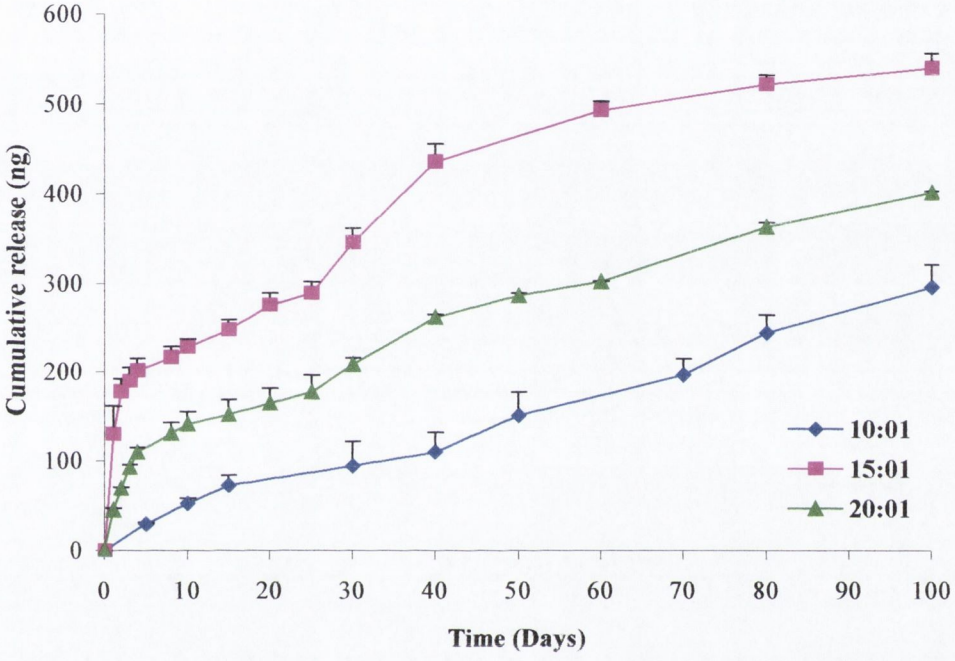


Figure 6- 29 Effect of aqueous volume ratio on cumulative release of DNA
(samples represent the mean of three determinations)

As can be seen the rate of release of plasmid from microspheres formed with an outer:inner ratio of 15:1 is faster then that from other batches. As was reported in the previous section RG 752 has a relatively long half-life, which would presumably have resulted in the continued release of plasmid DNA until the microspheres had been depleted had the study not been terminated. The pattern of plasmid release from microspheres formulated with outer:inner ratios of 15:1 and 20:1 appears to be biphasic with typical diffusion pattern. However in the case of microspheres formulated with an outer:inner ratio of 10:1 release appears to follow a more linear pattern of release with no burst release.

Fractional release from microspheres manufactured with varying aqueous volume ratios was fitted to Equation 1-14. Figure 6-30 illustrates the fractional release of DNA from microspheres fitted the model.

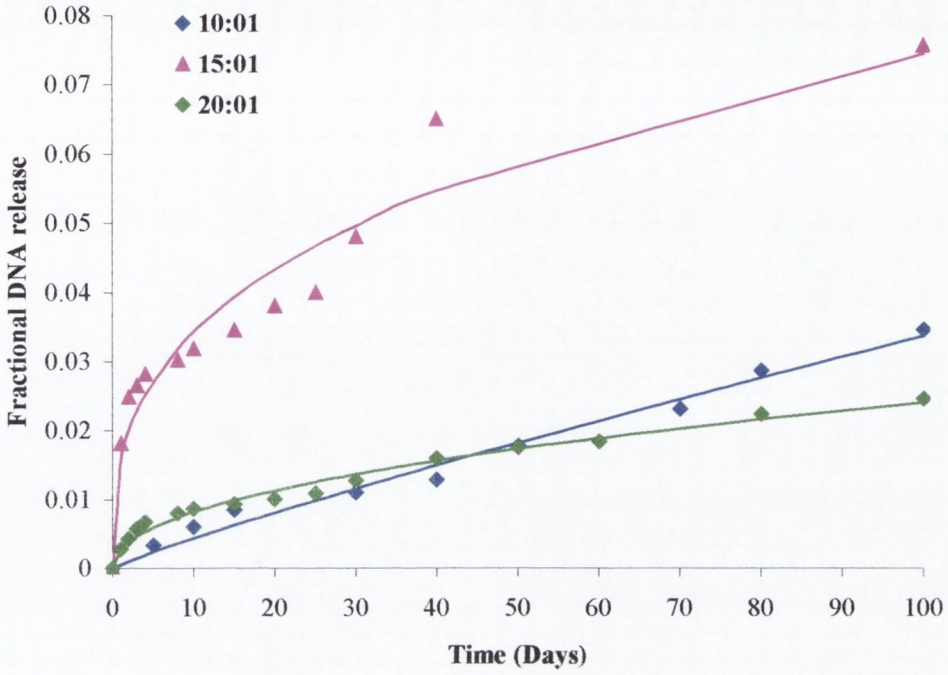


Figure 6- 30 Fractional release of plasmid DNA fitted to Equation 1-14

From Figure 6-30 it can be seen that microspheres manufactured with an outer inner ratio of 15:1 release a higher fraction of their encapsulated DNA over the period of the trial. These microsphere also displayed the lowest encapsulation of the three system and exhibited unusual thermal characteristics. Release data from DNA-loaded microspheres showed good fit with the model (Equation 1-14). Parameters associated with these plots are listed in Table 6-10.

Parameter	10:1 aqueous phase ratio	15:1 aqueous phase ratio	20:1 aqueous phase ratio
K	0.0057	0.0016	0.0028
n	0.178	0.335	0.467
CD	0.984	0.94	0.980
MSC	3.880	2.549	3.990

Table 6- 10 Parameters obtained for the fitting of release of DNA from microspheres manufactured with varying aqueous phase volumes to Equation 1-14

From Table 6-10 it can be seen that the DNA release from all batches was modelled successfully with Equation 1-14 resulting in MSC values of over 2.5 and coefficient of determination values close to 1. The values for n varied from 0.178 to 0.467. Microspheres manufactured with 15:1 and 20:1 aqueous phase ratios yielded n values of 0.335 and 0.467 respectively suggesting that release might be based on Fickian diffusion.

6.3. 6 pH

Fractional changes in buffer pH were analysed over an eighty-five day period.

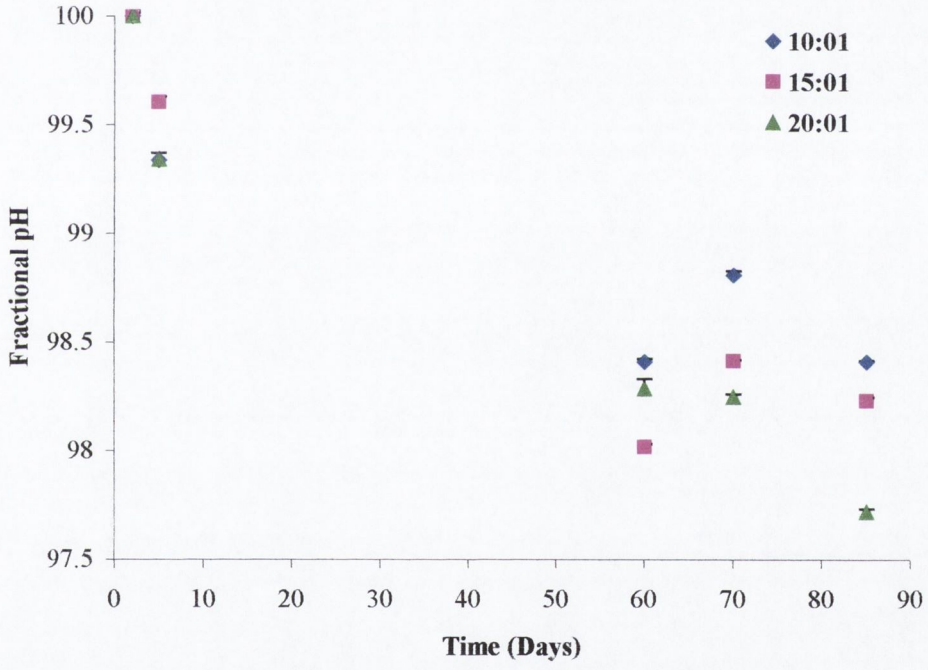


Figure 6- 31 Effect of aqueous volume on pH of buffer

(samples represent the mean of three determinations)

As can be seen from Figure 6-31 there is a decrease in buffer pH over eighty-five days when compared with the pH of the start buffer. The sampling technique used was chosen in an attempt to minimise pH changes during the trial. Theoretically replacement of complete sampling volume should minimise the effects of degradation products on the pH of the sampling environment. Figure 6-31 illustrates that the pH did decrease significantly during the time trial despite replacing 100% of the medium at each time point. The results show that while there is no significant difference between the two, the rate of decrease in pH for those made with 15:1 aqueous volume is steeper up to day 60 when compared with those

formulated with 20:1 aqueous volume. Hydrolytic degradation of the polymer is one of the possible mechanisms of release from a polymer matrix. Degradation occurs as the ester bonds of the polymer undergo hydrolytic scission into their various acidic components thus decreasing the pH of the surrounding medium (Li and Vert, 1999).

6.3. 7 Discussion

The current section examined the effect of the outer aqueous phase on the characteristics of DNA-loaded microspheres. Changing the ratio of outer to inner aqueous phases results in a change of the polymer: PVA ratio since the amount of polymer used was maintained. PVA acts as a stabiliser in the double emulsion and allows for the formation of stable polymer droplets. Scanning electron microscopy illustrated that decreasing the outer aqueous volume resulted in microspheres which were seen to have flocculated during emulsification leading to incomplete microsphere formation and incomplete dissociation of the microspheres from each other. This observation was seen to decrease as the volume was increased. Particle size analysis also showed a significant increase in particle size as the outer aqueous volume was increased. Encapsulation efficiency was higher for those microspheres manufactured with the highest outer aqueous volume with all batches exhibiting diffusion-based release of DNA. Percentage release was highest for those microspheres manufactured with a 15:1 outer to inner aqueous volume ratio however these microspheres also showed unusual thermal characteristics which might be due to the incomplete encapsulation of DNA.

In summary we have found that changes in the outer aqueous volume resulted in differences in particle size, microsphere morphology and encapsulation efficiency. Release patterns were also observed to be influenced by the outer aqueous volume. In the next section the influence of DNA loading was examined.

6. 4 Influence of DNA loading

It has been generally accepted that in order to cross mucosal surfaces, microspheres must be below 10 μm in size (Perez et al., 2001). DNA loaded microspheres have typically shown low loadings and encapsulation efficiency values (Walter et al., 1999). This is due to the particle size restriction, if microspheres are to be targeted to mucosal surfaces, and due to the molecular weight of plasmid DNA (approximately 3 to 5 $x10^3$ kDa), which is approximately a magnitude higher, when compared with commonly used proteins such as lysozyme (13.4 kDa), bovine serum albumin (66 kDa) and thyroglobulin (669 kDa). A further limitation to encapsulation efficiency is the sensitivity of plasmid DNA to several common encapsulation techniques such as sonication or high-speed homogenisation, which might otherwise result in higher loading values (Huang et al., 1999). Several studies have attempted to condense plasmid DNA with peptide in order to allow sonication of the emulsion to produce smaller microspheres or have included compounds, such as taurocholic acid, in their formulations in order to improve the efficacy of DNA microspheres (McKeever et al., 2002).

In an attempt to address the low encapsulation efficiencies associated with plasmid DNA further studies have examined the effect of increased theoretical loadings of DNA on the physicochemical properties of PLGA microspheres (Jong et al., 1997; Walter et al., 2001). Jong et al. (1997) compared microspheres loaded with 2% and 20% plasmid DNA and reported differences in some of their physicochemical properties such as release and morphological changes in the microspheres. Walter et al. (2001) similarly compared two DNA loadings and found that morphological and release profiles were dependent on loading. In this current study the influence of initial loadings on the physicochemical properties of DNA-loaded microspheres was investigated. Theoretical loadings used here were 2.4, 4.7 and 11.8 μg plasmid DNA/mg PLGA.

6.4. 1 Microsphere morphology

Scanning electron microscopy was used to examine the external morphology of the microspheres. Figures 6-32 to 6-34 illustrate typical microspheres with theoretical loadings of 2.4, 4.7 and 11.8 μg plasmid DNA/mg PLGA.

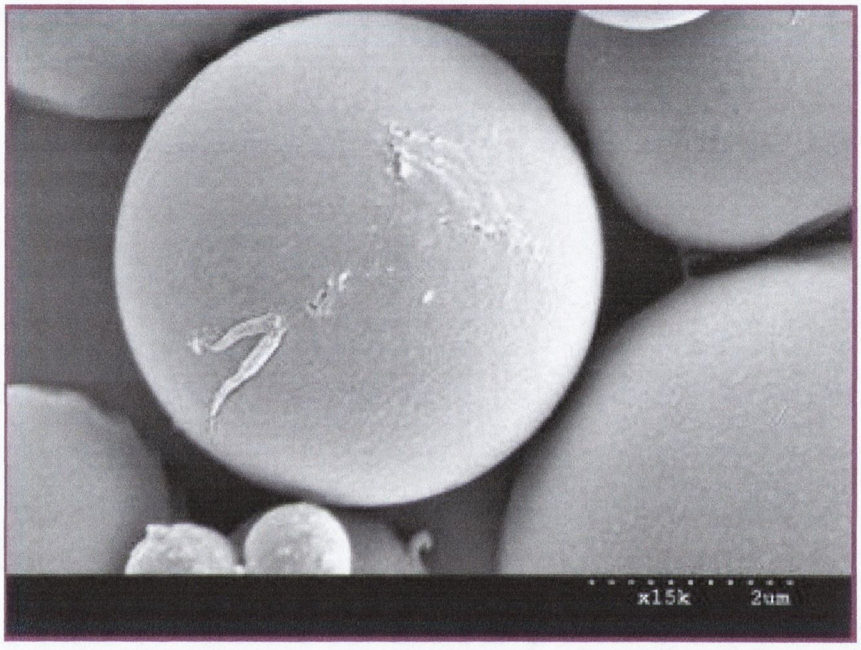


Figure 6- 32 2.4 μg plasmid DNA/mg PLGA Loaded microsphere

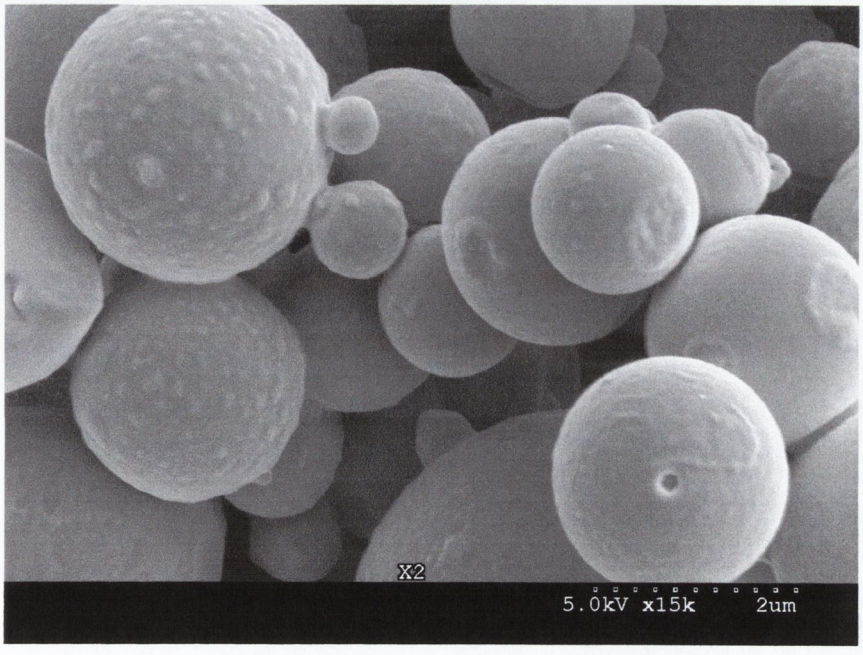


Figure 6-33 4.7 μg plasmid DNA/mg PLGA loaded microsphere

In the current study increasing the loading from 2.4 to 11.8 μg plasmid DNA/mg PLGA led to a more wrinkled microsphere surface and to an increase in frequency of pores on the outer surface as observed by scanning electron microscopy. Also from Figures 6-32 to 6-34 it can be seen that increasing the loading resulted in a decrease in microsphere size.

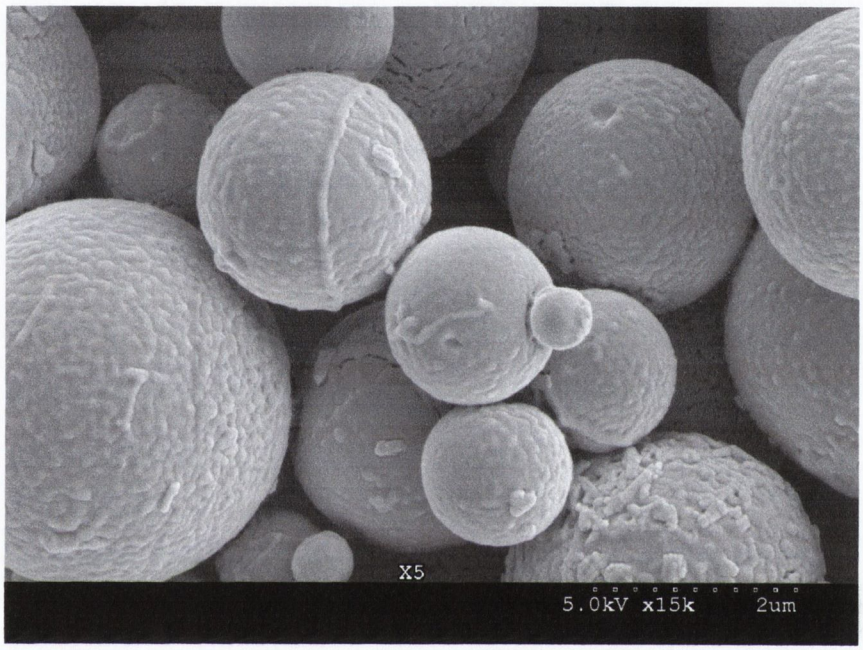


Figure 6-34 11.8μg plasmid DNA/mg PLGA loaded microsphere

Walter et al. (2001) observed that microspheres loaded with increasingly higher concentrations of DNA (from 2 to 20%) resulted in a more wrinkled microsphere surface. Aqueous DNA solutions have been reported to be highly viscous at relatively low concentrations, which would affect both loading and dispersion of the DNA during microsphere fabrication (Huang et al., 1999). Walter et al., (2001) suggested that the phenomenon of increased wrinkling in the surface of microspheres loaded with increasing highly concentrations of DNA could be due to the resultant higher viscosity which would cause slower evaporation of fluids during the formulation process.

6.4. 2 Particle size

Table 6-11 illustrates the effect of theoretical loading on particle size.

Theoretical loading μg plasmid DNA/mg PLGA	**D[v,0.5] μm**	**SD**	**D[v,0.9] μm**	**SD**	**Span**
2.4	4.48	± 0.51	5.77	± 0.65	2.52
4.7	3.53	± 0.01	7.18	± 0.04	2.58
11.8	3.10	± 0.02	6.24	± 0.01	2.56

Table 6- 11 Effect of DNA loading on particle size

(samples represent the mean of three determinations)

Particle size data for all batches manufactured with 2.4, 4.7 and 11.8 μg of DNA per mg PLGA were significantly different for both the D[v, 0.5] and D[v, 0.9] measurements ($p < 0.05$).

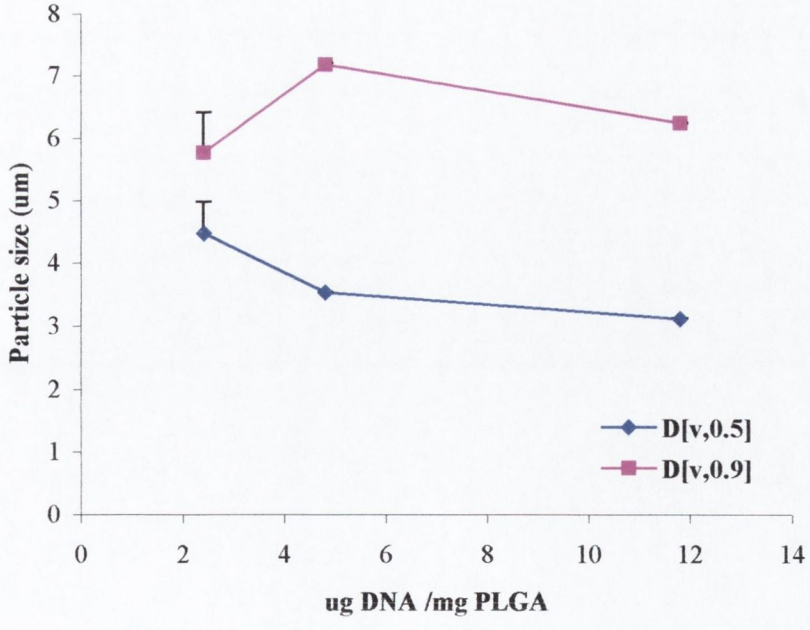


Figure 6- 35 Particle size data for microspheres manufactured with varying concentrations of DNA

(samples represent the mean of three determinations)

Figure 6-35 illustrates the effect of loading on D [v, 0.5] and D [v, 0.9] particle size. As can be seen there appears to be a relationship between D[v,0.5] particle sizes and loading.

As the loading value increased the D[v,0.5] particle size value was observed to decrease. However this observation was not seen at the D[v,0.9] interval.

6.4. 3 Differential Scanning Calorimerty(DSC)

Figure 6-36 illustrates the thermal characteristics of polymer powder, empty microspheres, and microspheres loaded with, 2.4, 4.8 and 11.8 μg DNA/mg PLGA.

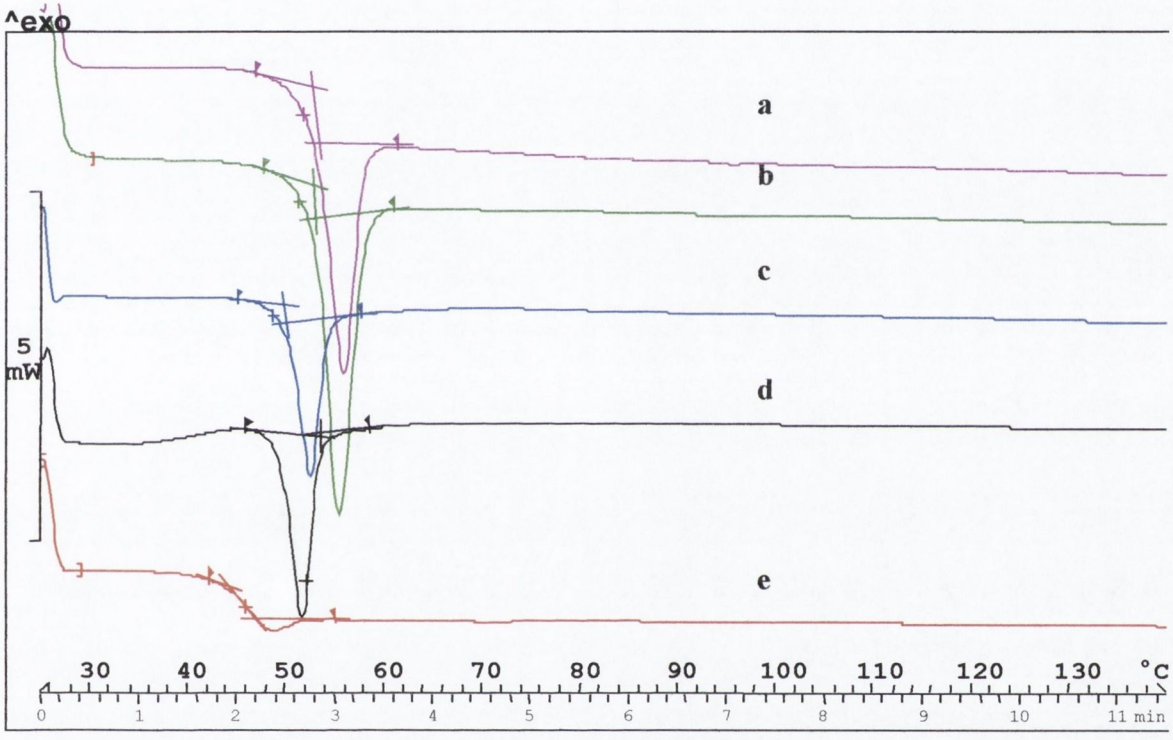


Figure 6- 36 Differential scanning calorimerty of (a) RG 752 polymer powder, (b) empty microspheres and microspheres loaded with (c) 2.4, (d) 4.8 and (e) 11.8 μg DNA/mg PLGA.

DSC scans showed endotherms with glass transition values of 48.71°C, 52.73°C and 45.87°C for microspheres loaded with 2.4, 4.8 and 11.8 μg DNA/mg PLGA. Glass transition temperatures for polymer powder and empty microspheres were 51.73°C and 51.43°C respectively. As can be seen there is no significant difference between the thermal characteristics of polymer powder and empty microspheres and DNA-loaded microspheres. This would indicate that the DNA has been encapsulated in the microsphere matrix and does not affect the thermal properties of the polymer.

6.4. 4 DNA loading

Table 6-12 illustrates the influence of theoretical loading on actual loading.

Theoretical loading μg plasmid DNA/mg PLGA	**Theoretical DNA load μg DNA/mg microsphere**	**Actual DNA load μg DNA/mg microsphere**	**Encapsulation efficiency**
2.4	2.86	0.817 ± 0.0492	28.513 %
4.7	7.56	0.682 ± 0.0562	9.461 %
11.8	17.69	1.674 ± 0.0395	9.021 %

Table 6-12 Effect of DNA concentration on loading

(samples represent the mean of three determinations)

As can be seen from the values shown, there appears to be higher loading with 11.8 μg plasmid DNA/mg PLGA concentration of DNA. However, microspheres formulated with 4.7 μg plasmid DNA/mg PLGA concentration show lower loading values when compared with 2.4 μg plasmid DNA/mg PLGA. Encapsulation efficiency indicates that although there is a higher concentration of DNA encapsulated in batches with 11.8 μg plasmid DNA/mg PLGA the efficiency of the incorporation is less than that for theoretical loadings of 2.4 μg plasmid DNA/mg PLGA and 4.7 μg plasmid DNA/mg PLGA. Pavanetto et al. (1992) reported that encapsulation efficiencies did not correlate with theoretical drug loading. They studied the effect of increasing the theoretical loading from 4.7% to 22.6% and observed that the encapsulation efficiency fell from 20% to 5.5%. They observed that frequently the lowest drug loadings corresponded to the highest level of encapsulation. This observation mirrors the results found in the current study where the lowest theoretical loading resulted in the highest encapsulation efficiency.

6.4. 5 DNA release

Figure 6-37 illustrates the effect of loading on DNA release over one hundred days.

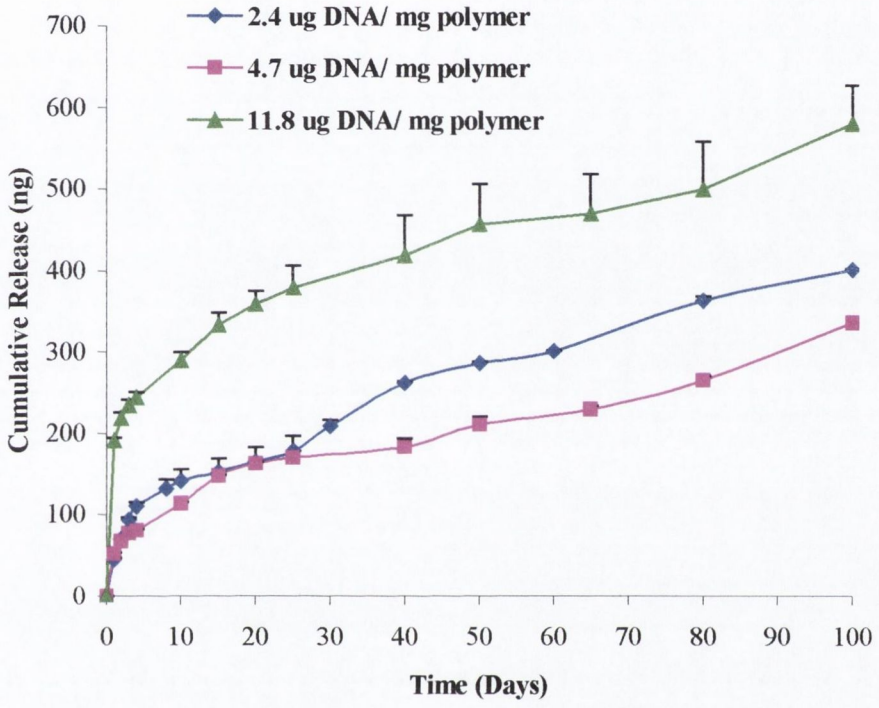


Figure 6- 37 Effect of loading on release of DNA

(samples represent the mean of three determinations)

As can be seen from Figure 6-37 there appears to be a higher amount of DNA released from microspheres formed with theoretical loadings of 2.4 μg DNA/mg polymer when compared with 4.7 μg DNA/mg polymer loading in the early sampling time points. Statistical analysis however, indicated that at a 95% confidence interval, there was no statistical difference between release from 2.4 μg DNA/mg polymer loaded microspheres and 4.7 μg DNA/mg polymer loaded microspheres ($p > 0.05$). When compared with the release from 2.4 μg DNA/mg polymer and 4.7 μg DNA/mg polymer loaded microspheres, release from 11.8 μg DNA/mg polymer loaded microspheres was shown to be statistically significant ($p < 0.05$). All batches followed a biphasic release pattern with an initial burst release followed by a longer slower phase of release. Due to the hydrophobic nature of the polymer and its long half-life it is presumed that release from these microspheres would have continued until the depletion of the microspheres had the study not been terminated.

Statistical analysis indicated that there was no significant difference between the release over the first ten days between particles loaded with 2.4 μg DNA/mg polymer DNA and those loaded with 4.7 μg DNA/mg polymer ($p > 0.05$), however there was between these microspheres and those formed with 11.8 μg DNA/mg polymer DNA ($p < 0.05$).

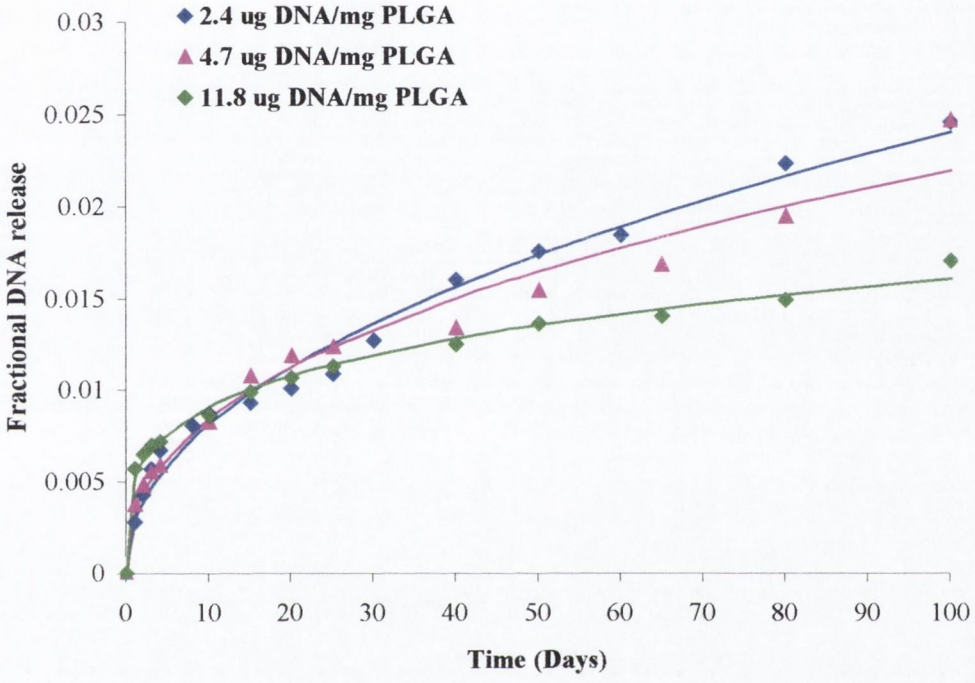


Figure 6- 38 Fractional release of DNA fitted to Equation 1-14.

Figure 6-38 illustrates the fractional release of DNA fitted to Equation 1-14. As can be seen the fraction of DNA released from microspheres loaded with 4.7 μg DNA/mg polymer is highest over the one hundred days. A higher percentage burst release was observed for the higher loaded microspheres. This could be attributed to the higher concentration of DNA at the surface of the microspheres. Following this burst release there is a slower rate of release from microspheres loaded with 11.8 μg DNA/mg polymer when compared to the other two batches. All batches followed a typical biphasic release profile as seen by a burst release followed by a slower rate of release.

Parameters obtained for DNA release from microspheres loaded with different theoretical loading values are listed in Table 6-13.

Parameter	2.4 μg DNA/mg PLGA	4.7μg DNA/mg PLGA	11.8μg DNA/mg PLGA
K	0.0028	0.0033	0.0058
n	0.467	0.413	0.245
CD	0.980	0.970	0.993
MSC	3.990	3.361	4.552

Table 6- 13 Parameters obtained for the fitting of release of DNA from microspheres manufactured with varying theoretical loadings to Equation 1-14

The model selection criteria (MSC) indicates the suitability of the equation fitted to the release data. All batches exhibit good MSC values and coefficient of determination results close to 1 indicating good fits between actual release data and the model. It can be seen that the MSC value is highest for the release from microspheres manufactured with 11.8 μg DNA/mg PLGA. The values of n for release from microspheres manufactured with 2.4 and 4.7 μg DNA/mg PLGA indicate that Fickian diffusion might be a possible release mechanism from these microspheres.

6.4.6 Degradation

Figures 6-39 (a) to (c) illustrate the outer morphology of microspheres loaded with varying concentrations of DNA following incubation in the release medium for one hundred days.

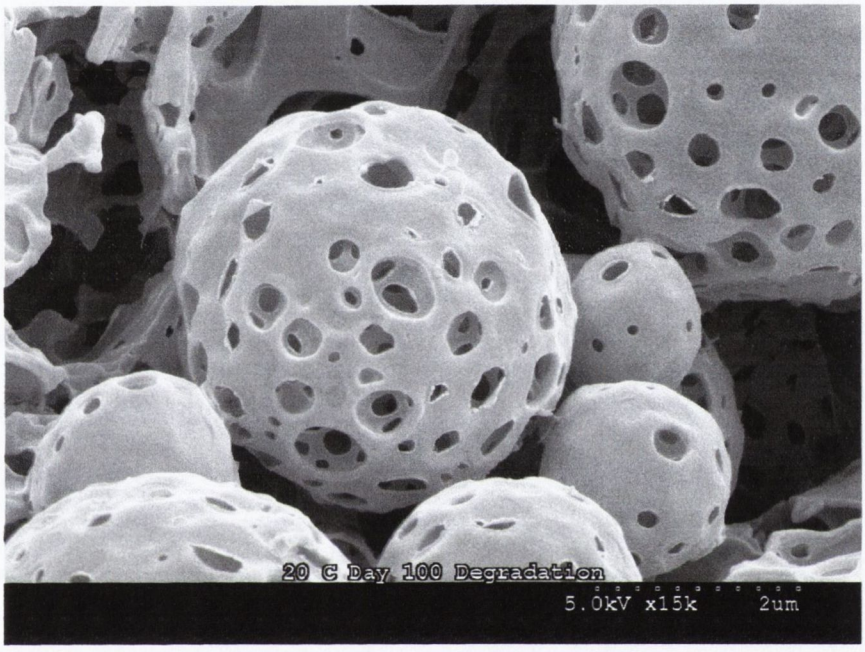


Figure 6- 39 a Day 100 degradation 2.4 μg DNA/mg PLGA

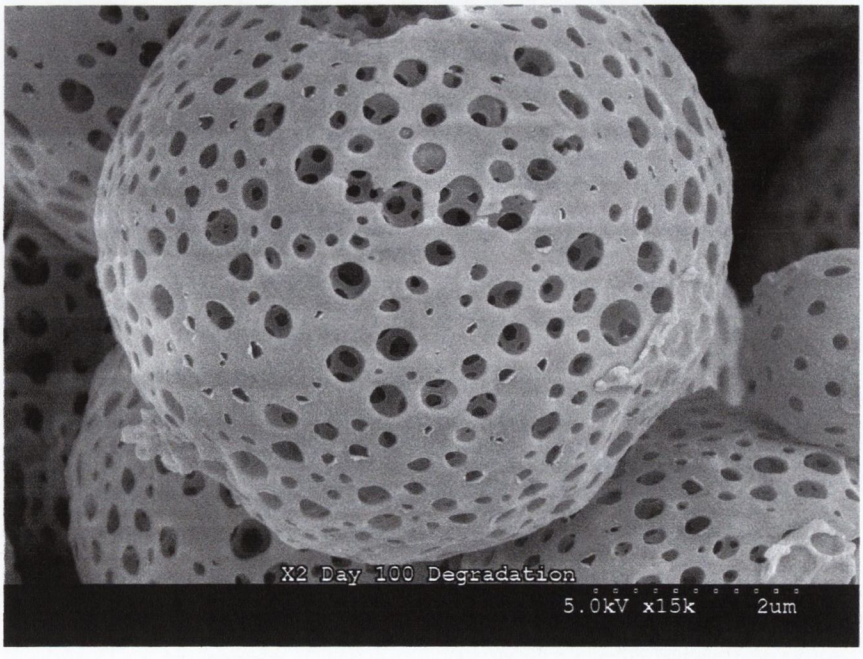


Figure 6- 39 b Day 100 degradation 4.7 μg DNA/mg PLGA

Scanning electron micrographs show that over the one hundred days of the release trial some of the microspheres were porous irrespective of loading.

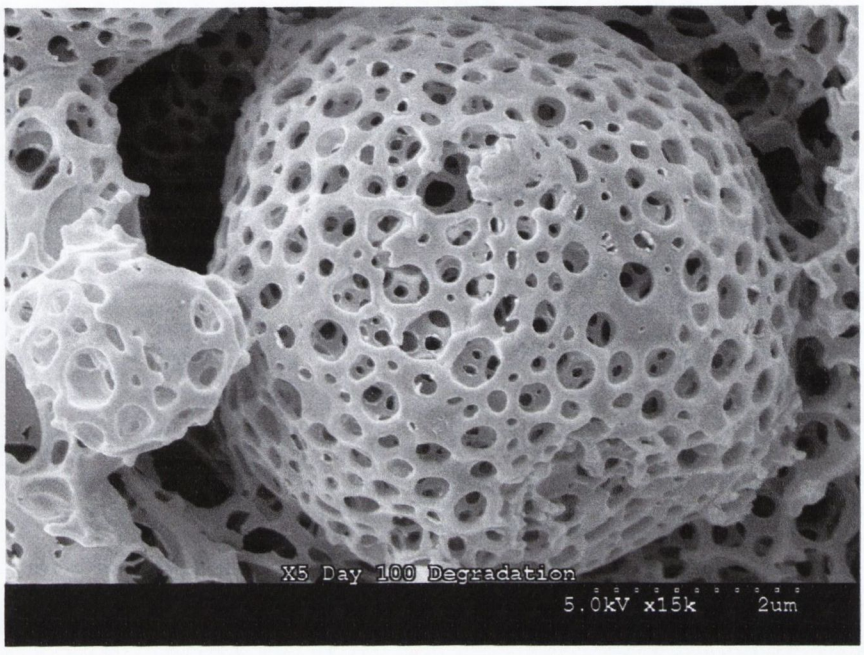


Figure 6- 39 c Day 100 degradation 11.8 μg DNA/mg PLGA

Comparing the three micrographs it appears that the microspheres with the highest theoretical loading have the more extensive internal porous network and a greater occurrence of outer pores.

6.4. 7 Discussion

In this section of work the influence of DNA loading was examined. Microspheres were manufactured with 2.8, 4.7 and 11.8 μg DNA/mg PLGA. These values were chosen because they represented the original loading value used in the previous sections, twice that value and five times the original loading value. In the current section it was observed that increasing the loading from 2.4 to 11.8 μg plasmid DNA/mg PLGA resulted in microspheres with a more wrinkled microsphere surface and an increase in surface pores. Particle sizes as determined by scanning electron microscopy were seen to increase as loading increased. However from particle size analysis via laser diffraction particles at the D[v,0.5] interval was seen to decrease with increasing loading. This might be explained by the encapsulation efficiency results where increasing the loading resulted in a decrease in

encapsulation efficiency. Percentage release profiles of DNA from these microspheres fitted to Equation 1-14 indicated that in the case of microspheres loaded with 2.8 and 4.7 μg plasmid DNA/mg PLGA release might be attributed to Fickian diffusion however results from microspheres loaded with 11.8μg plasmid DNA/mg PLGA were not informative.

In conclusion changes in DNA loading were observed to influence particle size, surface morphologies and encapsulation efficiencies. In the next section the processes of spray-drying and solvent evaporation were further investigated.

6. 5 Influence of manufacturing technique

One of the most commonly used techniques in the encapsulation of DNA is solvent evaporation (Jones et al., 1997; Wang et al., 1999; Perez et al., 2001), however other techniques such as heat extrusion (Jong et al., 1997), spray-drying (Walter et al., 2001) and phase inversion nanoencapsulation (Mathiowitz et al., 1997) have also been examined. The integrity and sequence of DNA is fundamental to its use as a viable vaccine. Middaugh et al. (1998) categorises the issue of DNA stability into physical stability and chemical stability. Changes in the physical structure of DNA i.e. from a supercoiled to a linear or open circular form are undesirable and as such must be avoided in the formulation of such systems. Common chemical processes such as oxidation and deamination could also lead to breakages in the phosphodiester backbone of the plasmid converting supercoiled DNA into open circular DNA. One of the major degradation pathways for DNA in vivo is the two-step process of depurination and β-elimination, leading to the cleavage of the phosphodiester backbone.

Methods that are used to protect DNA from the chemical and physical processes which could cause denaturation, include encapsulation in a polymer matrix. However many of these methods are potentially damaging to the integrity of the plasmid DNA. These include high-speed homogenisation, as is the case when using an Ultra Turrax homogeniser or the high temperatures employed during spray-drying. Microspheres were formulated following homogenisation with a Silverson mixer and were spray-dried and were compared with those manufactured following homogenisation with a Silverson mixer and solvent evaporation. Microspheres were also manufactured following homogenisation with an Ultra Turrax (T25) with 4.8 and 11.8 μg DNA/mg PLGA theoretical loadings.

6.5. 1 Influence of spray-drying on microsphere morphology

Figures 6-40 and 6-41 illustrate the influence of spray-drying on microsphere morphology.

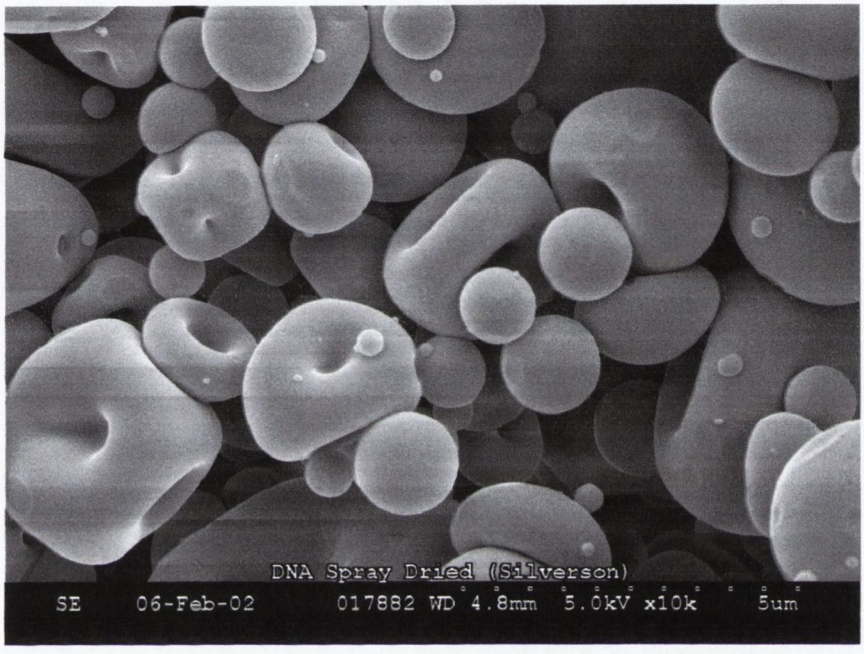


Figure 6-40 Typical scanning electron micrograph of DNA loaded spray-dried microspheres

Figure 6-40 shows typical microspheres formulated following spray-drying. These micrographs illustrate microspheres with a variety of shapes and sizes including doughnut shaped microspheres which are similar in morphology to spray-dried protein loaded microspheres as illustrated in Chapter 5. Microspheres formed following spray-drying are usually spherical but have been reported to be hollow (Jain et al., 2000). Broadhead et al. (1992) also reported that spray-dried microspheres were typically produced with hollow interiors. Hsu et al. (1996) presented a theory, which is based on the formation of a skin at high temperatures on the outer surface of the spray particles. They suggested that when the inner water phase is evaporated off the skin is destroyed and the outer surface collapses. The doughnut shape of microspheres observed here could be attributed to the collapse of the external microsphere wall at high temperatures.

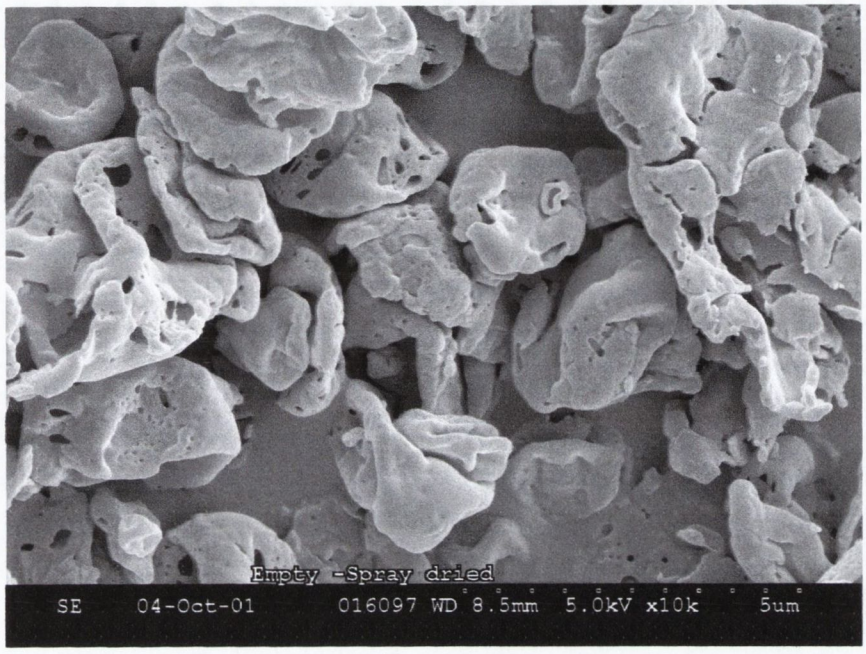


Figure 6-41 Typical scanning electron micrograph of empty spray-dried microspheres

Figure 6-41 shows typical empty microspheres formulated following spray-drying. Interestingly while empty microspheres manufactured following solvent evaporation were spherical and similar in shape and size to DNA loaded microspheres (Figure 6-42 and 6-43) those formulated following spray-drying are very different in shape to their loaded equivalents and do not have smooth outer surfaces (Figure 6-41). These were very similar in appearance to spray-dried microspheres reported by Ståhl et al. (2002).

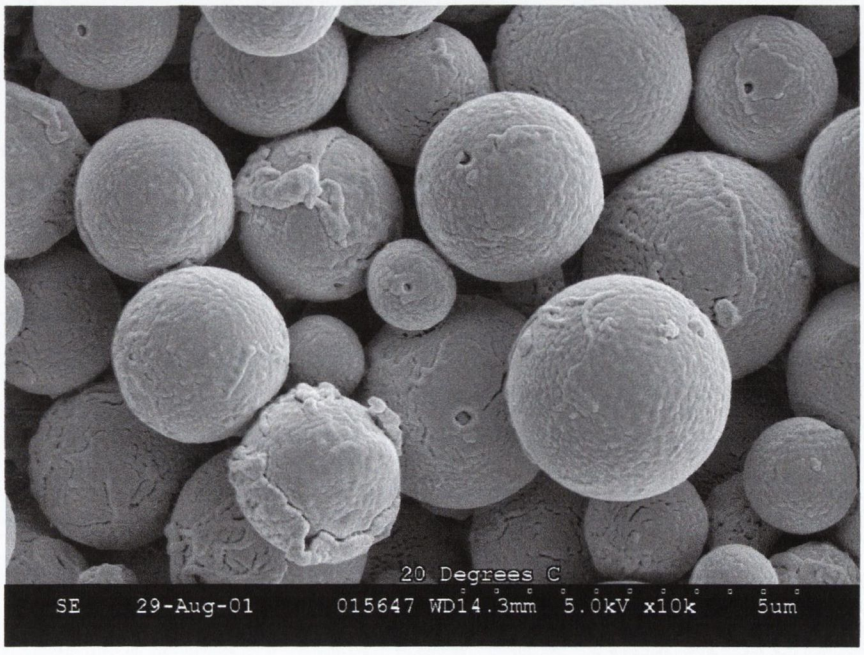


Figure 6-42 Scanning electron micrographs of DNA-loaded solvent-evaporated microspheres

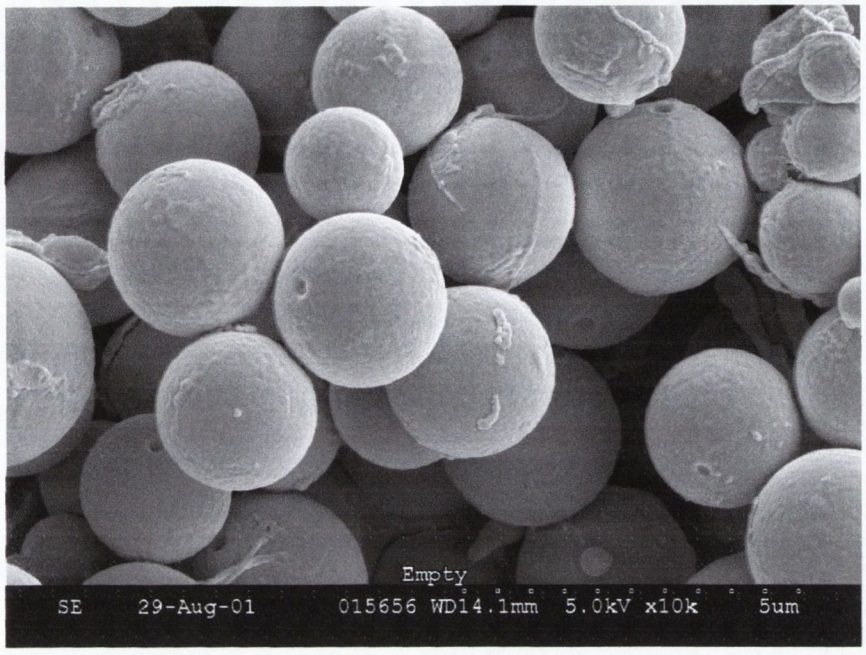


Figure 6- 43 Scanning electron micrographs of typical empty solvent-evaporated microspheres

6.5. 2 Influence of spray-drying on particle size

Table 6-14 illustrates the influence of manufacturing technique on particle size. Microspheres were manufactured following solvent evaporation and spray-drying.

Manufacture technique		D [v, 0.5] μm	S.D.	D [v, 0.9] μm	S.D.	Span
Spray-drying	Silverson (L4R)	2.92	± 0.00	5.96	± 0.03	1.25
Solvent-evaporation	Silverson (L4R)	4.48	± 0.51	5.77	± 0.65	2.52

Table 6- 14 Particle size data for spray-dried and solvent-evaporated microspheres
(samples represent the mean of three determinations)

Table 6-14 illustrates the particle size data for particles manufactured with a Silverson mixer (L4R) and produced following solvent evaporation or spray-drying. Statistical analysis showed that particle sizes were statistically different at both the D [v, 0.5] and the D [v, 0.9] interval ($p < 0.05$). Microspheres produced following solvent evaporation were larger than those prepared via spray-drying at the D [v, 0.5] interval but were smaller at the

D [v, 0.9] interval. This observation has been reported by several other researchers including Pavanetto et al. (1992) who studied the effect on particle size of solvent evaporation, solvent extraction and spray-drying. They reported that particles manufactured following solvent evaporation exhibited the largest particle sizes at all intervals examined (with 68% of particles falling below 5 μm). Ståhl et al. (2002) also examined the effect of spray-drying on particle size and reported mean particle sizes in the range of 1.6 to 4.4 μm. In Chapter 5 microencapsulation of proteins via spray-drying was examined.

6.5. 3 Influence of spray-drying on DNA loading

Table 6-15 illustrates the effect of manufacture technique on DNA loading.

Manufacture technique	**Theoretical DNA load μg DNA/mg microsphere**	**Actual DNA load μg DNA/mg microsphere**	**Encapsulation efficiency**
Solvent-evaporated	2.864	0.817 ± 0.051	28.513 %
Spray-dried	3.152	2.879± 0.086	91.111 %

Table 6- 15 Effect of manufacture technique on DNA loading

(samples represent the mean of three determinations)

Analysis showed that loading values were statistically different with respect to manufacture technique ($p < 0.05$). As can be seen from Table 6-15 spray-dried microspheres showed higher encapsulation efficiency when compared with solvent-evaporated microspheres with approximately 91% of loaded DNA successfully encapsulated. These results mirrored that reported in Chapter 5 in relation to protein loading. This level of encapsulation was the highest observed for any of the systems studied in the current work. This was in line with Pavanetto et al (1992) who reported higher encapsulation efficiencies for microspheres manufactured following spray-drying when compared with solvent-evaporated microspheres. Walter et al. (2001) also reported levels of encapsulation efficiency of between approximately 20 and 80% following spray-drying of PLGA 50:50 microspheres.

6.5. 4 Differential Scanning Calorimetry

Figure 6-44 illustrates the thermal characteristics of DNA-loaded microspheres manufactured following solvent evaporation and spray-drying.

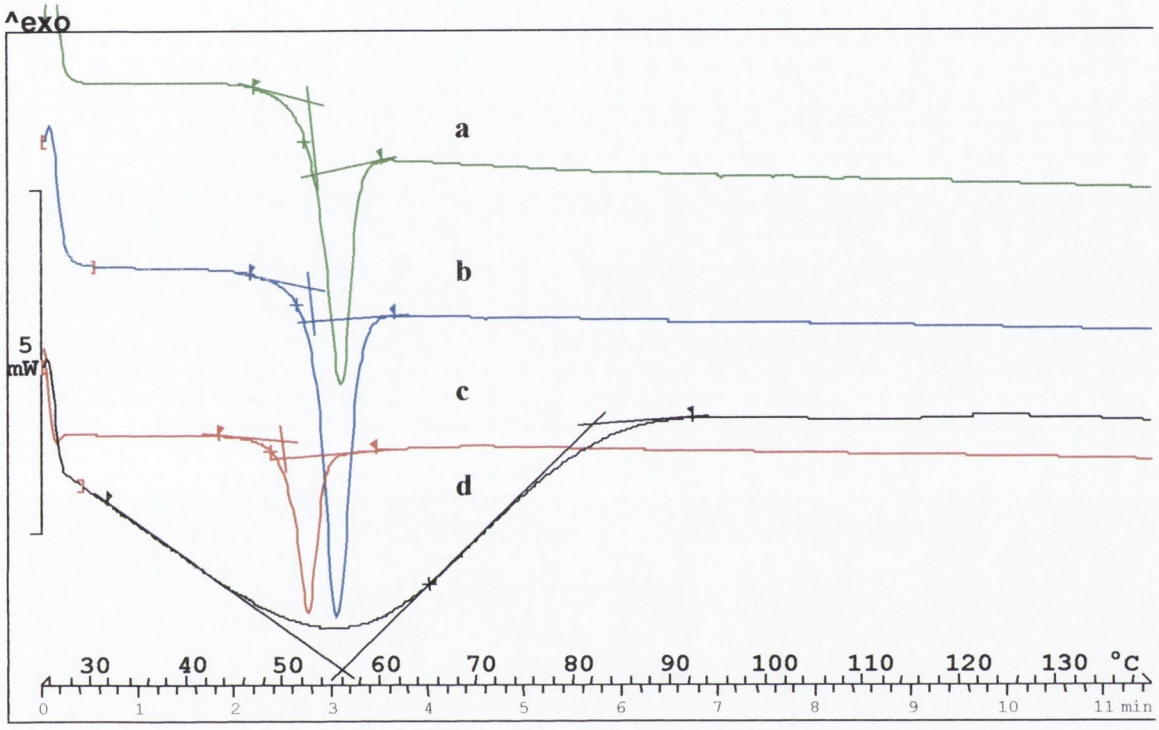


Figure 6- 44 DSC scans of (a) PLGA 75:25, (b) empty microspheres and DNA loaded microspheres manufactured following (c) solvent evaporation and (d) spray-drying

Differential scanning calorimerty show endotherms with glass transition temperatures of 52.68°C and 52.74°C for PLGA 75:25 and empty microspheres respectively. DNA-loaded microspheres manufactured following solvent evaporation exhibited transition temperatures of 49.87°C. In the case of solvent-evaporated microspheres the thermogram is a narrow peak similar to that seen in the case of the polymer and empty microspheres and is similar to the thermal characteristics of DNA loaded microspheres seen in previous sections. It has been attributed to the complete encapsulation of the DNA within the polymer matrix. However in the case of the spray-dried microspheres a broad band is seen with no glass transition temperature. These microspheres exhibited the highest DNA encapsulation efficiency of any system. The unusual thermal traces might be attributed to the incomplete encapsulation of the DNA.

6.5. 5 Influence of spray-drying on DNA release

Figure 6-45 compares the release of DNA from spray-dried microspheres with that from solvent-evaporated microspheres.

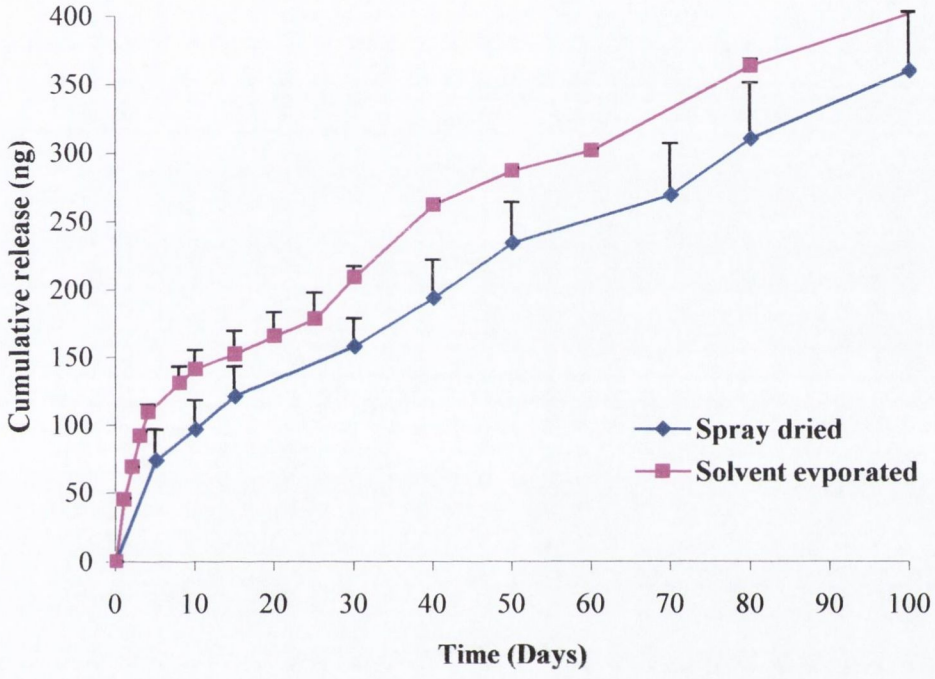


Figure 6-45 Comparison of cumulative release from microspheres manufactured following freeze drying and spray-drying
(samples represent the mean of three determinations)

In terms of actual release from microspheres there was a greater release of DNA from solvent-evaporated particles in comparison to spray-dried particles. Both systems exhibited a sustained release of DNA over the one–hundred days of the trial. Statistical analysis indicated that release from spray-dried and solvent-evaporated systems was not statistically different ($p > 0.05$). The fractional release of DNA from these systems was fitted to Equation 1-14 and the results illustrated in Figure 6-46.

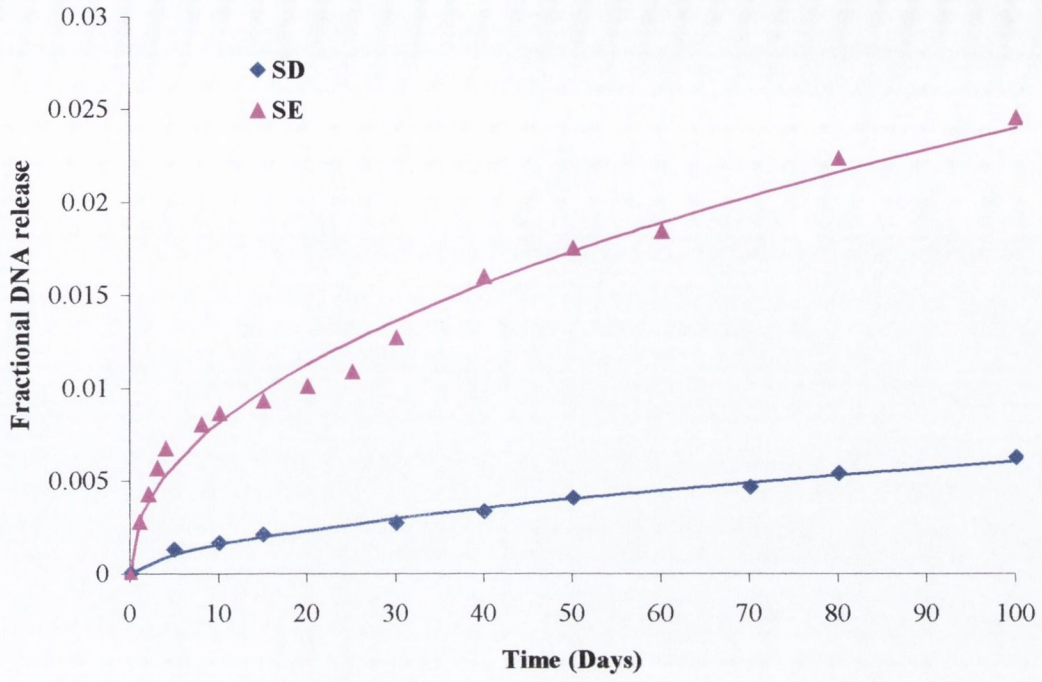


Figure 6- 46 Cumulative percentage release of DNA from spray-dried (SD) and solvent-evaporated (SE) microspheres fitted to Equation 1-14

As can be seen from Figure 6-46 microspheres manufactured following solvent evaporation exhibits a burst release followed by a second sustained release. However there does not appear to be any burst release from spray-dried microspheres. Parameters generated from the fitting of actual to generated results are tabulated in Table 6-16.

Parameter	Spray dry	Solvent evaporation
K	0.0004	0.0028
n	0.587	0.467
CD	0.99	0.980
MSC	4.37	3.990

Table 6- 16 Parameters obtained for the fitting of release of DNA from microspheres manufactured with varying theoretical loadings to Equation 1-15

The model selection criteria for both systems indicated that there was a good fit between release data and the model when the data was fitted to Equation 1-14. The coefficient of determination values are close to 1 which indicates good correlation between data and the model. The n values generated from the two sets of data suggested that Fickian or anomalous diffusion might be the possible release mechanisms in both cases.

6.5.6 Influence of spray-drying on DNA integrity

The integrity of DNA encapsulated in PLGA microspheres manufactured following spray-drying and solvent evaporation was examined. Prior to the extraction of DNA encapsulated in PLGA microspheres a spiking experiment was carried out to determine the effect of the extraction procedure on plasmid DNA.

Incubation time (minutes)	**Experimental [DNA] μg**	**% recovery**
Control	2.61μg	100
60	2.25μg	86.21
90	2.39μg	91.57
120	2.11μg	90.84

Table 6-17 Recovery of DNA following spiking experiment

As can be seen from Table 6-17 recovery of DNA following the spiking experiment indicated that almost 100% of the DNA was recovered following 60, 90 and 120-minutes incubation followed by chloroform extraction.

Figure 6-47 illustrates the agarose gel of the DNA extracted following spiking experiments. As can be seen from this figure the DNA extracted retains the isoform of the control DNA indicating that the extraction procedure does not affect DNA isoform.

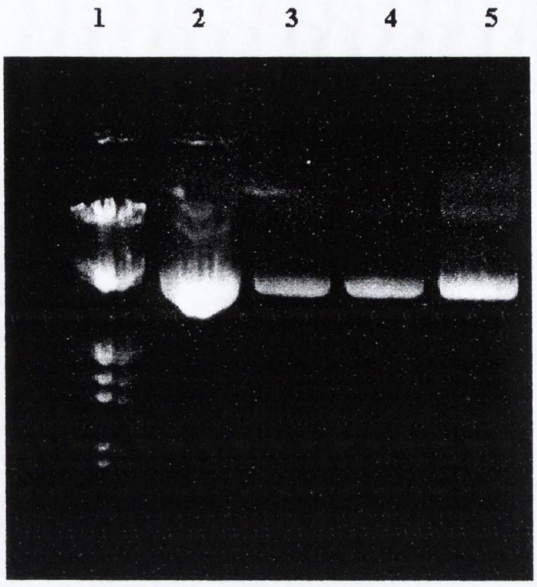


Figure 6- 47 Agarose gel electrophoresis of DNA extracted from spiking experiment

Lane 1: Molecular weight markers (M.W. range: 23,130 base pairs to 125 base pairs)
Lane 2 Control DNA
Lane 3: DNA extracted from microspheres rotated for 60 minutes
Lane 4: DNA extracted from microspheres rotated for 90 minutes
Lane 5: DNA extracted from microspheres rotated for 120 minutes

Samples of DNA-loaded microspheres produced following solvent evaporation and spray-drying were analysed. The results from agarose gel electrophoresis of the two samples analysed by this method is shown in Figure 6-44. The two samples analysed were chosen because of the possible effect of the manufacturing procedure on the integrity of the encapsulated DNA.

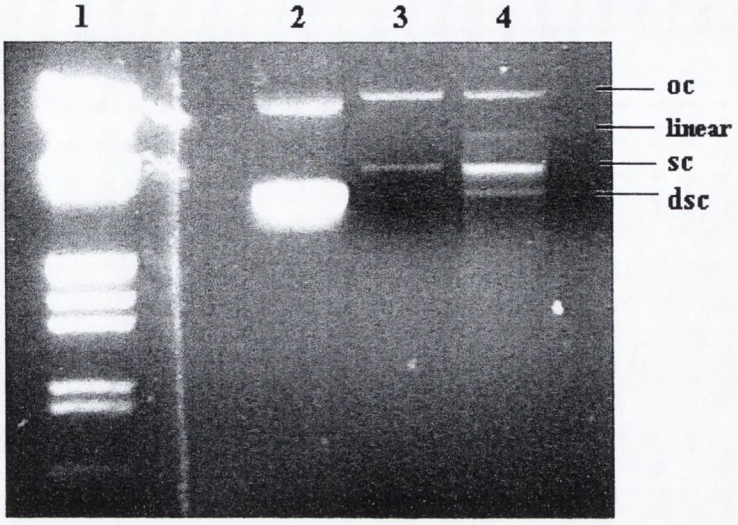


Figure 6-48 Agarose gel electrophoresis of extracted DNA where oc – open circular, sc – supercoiled and dsc- denatured supercoiled

Lane 1 Molecular weight markers (M.W. range: 23,130 base pairs to 125 base pairs)
Lane 2 Control DNA
Lane 3 DNA extracted from microspheres produced following solvent evaporation
Lane 4 DNA extracted from microspheres produced following spray-drying

From Figure 6-48 it can be seen that both lanes of extracted plasmid DNA contain both open circular (oc) and supercoiled (sc) DNA. Plasmid DNA extracted from the microspheres maintained most of its supercoiled form as determined by agarose gel electrophoresis and when compared with control plasmid DNA (lane 2). However DNA extracted from spray-dried microspheres also contained open circular (oc) and supercoiled (dsc) denatured products which is most likely due the spray-drying technique.

The migration patterns of the unencapsulated control and DNA extracted from solvent-evaporated microspheres appeared to be identical indicating that this encapsulation procedure did not degrade or linearise DNA. Unencapsulated DNA was primarily in supercoiled form (as indicated by the higher intensity of the supercoiled band when compared with the open circular band) while DNA extracted from solvent-evaporated microspheres show nearly equal intensities between the supercoiled and open circular band.

However extracted DNA from spray-dried microspheres shows some degree of conversion. This can be seen from the smearing that occurred in the gel during the run, which would indicate the presence of denatured DNA isoforms. Denatured DNA extracted from spray-dried microspheres could be as a result of the high inlet temperatures used during spray-drying (80°C). DNA extracted from Ultra Turrax (T25) homogenised microspheres was also examined by gel electrophoresis however no band was detected possibly indicating that the DNA was too badly sheared to allow for the detection of bands from this sample.

6.5. 7 Influence of high-speed homogenisation on microsphere morphology

Figure 6-49 and 6-50 show the effect of high-speed homogenisation on the morphology of microspheres loaded with 4.7 μg plasmid DNA/mg PLGA when compared with homogenisation with a Silverson mixer (L4R).

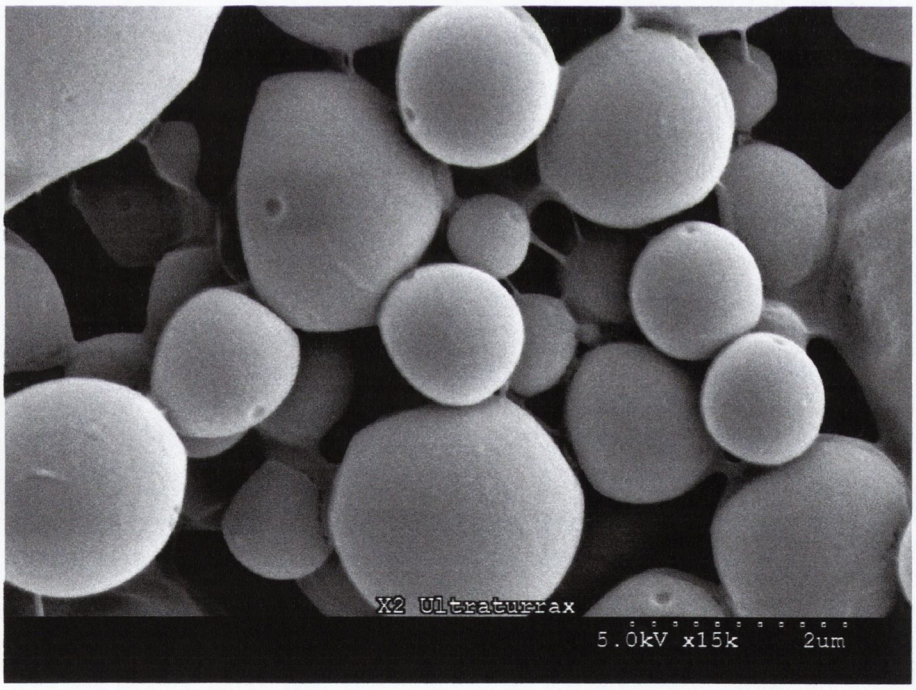


Figure 6-49 4.7 μg plasmid DNA/mg PLGA– high-speed homogenisation (Ultra Turrax T25)

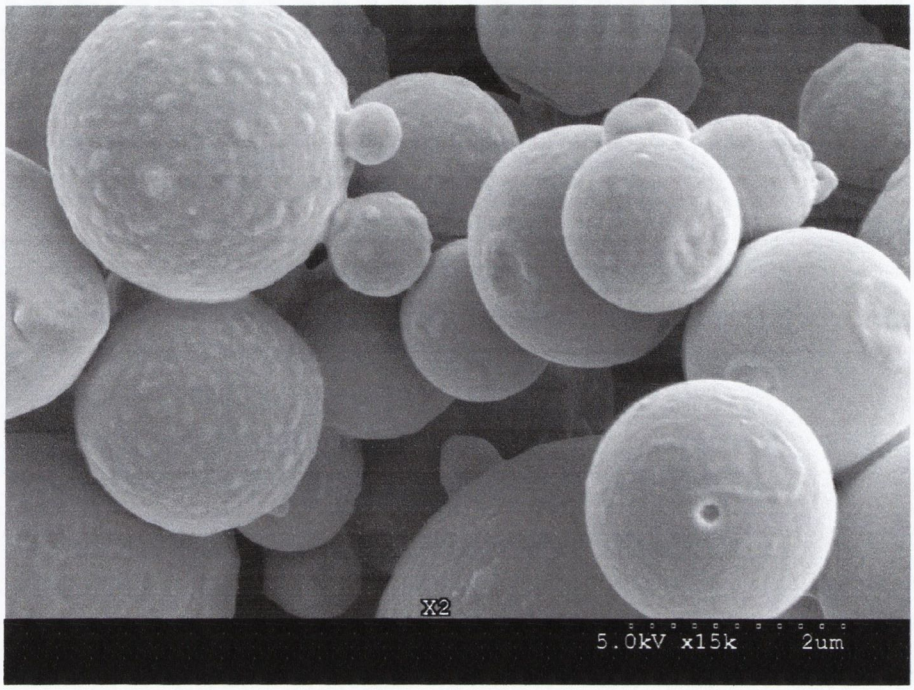


Figure 6-50 4.7 μg plasmid DNA/mg PLGA– Silverson (L4R)

Figures 6-51 and 6-52 illustrate the effect of manufacture technique on the morphology of microspheres loaded with 11.8 μg plasmid DNA/mg PLGA plasmid DNA.

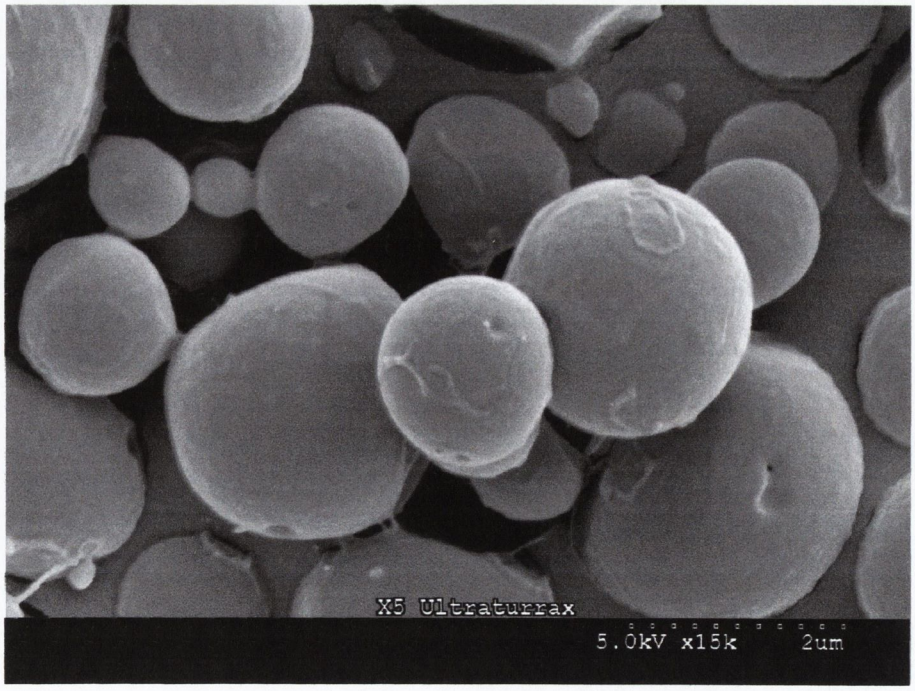


Figure 6-51 11.8 μg plasmid DNA/mg PLGA – high-speed homogenisation (Ultra Turrax T25)

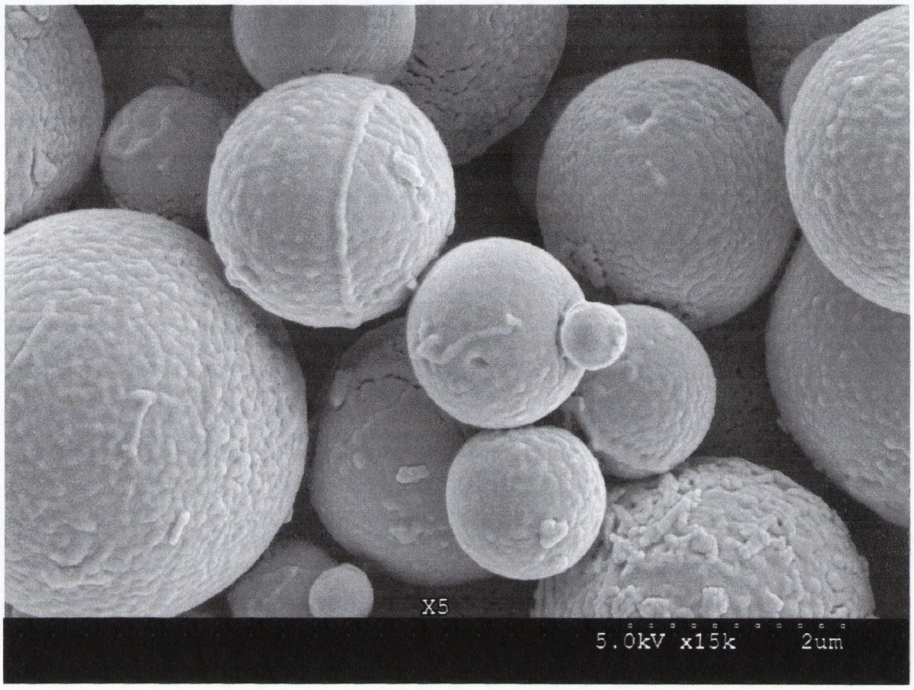


Figure 6-52 11.8 μg plasmid DNA/mg PLGA– Silverson mixer (L4R)

As can be seen from Figures 6-49, 6-50, 6-51 and 6-52 both manufacturing techniques produce spherical microspheres. However, microspheres formulated following Silverson homogenisation show a more wrinkled outer surface when compared with those formed following homogenisation with an Ultra Turrax (T25). High-speed homogenisation causes droplets to be formed faster than those formed following slower homogenisation speeds and thus would reduce precipitation of polymer onto the outer surface of the microsphere (McGee at al., 1997). Mass transfer of the solvent from the microsphere matrix would also occur at a faster rate again increasing the speed of polymeric hardening and microsphere formation (Yang et al., 2000 a).

6.5. 8 Influence of high-speed homogenisation on particle size

Table 6-18 illustrates the particle size of solvent-evaporated microspheres manufactured following homogenisation with a Silverson mixer (L4R) and Ultra Turrax (T25). Microspheres were manufactured with 0.47% and 1.18% theoretical loading.

Manufacture technique		Theoretical loading	D [ν,0.5] μm	S.D.	D [ν, 0.9] μm	S.D.	Span
Solvent evaporation	Silverson (L4R)	0.47%	4.48	± 0.51	5.77	± 0.65	2.58
		1.18%	3.10	± 0.02	6.24	± 0.01	2.56
	Ultra Turrax (T25)	0.47%	4.55	± 0.02	9.16	± 1.10	2.49
		1.18%	7.16	± 0.50	19.44	± 4.70	2.58

Table 6- 18 Particle size data for solvent-evaporated microspheres

(samples represent the mean of three determinations)

As can be seen from Table 6-18 in the case of microspheres manufactured with 0.47% theoretical loading, particle size appears dependent on manufacturing technique. However following statistical analysis these values were deemed not significantly different from each other ($p > 0.05$) at the D [v,0.5] interval but were significantly different at the D [v,0.9] interval. In this case particle size differences may be due to higher loadings in microspheres manufactured with a Silverson (L4R) when compared with values obtained from microspheres manufactured with an Ultra Turrax (T25). In the case of microspheres manufactured at the high loading (11.8 μg plasmid DNA/mg PLGA) microspheres manufactured following homogenisation with a Silverson mixer (L4R) were significantly smaller than those manufactured following Ultra Turrax (T25) homogenisation. Particle sizes as determined by laser diffraction were seen to be larger than those seen during scanning electron microscopy. This might be attributed to aggregation of the particles during laser diffraction particle sizing despite sonication of the sample.

6.5. 9 Influence of manufacturing technique on DNA loading

Table 6-19 illustrates the effect of manufacture technique of DNA loading.

Manufacture technique	**Theoretical loading μg plasmid DNA/mg PLGA**	**Actual DNA load μg DNA/mg microsphere**	**Encapsulation efficiency %**
Silverson (L4R)	4.7	0.683 ± 0.062	9.021
Ultra Turrax (T25)	4.7	0.304 ± 0.036	4.427
Silverson (L4R)	11.8	1.678 ± 0.0611	9.462
Ultra Turrax (T25)	11.8	0.4913 ± 0.0437	3.011

Table 6- 19 influence of manufacturing technique on loading

(samples represent the mean of three determinations)

As can be seen from Table 6-19 manufacturing technique appears to have an influence on DNA loading. In the case of microspheres loaded with theoretical levels of 4.7 μg plasmid DNA/mg PLGA microspheres manufactured with the Silverson mixer (L4R) appear to contain twice as much DNA as those manufactured with the Ultra Turrax (T25). In the case of microspheres manufactured with the Silverson (L4R) loaded with 11.8 μg plasmid DNA/mg PLGA approximately three times as much DNA was encapsulated when compared with microspheres manufactured with an Ultra Turrax (T25). This difference could be attributed to the shear speeds associated with the Ultra Turrax (T25), which may shear the DNA leading to lower levels of encapsulated plasmid DNA.

6.5. 10 Influence of high-speed homogenisation on DNA release

Figures 6-53 and 6-54 illustrate the influence of manufacturing technique on the release of DNA.

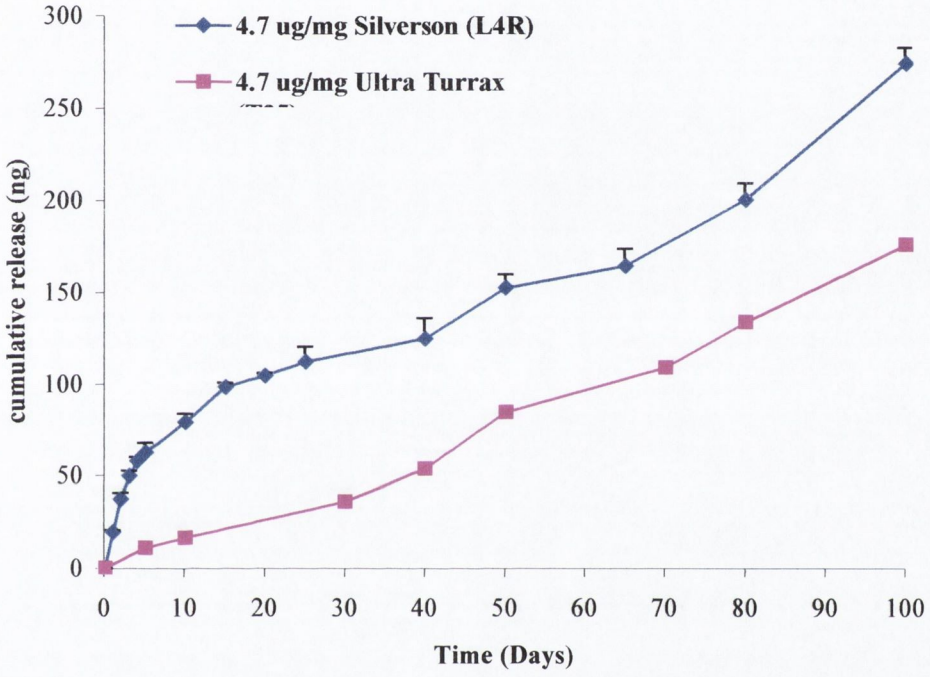


Figure 6- 53 Cumulative release from microspheres formulated with 4.7 μg plasmid DNA/mg PLGA following homogenisation with a Silverson mixer (L4R) and an Ultra Turrax (T25)

(samples represent the mean of three determinations)

Figure 6-53 illustrates the release profiles of DNA from microspheres formed following high speed and Silverson homogenisation and solvent evaporation with 4.7 μg plasmid DNA/mg PLGA. As can be seen from the graph the cumulative release of DNA from microspheres formulated following homogenisation with the Silverson mixer is higher then that from microspheres formulated following high-speed homogenisation.

Fractional release from microspheres with theoretical loadings of 4.7 μg plasmid DNA/mg PLGA was fitted to Equation 1-14 (Figure 6-54).

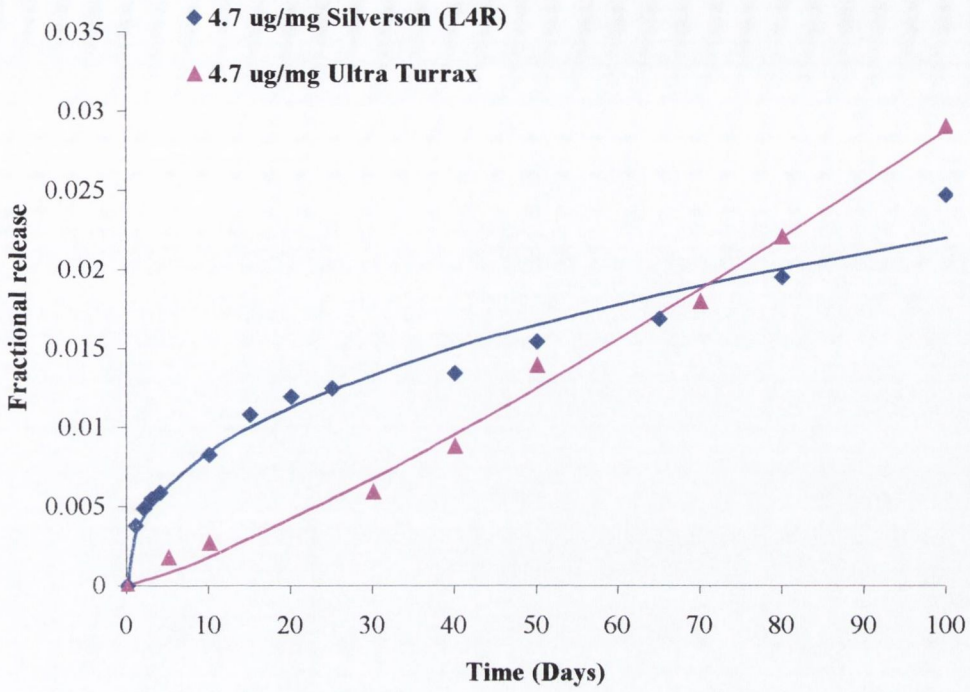


Figure 6- 54 Fractional release of DNA fitted to Equation 1-14

Figure 6-54 illustrates the fitting of the model to the release data points. In the case of microspheres manufactured following homogenisation with a Silverson mixer (L4R) two phases can be distinguished for the release of DNA: the initial burst release and a second release phase. Microspheres manufactured following homogenisation with an Ultra Turrax (T25) exhibited no defined initial burst release. Parameters generated by the model are illustrated in Table 6-20.

Parameter	Silverson mixer (L4R)	Ultra Turrax (T25)
K	0.0033	0.0001
n	0.413	1.196
CD	0.970	0.990
MSC	3.361	4.510

Table 6- 20 Parameters obtained from fitting of release of DNA from microspheres fitted to Equation1-14

As can be seen from Table 6-20 release from both systems correlated well with data generated from the model. Both systems exhibited high MSC values indicating the suitability of the model. The correlation of determination values were both close to 1 further suggesting the suitability of the model for the release from these systems.

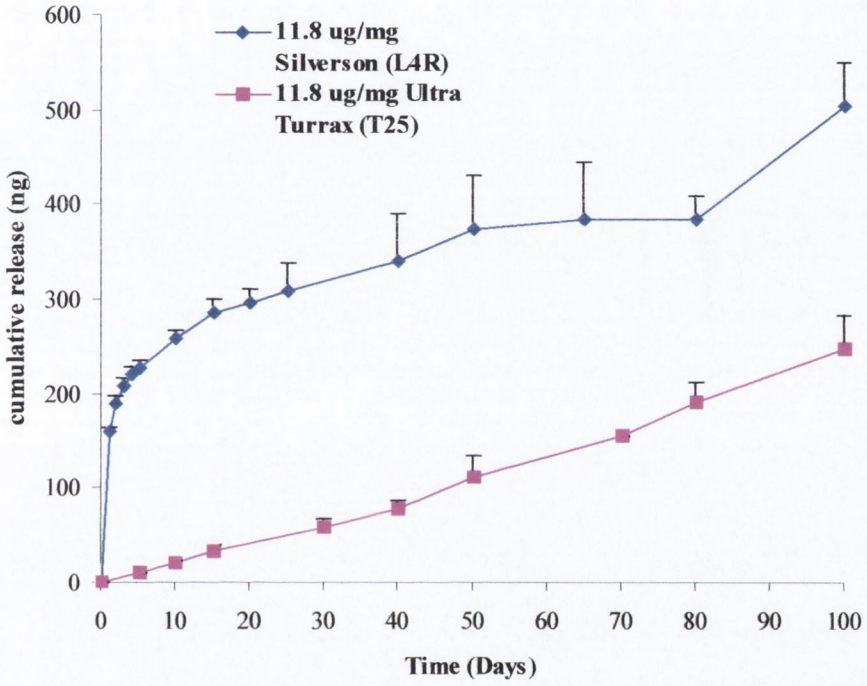


Figure 6-55 Cumulative release from microspheres formulated with 11.8 μg plasmid DNA/mg PLGA following homogenisation with a Silverson mixer (L4R) and an Ultra Turrax (T25)

(samples represent the mean of three determinations)

Figure 6-55 illustrates the cumulative release from microspheres formulated following Silverson homogenisation and high-speed homogenisation. A similar pattern is evident here when compared with the case of microspheres loaded with 4.7 μg plasmid DNA/mg PLGA in the previous figure. There is a much greater extent of release from microspheres formed following Silverson homogenisation when compared with microspheres manufactured following Ultra Turrax homogenisation.

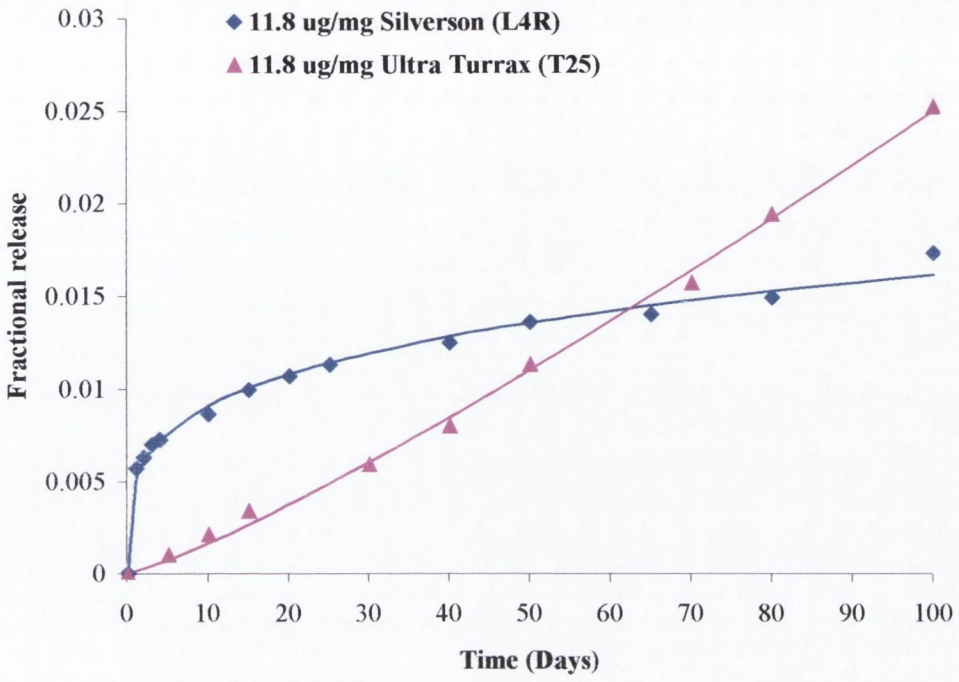


Figure 6- 56 Fractional release of DNA fitted to Equation 1-14

Figure 6-56 illustrates the actual release data fitted to those generated by the model. Both systems fit well to Equation 1-14. Two mechanisms can be seen in the case of microspheres manufactured following homogenisation with a Silverson mixer (L4R). A burst release can be seen which lasts until approximately day 10 of release. Following this is a sustained release. In the case of microspheres manufactured using an Ultra Turrax (T25) no burst release is exhibited and release appears to follow a simple diffusion pattern. Table 6-21 outlines the parameters generated by the model.

Parameter	Silverson mixer (L4R)	Ultra Turrax (T25)
K	0.0058	0.0001
n	0.245	1.175
CD	0.993	0.997
MSC	4.552	5.557

Table 6-21 Parameters generated from fitting Equation 1-14 to release of DNA

Table 6-21 outlines the parameters generated from the release of DNA. As can be seen in the case of both systems the release from microspheres manufactured following homogenisation with a Silverson Mixer (L4R) and an Ultra Turrax (T25) fit well to Equation 1-14. In both cases the model selection criterion (MSC) and the coefficient of determination indicated good suitability between the data and the model.

6.5. 11 Discussion

In this section three manufacturing techniques were examined. These were homogenisation of the emulsion by a Silverson mixer (L4R) or an Ultra Turrax followed by solvent evaporation and microspheres manufactured following homogenisation with a Silverson mixer (L4R) and spray-dried.

Firstly solvent-evaporated microspheres manufactured following homogenisation with a Silverson mixer (L4R) and spray-dried microspheres manufactured following homogenisation with a Silverson mixer (L4R) were examined. It was observed following scanning electron microscopy that both batches exhibited very different morphologies. Those manufactured following solvent evaporation were spherical particles while those manufactured following spray-drying were doughnut shaped with exterior walls which in the case of empty microspheres were seen to collapse inwards. Microspheres produced following solvent evaporation were observed to be larger following particle size analysis via laser diffraction than those prepared via spray-drying at the D [v, 0.5] interval but were smaller at the D [v, 0.9] interval. In relation to encapsulation efficiency spray-dried microspheres showed higher encapsulation efficiencies when compared with solvent-evaporated microspheres with approximately 91% of loaded DNA successfully encapsulated. This level of encapsulation was the highest observed for any of the systems studied in the current work. Parameters generated from fitting release from the two systems to Equation 1-14 suggested that Fickian or anomalous diffusion might be the possible release mechanisms in both cases Gel electrophoresis of the extracted DNA showed that while solvent-evaporated DNA exhibited open circular and supercoiled isoforms spray-dried DNA exhibited open circular, linear, supercoiled and possibly some degree of denatured supercoiling. Open circular and supercoiled forms of DNA have been reported to transfect cells successfully however linear forms do not. This would indicate that solvent

evaporation of DNA microspheres might conserve the original DNA isoform. In this section there appeared to be a compromise between encapsulation efficiency and DNA integrity with higher encapsulation efficiencies associated with spray-drying but better structural integrity associated with solvent evaporation. This might be overcome through the use of lower inlet temperatures than the one used in this study.

Microspheres manufactured following Ultra Turrax (T25) homogenisation showed spherical particles with larger particle sizes than those manufactured following homogenisation with a Silverson mixer (T25). This slightly unusual result, given the speed differences between the two systems, might be attributed to aggregation of particles homogenised with an Ultra Turrax (T25) despite sonication of the sample prior to particle sizing. In relation to release of DNA the amount released loading was higher in both cases following homogenisation with the Silverson mixer (L4R). This might be attributed to the conservation of structural integrity seen following homogenisation with a Silverson mixer (L4R) which appears to be lost during Ultra Turrax homogenisation (T25). DNA release was determined using a PicoGreen double stranded DNA quantitation kit which specifically detects double stranded DNA. If the DNA released from microspheres manufactured following Ultra Turrax homogenisation is damaged by the technique to the extent that it converts to single stranded DNA or fragments it would not have been detectable by this method.

In conclusion it appears that DNA integrity is conserved in microspheres manufactured following homogenisation with a Silverson mixer. The encapsulation efficiency was observed to be highest for microspheres manufactured following spray-drying and lowest for microspheres manufactured following homogenisation with an Ultra Turrax (T25).

Chapter 7

In vitro methods for evaluation of nucleic acid vaccines

7. Introduction

In recent years a number of recombinant proteins, synthetic peptides and nucleic acids have been developed as potential therapeutic agents. The most commonly employed systems for the delivery of such agents, comprises of microparticles developed from the poly(lactide-co-glycolide) polymers. A crucial issue in the development of such systems is the determination of the levels and the structure of the released agent. The available assay methods for conventional drug molecules are not applicable to the quantitation of DNA. Plasmid DNA has the potential to become a valuable therapeutic treatment for diseases such as allergy, cancer and a wide range of infectious diseases (Tighe et al., 1998). Compared to conventional immunisation techniques DNA vaccination has the advantage of providing prolonged expression of antigens and eliciting both humoral and cellular responses (Donnelly et al., 1997).

Plasmid DNA exists in three major isoforms: (1) supercoiled representing the most compact stable isomer, (2) nicked or open circular occurring when there are breaks in the phosphate backbone of a single strand of the double-stranded molecule and (3) linear indicating breaks at a single site in both strands of the circular plasmid. Beyond this, the plasmid can be degraded into smaller fragments by enzymes. The most obvious manifestation of this process is the progressive degradation of plasmid from a supercoiled to nicked then linear isoform and finally into smaller DNA fragments represented by a smear in the agarose gel (Hao et al., 2000).

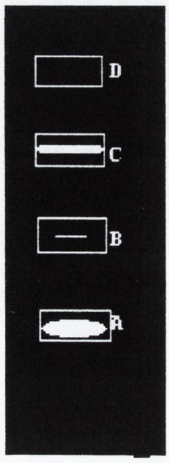


Figure 7- 1: Schematic representation of agarose gel electrophoresis of DNA: (a) supercoiled DNA , (b) linear DNA, (c) open circular DNA, (d) background (adapted from Ando et al. (1998))

The ultimate aim of a nucleic acid vaccine is the stimulation of a specific immune response through the translation of a specific protein. Although plasmid DNA is quite stable in vitro, it is subjected to degradation by nucleases once injected in vivo (Kawabata et al., 1995). Encapsulation of plasmid DNA in PLGA microspheres offers a means of protection against degradation and promotes the controlled release of the DNA. The most common technique used in the encapsulation of DNA is the double-emulsion solvent evaporation technique used in this study. However the process involves two rounds of homogenisation during which the shear forces can induce plasmid nicking. The resultant microspheres are then solvent-evaporated or spray-dried which can also denature the DNA (Hao et al., 2000). DNA is also sensitive to the organic solvents such as dichloromethane, which are commonly used in the formation of the double emulsion.

Microencapsulation is a popular technique in the development of controlled release systems especially in the area of DNA encapsulation. It is of interest to analyse and visualise the deposition of the DNA within the microsphere. Commonly used visualisation techniques for microparticles include conventional light microscopy and scanning electron microscopy, which was used in Chapter 5 and 6 to examine the exterior morphology of the microspheres. However light microscopy is always impeded by the scattered or emitted light from structures outside the focal plane and SEM does not allow the visualisation of inner microsphere structure without possible damage to the encapsulated phase (Lamprecht et al., 2000). It is therefore impossible to visualise the deposition of an encapsulated agent via these routes. Confocal laser scanning microscopy (CLSM) is a non-destructive method of visualising inner microsphere structures while fading out the light from out-of-focus structures. Through the use of different fluorescence labels and CLSM, marked compounds can be identified unambiguously. CLSM has been used by other researchers including Lamprecht et al. (2000) who examined polymer distribution in microcapsules by labelling the polymer used with a fluorescent marker. Confocal microscopy has also been used in studying the efficiency of cationic solid-lipid nanoparticles to bind and transfect DNA (Olbrich et al., 2001). In the current study plasmid DNA was intercalated with ethidium bromide and encapsulated in PLGA 75:25 microspheres. These were then examined using CLSM and the distribution of DNA within the microspheres studied.

The stability and potency of DNA encapsulated products must be assessed by reliable assay methods if they are to become drug candidates (Hao et al., 2000). Such methods

include the assessment of DNA isoform (i.e. supercoiled, nicked, linear) through agarose gel electrophoresis, which has been examined in Chapter 6. Transformation of bacterial cells such as E. coli has been shown to be a reasonable method for qualitative assessment of DNA since linear DNA does not confer antibiotic resistance to transformed cells, which would allow them to grow on antibiotic agar plates. Another assay method routinely used includes the assessment of the encoded gene product through transfection of eukaryotic cells with the encapsulated plasmid and transcription into the reporter protein, which in this case was luciferase enzyme.

Since the ultimate target of these microspheres are immune system cells such as macrophages uptake by these cells in an *in vitro* model was examined. The immune system cells used are macrophage cells, which are mature mononuclear phagocyte cells. When a foreign substance first comes in contact with the human body an inflammatory response is initiated by the host immune system. This is mediated by the mononuclear phagocyte system (MPS) in which macrophages are the most important cell (Tabata and Ikada, 1990). Mononuclear phagocytes are all derived from bone marrow precursors, which first circulate as monocytes before entering tissues under physiological or inflammatory conditions, where they mature into macrophages.

Biodegradable PLGA microspheres have been shown to be effective in the delivery of proteins and nucleic acids and have been suggested to be ideal carrier systems for phagocytotic immune system cells such as macrophage cells (MΦ) (Walter et al., 2001). Macrophage cells play a crucial role in host defence against many infectious agents including bacteria and viruses. They are attracted to a site of infection by a number of different substances including bacterial endotoxins and cellular components where they may phagocytose and kill infectious agents by a variety of mechanisms (Chapter 2). For phagocytosis to occur particles are connected by the pseudopods of macrophage cells and are then engulfed into the macrophage cytoplasm. The ability to develop macrophage targeting drug-loaded microspheres is an area of major interest for many pharmaceutical companies (Ahsan et al., 2002).

DNA and protein-loaded microspheres have been shown to effectively transport DNA into the body inducing antibody responses (McKeever et al., 2002, Challacombe et al., 1997). The effect of microparticulate size on uptake into macrophage cells has been well

documented and is explained in detail in Chapter 2 and hydrophobic particles have been shown to be more susceptible to phagocytosis then hydrophilic particles. However further information concerning the interaction and targeting of these microspheres to specific immune system cells is still lacking.

In this chapter we have evaluated the distribution, uptake and efficacy of encapsulated DNA and attempt to illustrate how such systems can provide predictive reproducible information to assist formulators in the characterisation of such vaccine systems. Confocal laser microscopy was used to examine the distribution of encapsulated ethidium bromide labelled DNA. The biological activity of samples of DNA extracted from microspheres manufactured following the three main manufacturing techniques, solvent-evaporated microspheres manufactured following homogenisation with a Silverson mixer (L4R) and an Ultra Turrax (T25) and spray-dried microspheres were assessed following cell transfection experiments and bacterial cell transformation. The ability of macrophage cells to take up protein and DNA-loaded microspheres with respect to their surface charge, polymer composition, encapsulated product and microsphere size was also studied.

7. 1 Confocal microscopy

Confocal imaging was used as a tool to determine the distribution of the encapsulated DNA with RG 752 microspheres. The DNA used was intercalated with ethidium bromide prior to encapsulation. Ethidium bromide is a DNA intercalator which has been reported to be relatively unaffected by differences in the base composition of DNA.

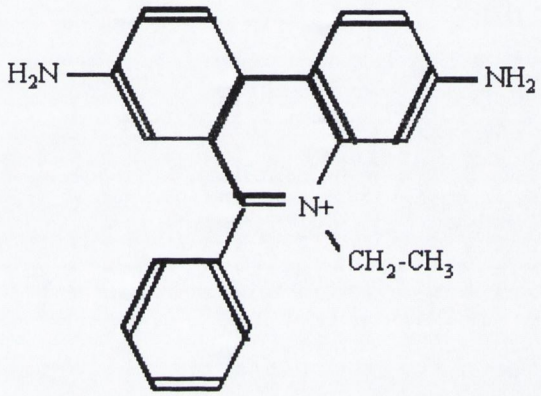


Figure 7- 2 Structure of ethidium bromide

Ethidium bromide intercalates between base pairs in the supercoiled DNA and generates positive superhelical turns in closed DNA molecules such as plasmid DNA. In a natural closed DNA that is negatively supercoiled the intercalation of ethidium bromide first removes the negative supercoils and then introduces positive ones. In open circular DNA the DNA can rotate freely in response to the intercalation thus relieving the tension (Lewin, 1994). Excitation of bound ethidium bromide occurs at 518 nm and is 20 to 30-fold higher then non-bound ethidium bromide. The emission wavelength of ethidium bromide is 605 nm.

Lamprecht et al. (2000 a) quantified the distribution of different polymers within a capsule wall. They prepared microcapsules with two fluorescently labelled polymers, gelatin and arabic gum. Using CLSM the distribution of the two polymers within the capsule wall was determined using computational image analysis and the fluorescence intensity across the imaged capsule wall was determined quantitatively. In order to quantify the distribution of the polymers, casein was added as a third component to the coacervation process. Casein

was fluorescently labelled with a FITC probe. By varying the concentration of label used they were able to quantify the levels of polymer in the microcapsule.

In the current study plasmid DNA was intercalated with ethidium bromide prior to encapsulation. Initial objectives in this section included the examination of the distribution of intercalated DNA within the microsphere.

Figures 7-4 and 7-5 illustrate low power confocal micrographs of the typical distribution of ethidium bromide stained DNA within the microspheres. In this study a z-section comprising nine sections was taken each 0.41 μm in depth transversely through a single microsphere (Figure 7-6). Intensity readings were carried out on each section as a function of μm across the section (Figure 7-7). Figure 7-3 illustrates a z-section through a single microsphere.

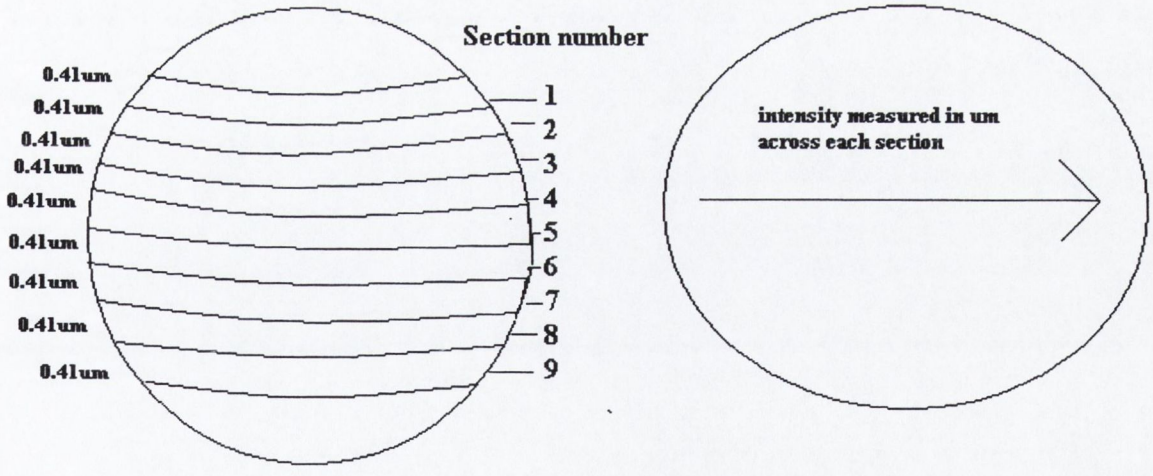


Figure 7- 3 Schematic of z-section and intensity measurements

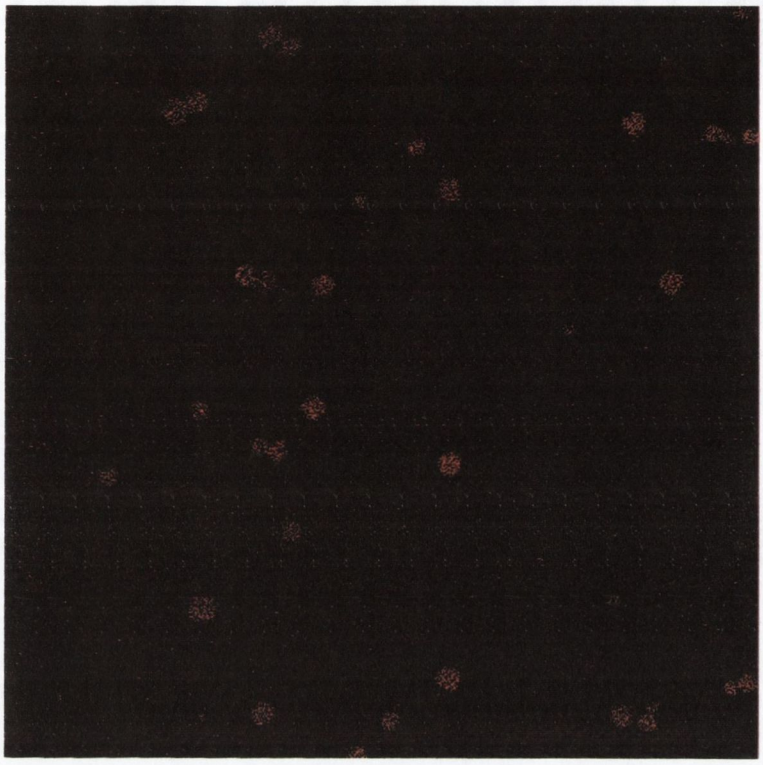


Figure 7- 4 Low powered confocal image of the typical distribution of ethidium bromide labelled plasmid DNA in PLGA microspheres

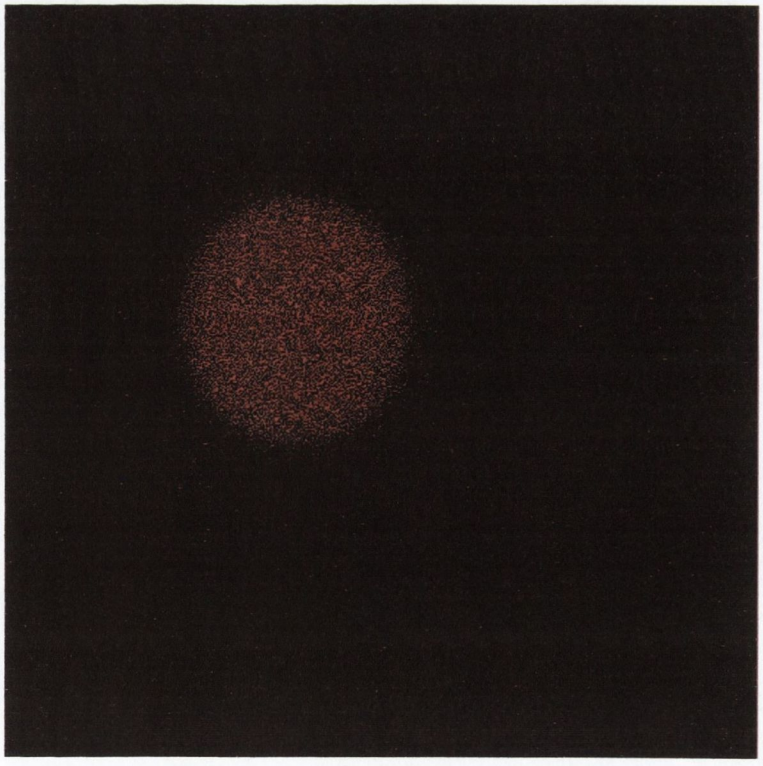


Figure 7- 5 Confocal image of the distribution of ethidium bromide labelled plasmid DNA in a single PLGA microsphere

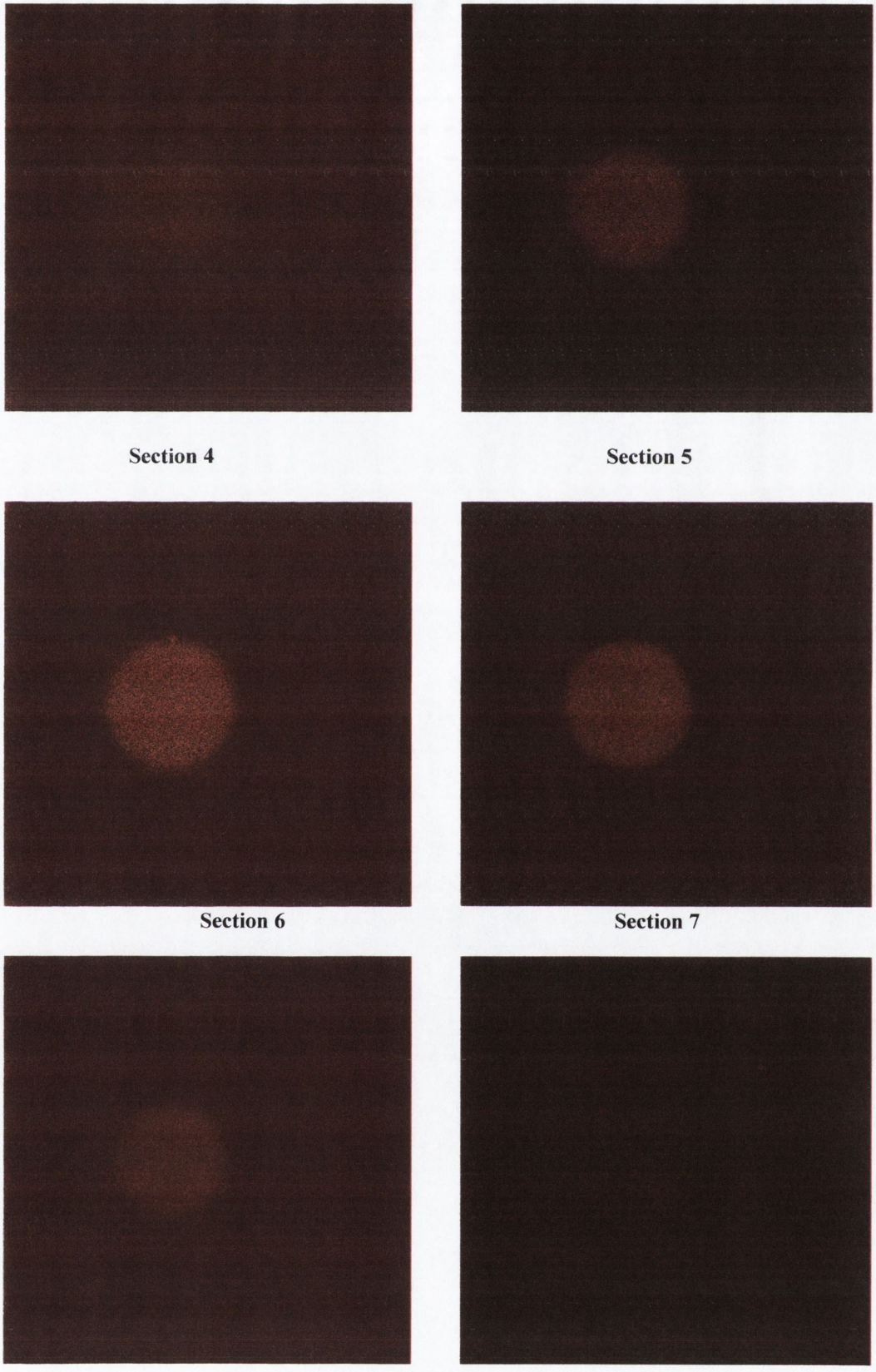


Figure 7-6 Z-sections through a single ethidium bromide labelled plasmid DNA-loaded microspheres

Figure 7-7a illustrates the intensity measurements through section 4, 6 and 8 and Figure 7-6b illustrates the intensity measurements through the odd numbered sections.

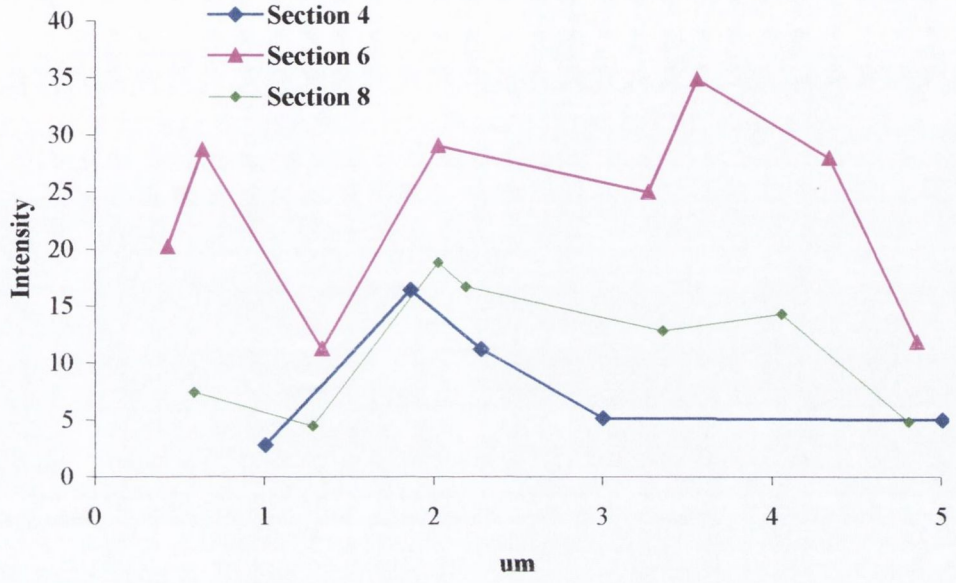


Figure 7- 7a Intensity of label vrs μm longitudinally through even numbered sections

As can be seen section 6 illustrated the highest intensity readings indicating that ethidium bromide labelled DNA was at its most concentrated in this section.

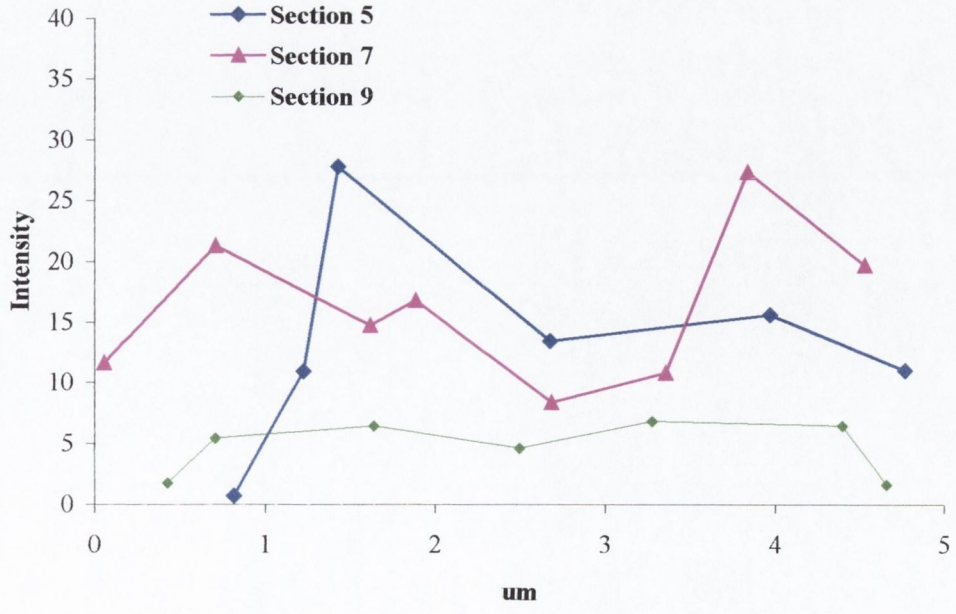


Figure 7- 7b Intensity of label vrs μm longitudinally through odd numbered sections

From Figure 7 a and 7 b it can also be seen that as sectioning progresses through the microsphere the width of the sections increases with sections 6 and 7 showing intensity readings across 4 to 5 μm. This illustrates that these sections were taken at the widest part of the microsphere or the central portion. Confocal imaging shows that the DNA is encapsulated within the microsphere and is held predominantly within the internal core of the microsphere as opposed to the periphery of the microsphere. Sections 4, 5, 7, 8 and 9 also display some fluorescence. This would indicate that the microsphere is not hollow and that while the DNA appears to be concentrated within the core it is also dispersed throughout the microsphere.

Lamprecht et al. (2000 b) urged caution however when using CLSM. Non-fluorescent material cannot be detected by CLSM without prior fluorescence labelling. They also reported that if a fluorescent label is required for detection of a given compound it should be taken into account that the label itself may alter the physiochemical properties of the encapsulated agent. If the drug and the marker have similar solubility properties mixing of the marker and the drug may be sufficient and the incorporation of the drug will not be altered by the marker. However if there is covalent coupling of the marker and drug the physicochemical properties of the drug may also be considerably changed.

Ethidium bromide intercalates between the DNA base pairs and in so doing causes the DNA to unwind. In the case of plasmid DNA, such as is the case in this study, there are no free ends and as such the molecule can only unwind to a limited extent. This limits the amount of ethidium bromide which can bind to the DNA (Old and Primrose, 1980).

7.2 Biological activity of extracted DNA

The CLSM studies outlined in the previous study confirm the presence of DNA in PLGA microspheres. However what was not established was the efficacy of the DNA once released. In the current study two methods were used to assess the biological potency of plasmid DNA extracted from solvent-evaporated and spray-dried microspheres. These methods used extracted DNA from microspheres manufactured following homogenisation with an Ultra Turrax (T25) and a Silverson mixer (L4R) with solvent evaporation and following homogenisation with a Silverson mixer (L4R) and spray drying. DNA extracted

from the three systems was transformed into *E. coli* bacterial cells and transfected into COS-7 cells.

7.2. 1 Bacterial transformation

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognised by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own DNA. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation. Bacteria which are able to uptake DNA are called "competent" and are made so by treatment with calcium chloride in the early log phase of growth. The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown. Following treatment with calcium chloride the cells are subjected to heat shocking at 42°C. When *E. coli* cells are incubated at 42°C, a set of genes are expressed which aid the bacteria in surviving at such temperatures. This set of genes are called the heat shock genes.

Heat shock opens the channels in the outer cell membrane allowing the DNA molecules to enter the bacterial cell (Figure 7-8). To help the bacterial cells recover from the heat shock, the cells are briefly incubated with non-selective growth media. As the cells recover, plasmid genes are expressed, including those that enable the production of daughter plasmids which will segregate with dividing bacterial cells. However, due to the low number of bacterial cells which contain the plasmid and the potential for the plasmid not to propagate itself in all daughter cells, it is necessary to select for bacterial cells which contain the plasmid.

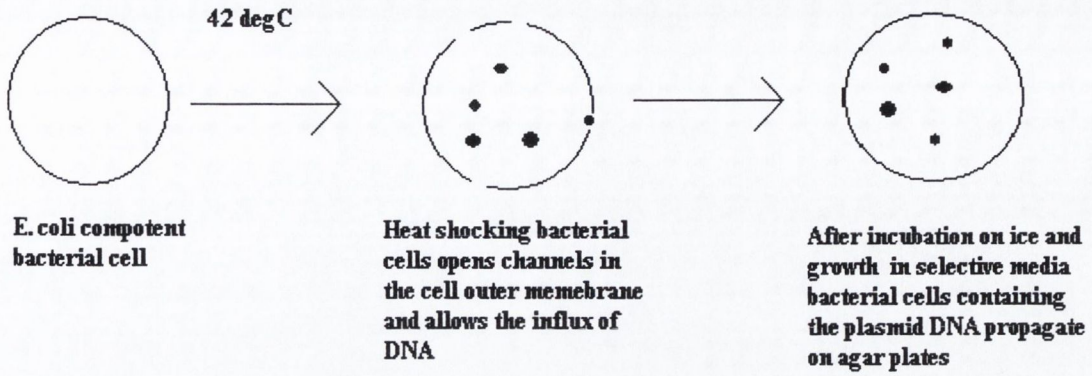


Figure 7- 8 schematic of transformation into *E. coli* cells

Bacterial transformations are based on the principle of antibiotic resistance. Within the plasmid DNA used in this study is an ampicillin resistant gene which allows successfully transformed bacterial cells to grow on agar plates containing ampicillin. It has been shown that linear forms of plasmid DNA do not successfully transform *E. coli* cells and that this type of assay method is more sensitive to DNA isoform than cell transfections (Hao et al., 2000).

The technique of transforming bacterial cells is a commonly used step in the preparation of plasmid DNA (Qiagen, 2000). It has also been used as a tool in the determination of the potency of encapsulated DNA by Hao et al. (2000) who transformed competent bacterial cells with DNA extracted from RG 502 microspheres. These researchers reported that DNA activity remained constant up to 50 days post-lyophilisation. Limitations to this procedure include operator sensitivity since this procedure must be carried out under strictly standardised incubation conditions including incubation time and antibiotic concentration.

It has been reported that the number of colonies obtained from each transformation varies and is dependent on several factors, such as the amount of DNA added and the efficiency of transformation (Tomley, 1996). As a result there is no direct relationship between amount of DNA used and the number of colonies observed. Figure 7-9 illustrates the transformation of 1 μg of extracted DNA from microspheres manufactured following homogenisation with an Ultra Turrax (T25) and a Silverson mixer (L4R) with solvent evaporation and following homogenisation with a Silverson mixer (L4R) and spray drying.

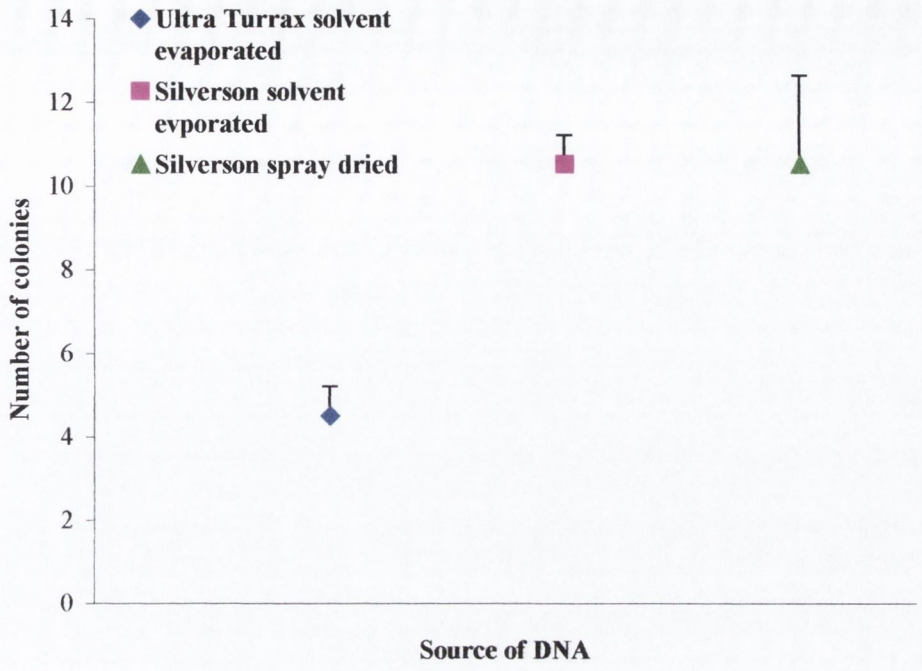


Figure 7- 9 Transformation efficiency of extracted DNA

(samples represent the mean of three determinations)

In the current study it was observed that DNA extracted from all three batches of microspheres transformed *E. coli* cells successfully. Since there is no relationship between amount of DNA used and number of colonies a quantitative statement regarding the efficiency of transformed DNA cannot be made. However the results allow for the observation that the extracted DNA does transform bacterial cells successfully and hence extracted samples contain a proportion of supercoiled or open circular DNA.

7.2. 2 COS-7 cell transfection

Hao et al. (2000) stated that, "the transfection of eukaryotic cells with plasmids containing reporter genes can be used to evaluate the potency of gene delivery vehicles". In this study COS-7 cells were transfected with DNA extracted from microspheres manufactured following homogenisation with an Ultra Turrax (T25) and a Silverson mixer (L4R) with solvent evaporation and following homogenisation with a Silverson mixer (L4R) and spray drying. The transfection reagent used in the study was DOTAP which is a liposome formulation of the cationic lipid DOTAP. It can be used for the highly efficient

transfection of DNA into eukaryotic cells for transient or stable gene expression. Mixing DOTAP Liposomal Transfection Reagent with DNA results in spontaneously formed stable complexes that can directly be added to the tissue culture medium with or without serum. These complexes adhere to the cell surface, fuse with the cell membrane and release the DNA into the cytoplasm. This method of DNA transfer is very gentle, avoiding cytotoxic effects, so that cells can be transfected with high efficiency. Cells were lysed and luciferase levels were determined using a luminometer and were measured as relative luciferase units.

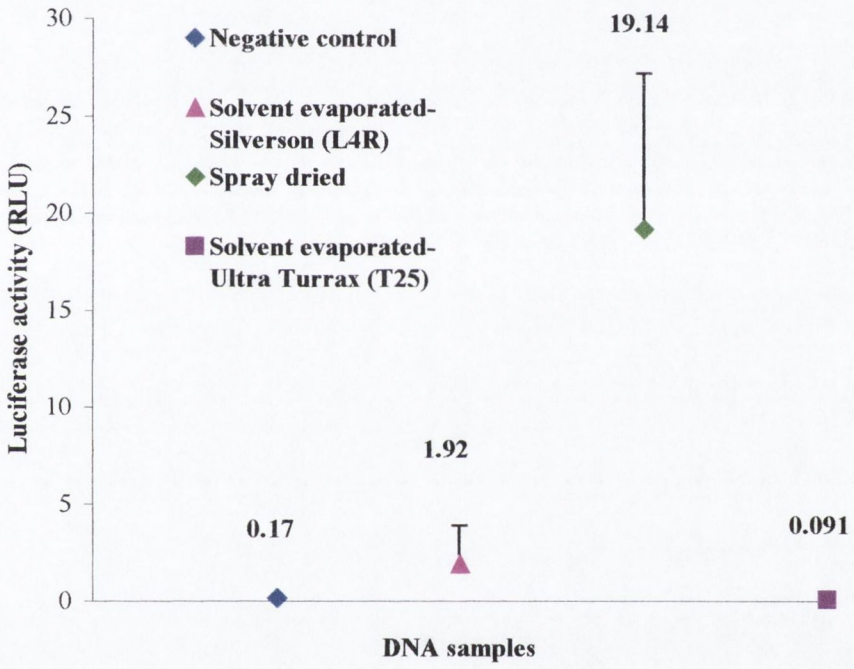


Figure 7- 10 Graph of luciferase activity against source of DNA

(samples represent the mean of three determinations)

Figure 7-10 illustrates that levels of luciferase were produced from DNA extracted from DNA-loaded solvent evaporated microspheres manufactured following homogenisation with a Silverson mixer (L4R) and an Ultra Turrax (T25) and from DNA-loaded spray-dried microspheres. Naked DNA controls resulted in the production of 171.50 $\pm$ 44.72 RLU. As can be seen transfected DNA extracted from spray-dried microspheres resulted in approximately eight times more luciferase activity when compared with the DNA extracted from solvent evaporation.

Levels of luciferase were also calculated in terms of relative luciferase units (rlu) per milligram of total cell protein and are illustrated in Figure 7-11. As can be seen plasmid DNA extracted from spray-dried microspheres (SD) produced the highest levels of rlu per mg of cell protein followed by DNA extracted from Silverson homogenised solvent-evaporated particles (SIL SE). Plasmid DNA extracted from microspheres manufactured with an Ultra Turrax (T25) (UT SE) produced the lowest rlu per mg of cell protein.

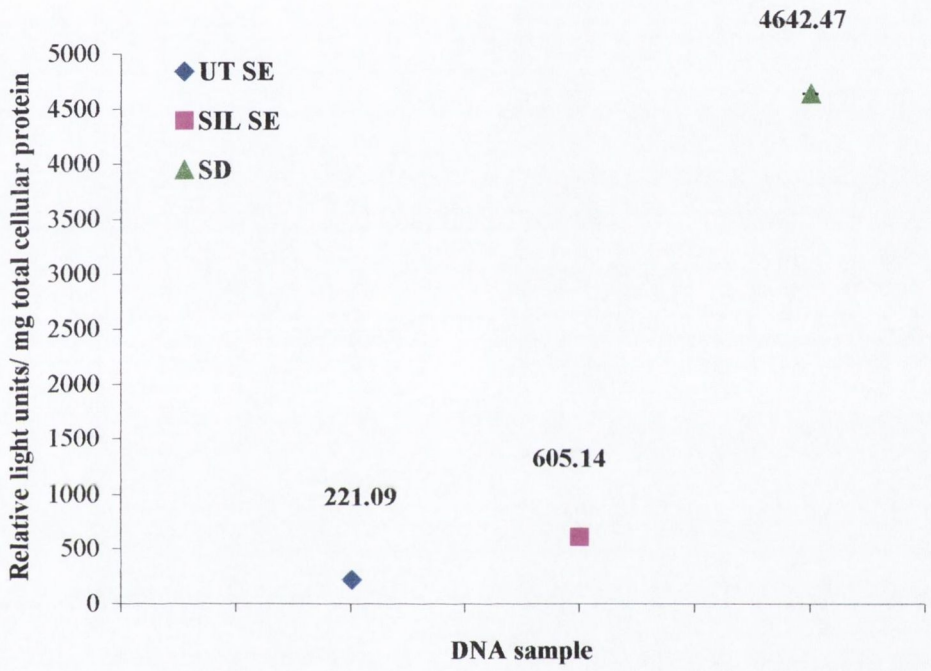


Figure 7- 11 Rlu/ mg total cellular protein

(samples represent the mean of three determinations)

Hao et al (2000) carried out similar transfection experiments on DNA extracted from RG 502 microspheres and reported that transfection efficiency was not affected by DNA isoform. The researchers found that a large fraction of the encapsulated plasmid DNA was converted to the nicked isoform but that transfection efficiency with this form of DNA was identical to transfection with supercoiled DNA. Linear DNA on the other hand was reported to be less active in this assay system.

Walter et al. (1999) reported that the presence of organic solvents such as ethyl formate or dichloromethane during ultrasonication, as is used in the current study, prevented degradation of the DNA in aqueous solutions. They also examined the impact of different processes on the plasmid DNA transfection efficiency. They reported that exposure of

plasmid DNA to pH ≤ 3 resulted in a rapid loss of its transfectivity as did ultrasonication of the aqueous solution of DNA. However it was reported that the addition of physiological concentrations of ions such as is present in phosphate buffer solution prevented degradation during sonication.

Wang et al. (1999) carried out transfection experiments using β-galactosidase plasmid DNA extracted from PLGA 50:50 microspheres. They reported similar levels of activity detected per mg of cellular protein as was observed in this study.

7. 3 Microsphere delivery to murine macrophage cells

7.3. 1 Factors influencing the phagocytosis of polymer microspheres

Phagocytosis of particles is a specialised feature of macrophages and dendritic cells, belonging to the group of Antigen Presenting Cells (APC). Employment of their phagocytic activity offers an attractive opportunity to target antigen in microparticles directly to these potent stimulators of the immune system. The uptake into these cells has been extensively studied with regard to size and charge of the phagocytosed particles (Lutsiak et al., 2002)

Murine monocyte-macrophages of the cell line J774.2 were employed to study the phagocytic uptake of PLGA microspheres.

The main aim of this section was to examine the effect of polymer composition on particle phagocytosis through the use of protein-loaded RG 502 microspheres manufactured following homogenisation with an Ultra Turrax (T25) and solvent evaporation. The effect of the encapsulated entity was also examined through the comparison of solvent-evaporated protein and DNA-loaded RG 752 microspheres. Finally the effect of different encapsulation techniques on the uptake of microparticles was studied by examining the uptake of solvent-evaporated DNA-loaded RG 752 microspheres when compared to the uptake of spray-dried DNA-loaded RG 752 microspheres. The effect of surface charge and particle size was also examined.

Table 7-1 illustrates the physicochemical properties of the microspheres used in the J774 MΦ cell study.

Encapsulated agent	**Protein**		**DNA**	
Manufacturing technique	**Ultra Turrax (T25) Solvent-evaporated**		**Silverson (L4R) Solvent-evaporated**	**Silverson (L4R) Spray-dried**
Polymer	**RG 502**	**RG 752**	**RG 752**	**RG 752**
Zeta potential (mV) (n=5)	-23.7 ± 3.90	-40 ± 2.30	0.9 ± 0.40	-5.2 ± 3.20
Particle size D[v,0.5] (μm) (n=3)	4.92 ± 0.15	4.55 ± 0.02	4.48 ± 0.51	3.16 ± 0.36
Loading /mg microsphere (n= 9 for protein) (n=3 for DNA)	11.38 ± 6.23μg	9.20 ±3.15μg	81.65 ± 5.14 ng	287 00± 8.56ng

Table 7- 1 Physicochemical properties of PLGA microparticles

Phagocytosis of microspheres by macrophage (MΦ) cells has been reported to be greatly influenced by the physicochemical properties of the microsphere surface and especially by the surface charge and the particle size (Tabata and Ikada, 1990).

As can be seen in the case of protein-loaded microspheres the more hydrophobic polymer, RG 752, resulted in microspheres with higher zeta potential values (-40 mV) when compared with those formed with the more hydrophilic RG 502 (-23.7 mV). As both

protein-loaded batches produced microspheres of a comparable size and loading and were manufactured following the same technique it is likely that the difference in zeta potential values can be attributed to the difference in physicochemical properties of the two polymers.

DNA-loaded microspheres were formulated with RG 752 but showed zeta potential values of –5.2 and 0.9 mV. The values reported for the DNA-loaded microspheres would suggest that the uptake of these particles might be expected to be less than for the protein microparticles (Tabata and Ikada, 1990).

Lacasse et al. (1998) reported that spray-dried microspheres exhibited higher zeta potential values when compared with solvent-evaporated microspheres. In the current work it was found that DNA-loaded microspheres manufactured following spray drying did exhibit higher zeta potential values when compared with solvent evaporated microspheres. However protein-loaded microspheres homogenised using an Ultra Turrax (T25) followed with solvent evaporation exhibited higher zeta potential readings than DNA-loaded batches manufactured following homogenisation with a Silverson mixer (L4R).

In the current study particle size values for all batches were found to be below 10 μm with the exception of DNA-loaded solvent evaporated microspheres manufactured following homogenisation with an Ultra Turrax (T25). The dependence of macrophage phagocytosis on the size of microspheres was examined by Tabata and Ikada (1990). They used monodispersed polystyrene to study this and reported that the number of microspheres phagocytosed per macrophage cell had a maximum at a diameter between 1 and 2 μm in diameter. Since the D[v,0.5] readings of the majority of the batches manufactured during this study fell below approximately 4 μm in diameter it was expected that there would be a large percentage available for phagocytosis.

The attachment of particles to MΦ cells has been described as a physicochemical rather than an active energy–dependent biological reaction. Tabata and Ikada, (1990) reported that phagocytosis was enhanced as the absolute values of the zeta potential increases for both the negatively charged and the positively charged surfaces of microspheres. It was also noted that the lowest phagocytosis was found for the microspheres with a surface zeta

potential of zero. Sahoo et al. (2002) reported that residual PVA associated with PLGA microparticles has an effect on the zeta potential and phagocytosis of nanoparticles. PVA is a commonly used emulsifier in the formation of PLGA microparticles and a fraction of PVA has been shown to remain associated with microparticles. The mechanism of association has been proposed to be due to the penetration of the hydrophobic segments of PVA into the organic phase of the double emulsion and the entrapment of the PVA into the polymeric matrix. It was reported that as the concentration of PVA was decreased from 5% w/w to 0.5% w/w the zeta potential value increased from –0.8 mV to –15.4 mV with nanospheres manufactured in the absence of PVA having a zeta potential of approximately –45 mV (Sahoo et al., 2002). In the current study a low PVA concentration (0.27% w/v) was used during the emulsification process in an attempt to overcome any potential effect between residual PVA and surface charge.

The selection of the cell culture model is critical (Roser et al., 1998). Mouse peritoneal macrophages have been previously employed and were found to discriminate for particles with different surface characteristics (Illum et al., 1987; Tabata and Ikada, 1990; Zhang et al., 1998). One should also keep in mind that the source (Zhang et al., 1998), type and viability state of phagocytic cells, as well as the culturing and incubation procedures may all influence particle-to-cell interactions. Therefore, it may not necessarily be conclusive to compare particle uptake and cell activation data from different studies with each other.

7.3. 2 Phagocytosis of microparticles by J774 macrophage cells

Figure 7-12 illustrates a schematic of drug-loaded microparticulate uptake. Briefly, the microparticle becomes associated with the macrophage cell and is taken up into the cell by endocytosis into an endocytotic vesicle. This vesicle becomes associated with a lysosome and the drug is released from the particle as it degrades.

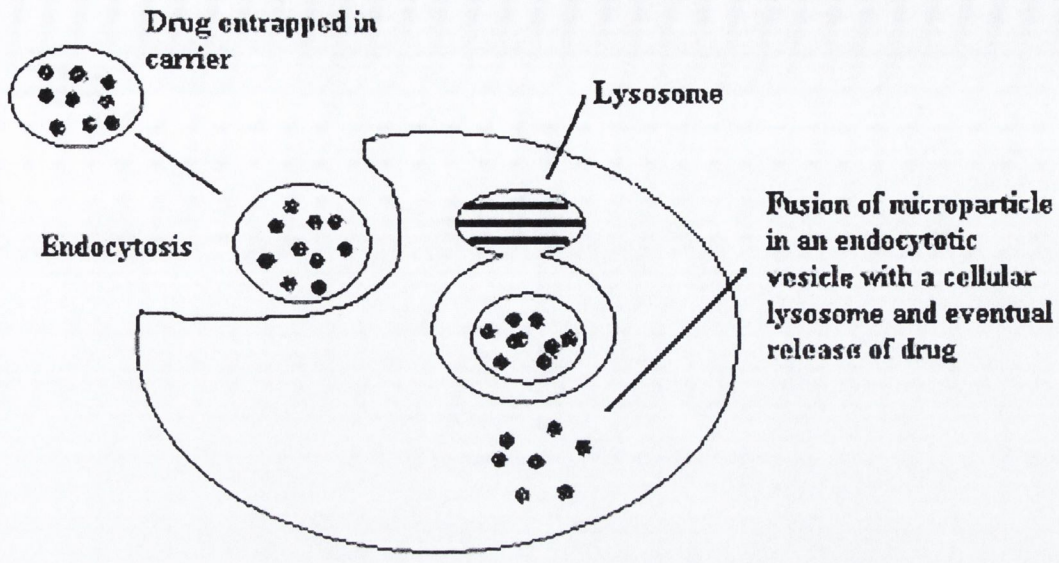


Figure 7- 12 Schematic of particulate uptake into a macrophage cell (adapted from Ahsan et al., 2002)

In the current study macrophage cells were incubated with a predetermined weight of microspheres and were incubated at 37°C for 30 minutes and 24 hours. Figure 7-13 are phase contrast photographs taken during the study following incubation of J774 macrophage cells with protein-loaded RG 752 particles, cytoplasm staining and gravity centrifugation of cells onto a glass slide. It illustrates the sequence of phagocytosis in J774 MΦ cells treated with these particles in this study.

The events of phagocytosis are considered to take place in three subsequent stages; attachment, ingestion and digestion (Tabata and Ikada, 1990). The J774 MΦ cell moves with flapping ruffles at its leading edge and push out processes towards the foreign particulate substance (Figure 7-13 1). Once a particle is attached to a cell surface, there is a local perturbation of the membrane beneath the attachment site (Figure 7-13 2). Structures called pseudopods are formed which enclose the particle (Figure 7-13 3 and 7-13 4) and the formation of a phagosome is triggered so that the particle is internalised (Figure 7-13 5).

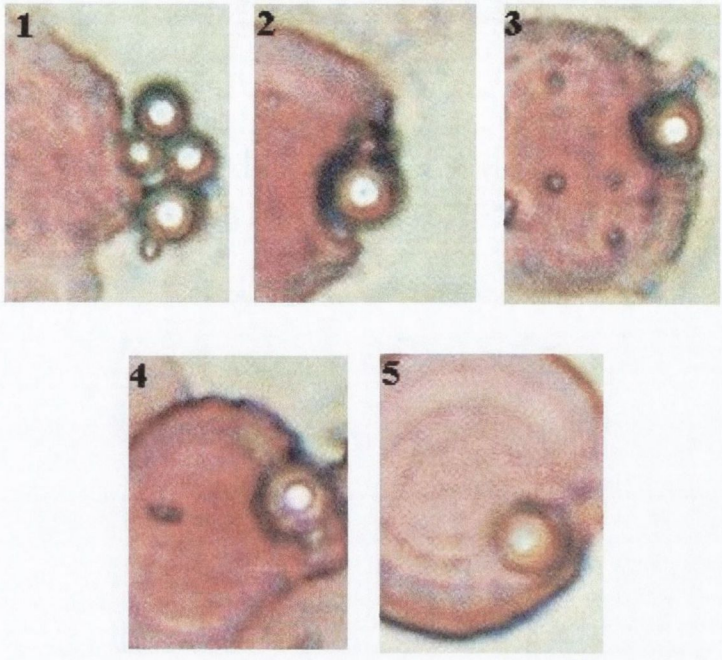


Figure 7- 13 Phagocytosis of protein–loaded RG 752 microsphere

This figure illustrates that the microspheres were successfully taken up into the cells through the process which was described by Tabata and Ikada (1990).

The process of phagocytosis is dependent on the actin and myosin microfilaments, which are concentrated in the cytoplasm of the cell immediately beneath the particle-associated plasma membrane. When small particles become associated with the plasma membrane of the cell they are swept by active ruffling of the cell membrane into vacuoles, which then pass deep into the cell. After ingestion of the vesicle which forms around the particle the phagosome fuses with one or more lysosomes to form a secondary lysosome and the hydrolytic enzymes contained in the lysosome are discharged degrading the contents of the vacuole. It has also been reported that the engulfing plasma membrane, which surrounds the particles during their entry into the J774 MΦ cell, is very closely apposed to the surface of the particle in order to minimise fluid intake from the surrounding medium (Tabata and Ikada, 1988).

In this study microspheres, formulated with different molecular weight polymers, which contained both protein and DNA encapsulated following different processes were targeted

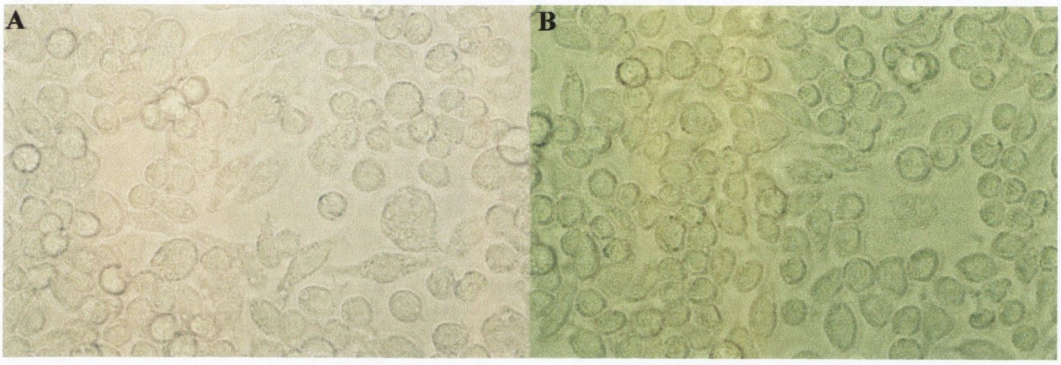
to murine J774 MΦ cells. Microspheres were suspended in cell culture medium and added to the cells at a concentration of approximately 7.3 x 10^5 particles per well (Walter et al., 2001). Incubation was carried out at 37°C for specific time points prior to phase contrast microscopic analysis. Walter et al (2001) studied the effect of temperature (4°C and 37°C) on phagocytosis and found that at 4°C no uptake occurred. Cells were photographed prior to and after washing with phosphate buffer (pH 7.4). Selected batches of microspheres used were those formulated following solvent evaporation with RG 502 and RG 752 containing protein to examine the effect of polymer composition and surface charge. RG 752 DNA-loaded microspheres formulated following solvent evaporation and spray drying were also studied to examine the effect, if any, manufacture technique had on the surface charge and the phagocytosis of particles.

Cells were collected onto glass slides and were stained using a cytoplasm stain in order to examine the cells in greater detail. Cells were dehydrated prior to analysis with transmission microscopy

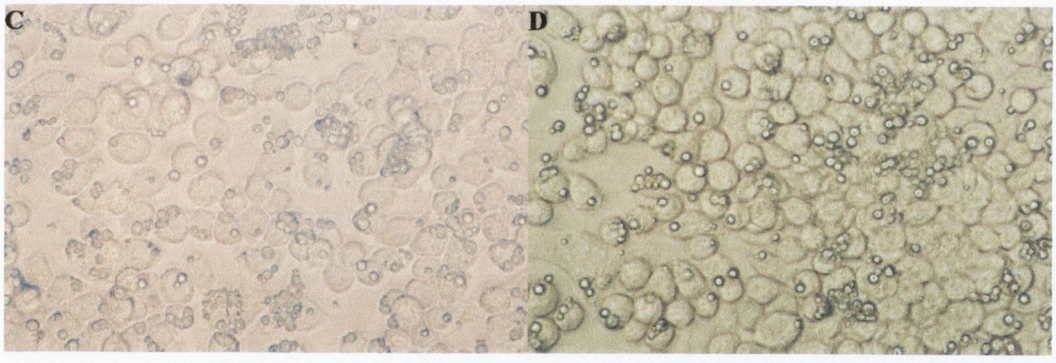
From Figure 7-14 it can be seen that unwashed cells show particles both outside the cell and associated with the cells. However once washed the remaining particles are associated with the J774 MΦ cells. Photographs of the control cells (A and B) show cells which are irregular in shape and size with no evidence of any extracellular structures. Cells which have been treated with protein-loaded RG 752 particles show a large number of spherical particles both in the extracellular space in the case of the unwashed system and particles associated with the cells as in the case of both unwashed and washed (C and D). J774 MΦ cells treated with protein-loaded RG 502 particles homogenised with an Ultra Turrax (T25) (UT) show a smaller amount of particulate uptake (E and F). These findings were similar to Prior et al. (2002) who reported more efficient phagocytosis with the more hydrophobic PLGA 75:25 which was attributed to the higher zeta potential value of these particles when compared with PLGA 50:50 particles. DNA-loaded RG 752 particles were also used to examine the effects of processing on particulate uptake. Spray-dried (SD) microspheres (I and J) appeared to aggregate more in cell culture medium when compared with (SE) solvent-evaporated microspheres. (Figure 7-11 I also shows some excessive light exposure during the study.) This aggregation may be attributed to the observation that spray-dried

microspheres appeared to agglomerate at increased inlet temperature during spray drying (Ståhl et al., 2002).

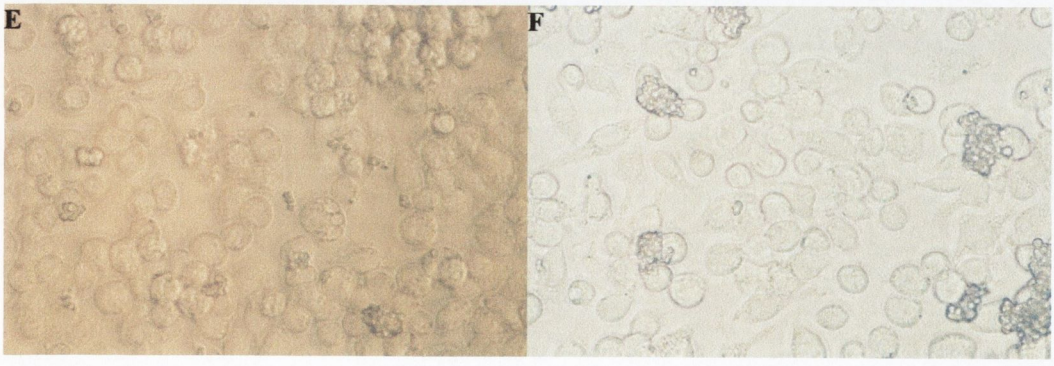
Negative control: A unwashed B washed



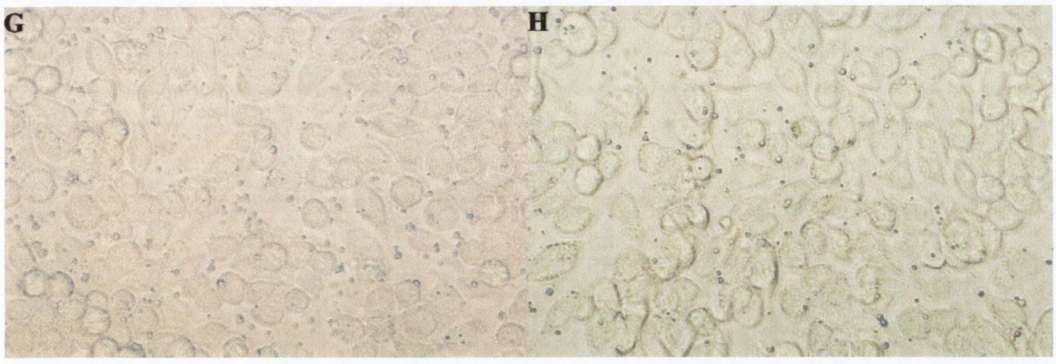
RG 752 protein-loaded UT, SE: C unwashed D washed



RG 502 protein-loaded UT, SE: E unwashed F washed



RG 752 DNA-loaded Silverson mixer (SIL), SE: G unwashed H washed



RG 752 DNA-loaded SIL, SD: I unwashed J washed

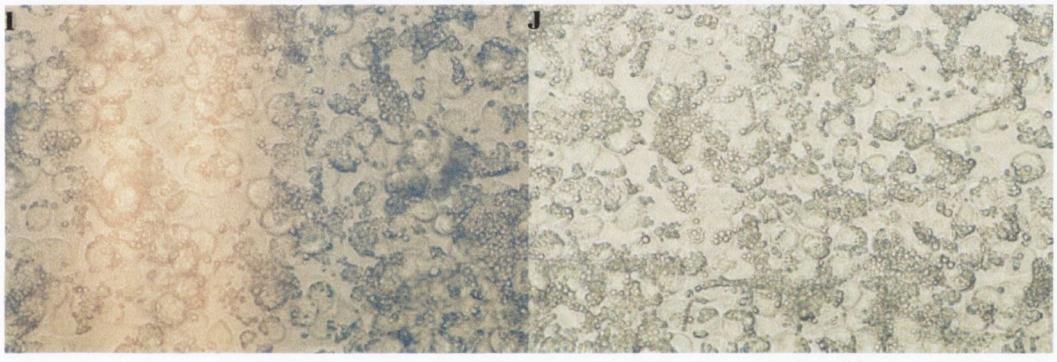
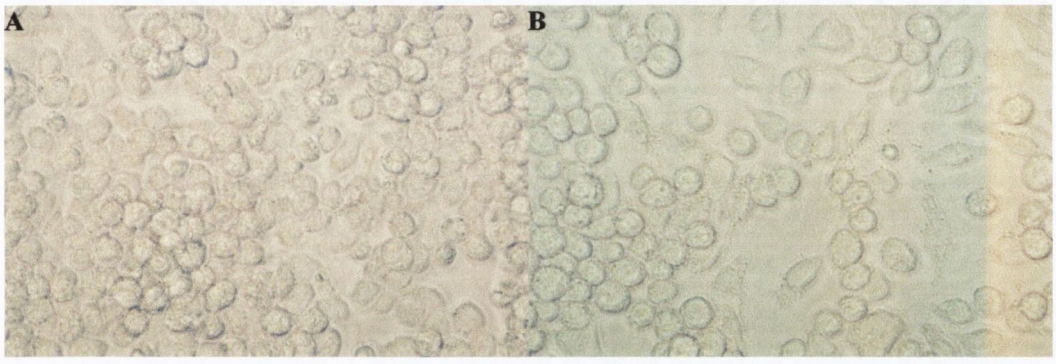


Figure 7- 14 30 minute time point phagocytosis

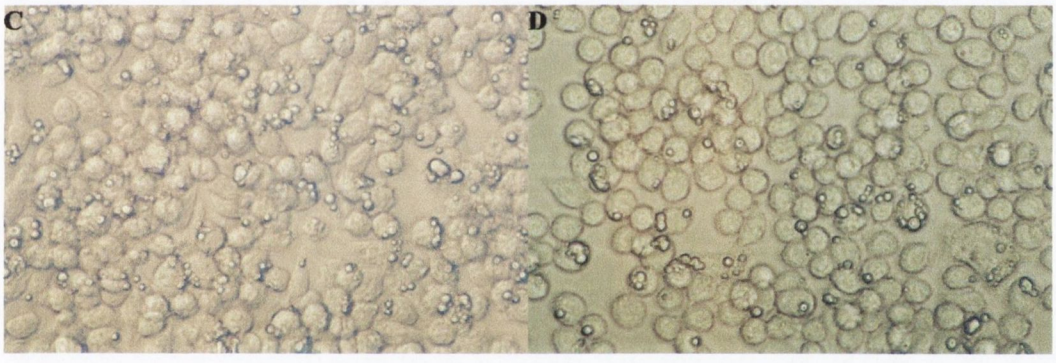
Negative control	**A** unwashed **B** washed
RG 752 protein-loaded solvent-evaporated	**C** unwashed **D** washed
RG 502 protein-loaded solvent-evaporated	**E** unwashed **F** washed
RG 752 DNA-loaded solvent-evaporated	**G** unwashed **H** washed
RG 752 DNA-loaded spray-dried	**I** unwashed **J** washed

Figure 7-15 illustrates the phagocytosis of particles following 24 hours incubation. There appears to be extensive uptake of RG 752 particles, which are hydrophobic, when compared with RG 502 particles. Washed cells show a decrease in particle number not associated with cells. It can be seen in the case of both batches of DNA-loaded cells that the number of particles, which remain associated with the J774 MΦ cells, is relatively high compared with both batches of protein-loaded microspheres despite the higher zeta potential readings (0.9 $\pm$ 0.4 mV solvent-evaporated DNA-loaded microspheres and -5.2 $\pm$ 3.2 mV in the case of the spray-dried microspheres).

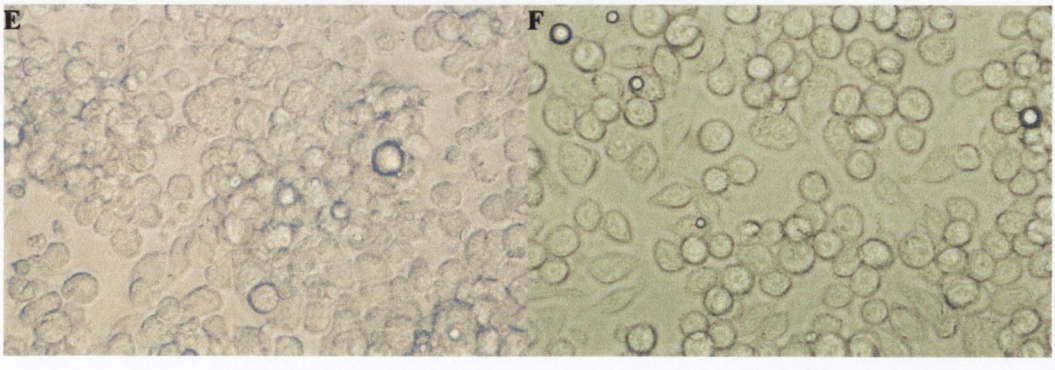
Negative control: A unwashed B washed



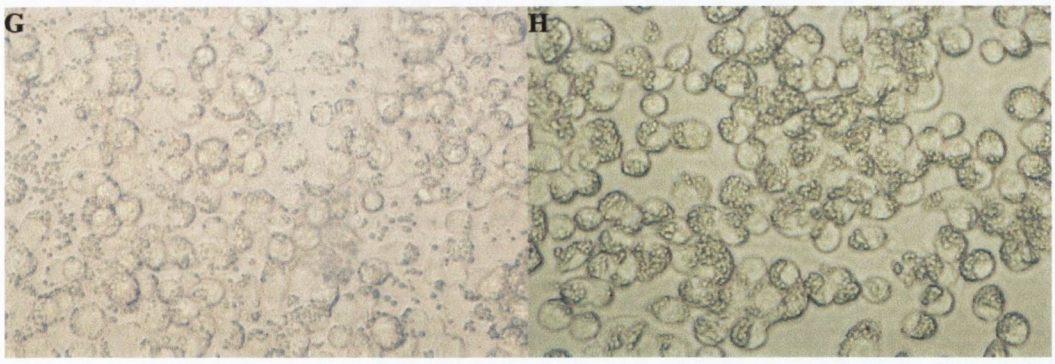
RG 752 protein-loaded UT, SE: C unwashed D washed



RG 502 protein-loaded UT, SE: E unwashed F washed



RG 752 DNA-loaded SIL, SE: G unwashed H washed



RG 752 DNA-loaded SIL, SD: I unwashed J washed

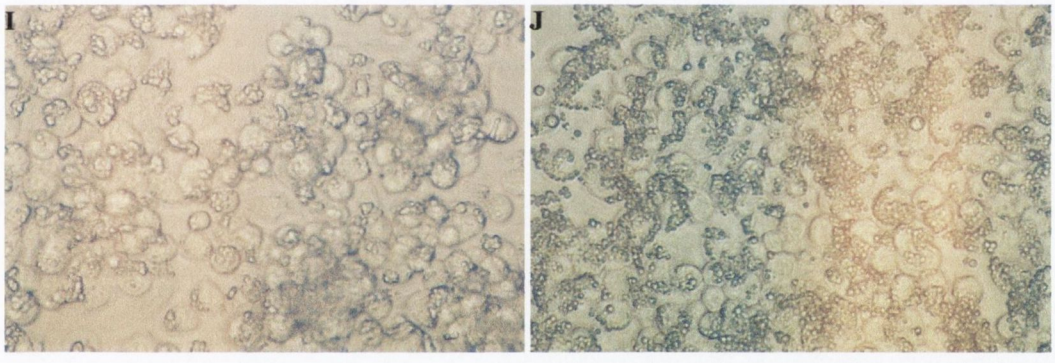


Figure 7- 16 24 hour time point phagocytosis

Negative control	**A** unwashed **B** washed
RG 752 protein-loaded solvent-evaporated	**C** unwashed **D** washed
RG 502 protein-loaded solvent-evaporated	**E** unwashed **F** washed
RG 752 DNA-loaded solvent-evaporated	**G** unwashed **H** washed
RG 752 DNA-loaded spray-dried	**I** unwashed **J** washed

The number of particles in each cell was counted in an effort to examine the effect of longer incubation periods on particle uptake. A pre-determined area was chosen and cells containing one or more particles counted. Cells containing ten or more particles were classified as having 10+ particles.

Figure 7-17 illustrates the number of RG 502 protein-loaded particles taken up into macrophage cells. As can be seen in comparison with RG 752, few RG 502 microspheres were taken up. In this case there was no statistical difference between time of incubation or

samples which had been unwashed or washed. Walter et al. (2001) reported similar RG 502 particle numbers associated with cells.

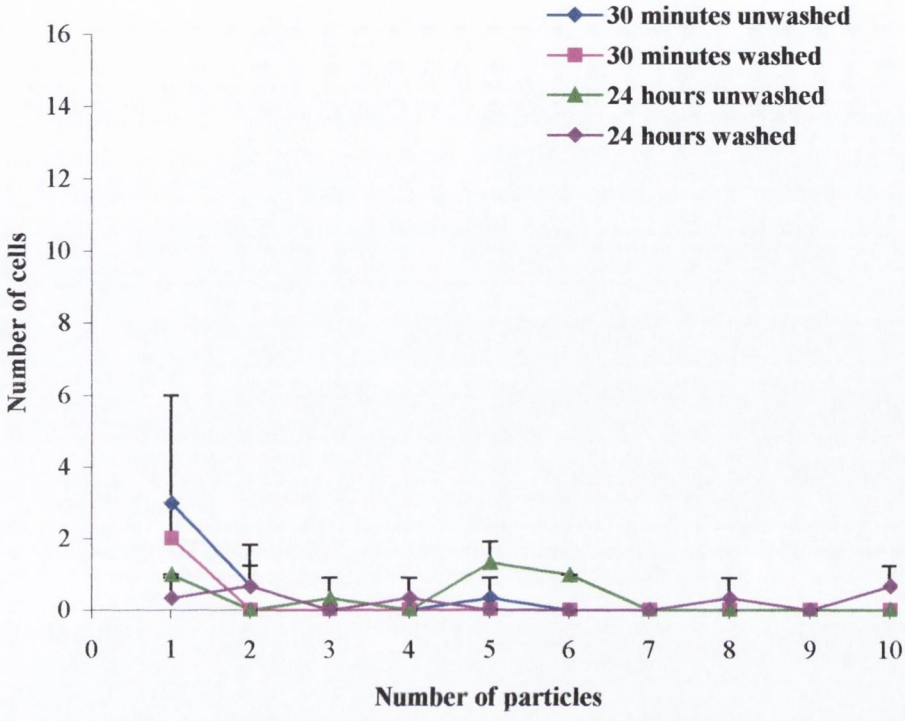


Figure 7- 17 Number of RG 502 protein-loaded particles taken up into macrophage cells

(samples represent the mean of three determinations)

Figure 7-18 illustrates the number of RG 752 protein-loaded microspheres which were associated with macrophage cells. As can be seen there was no statistical difference between the number of particles taken up by macrophage cells following 30 minutes and 24 hours. Nor was there any statistical difference between uptake in cells which had been washed and unwashed. The level of particle uptake was in line with that observed by Walter et al. (2001).

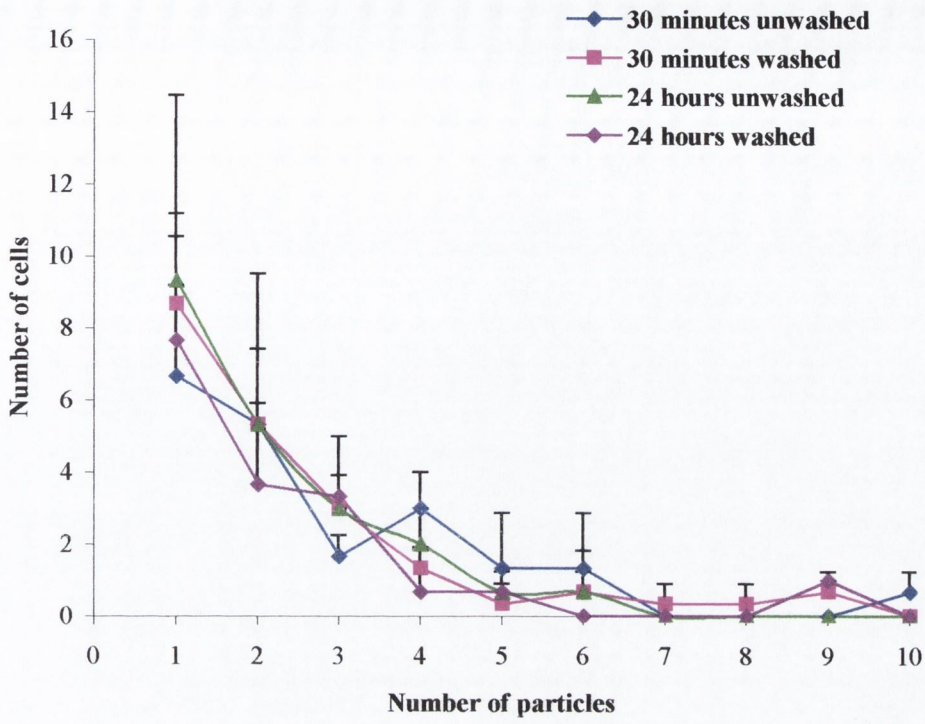


Figure 7- 18 Number of RG 752 protein-loaded particles taken up into macrophage cells

(samples represent the mean of three determinations)

From both Figure 7-17 and Figure 7-18 it appears that phagocytosis of these particles occurred within the first 30 minutes of incubation and that the particles seen associated with cells were not removed following washing three times with PBS buffer.

Figures 7-19 and 7-20 illustrate the number of RG 752 solvent-evaporated microspheres manufactured following Silverson homogenisation and spray drying which were taken up over a 24 hour period. From Figure 7-19 it can be seen that unlike the case for the protein-loaded microspheres the number of solvent-evaporated DNA-loaded particles taken up by the cells increases over the 24 hours of the study. Initially the number of cells with one or two particles associated with them is high when compared with those cells which had 4 or more particles. However following 24 hours incubation the number of cells with 7 particles or more associated with them rose significantly.

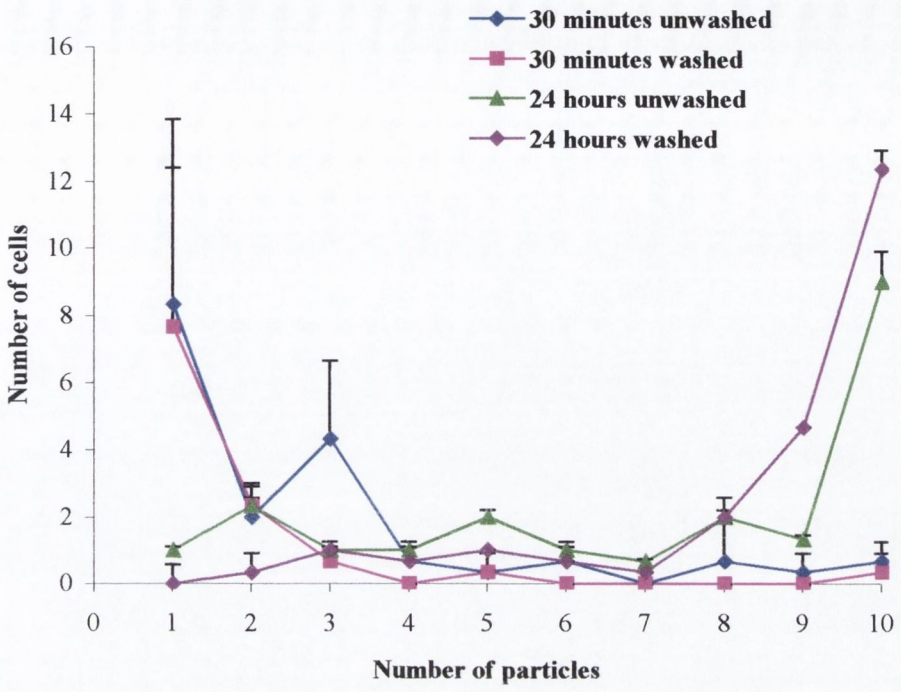


Figure 7- 19 Number of RG 752 solvent-evaporated DNA-loaded particles taken up into macrophage cells

(samples represent the mean of three determinations)

Figure 7-20 illustrates the number of spray-dried DNA-loaded RG 752 particles associated with macrophage cells. In the case of spray-dried DNA particles, uptake did not appear to have altered significantly between 30 minutes and 24 hours indicating that the majority of particles phagocytosed do so in the first 30 minutes of incubation. What is not shown is the large percentage of cells which were associated with 10+ particles, since these were classified for the sake of counting as 10 particles. Following examination of the cells this was found to be approximately 90% of cells counted which would indicate that these particles appear to be more readily phagocytosed when compared with particles of the other three systems.

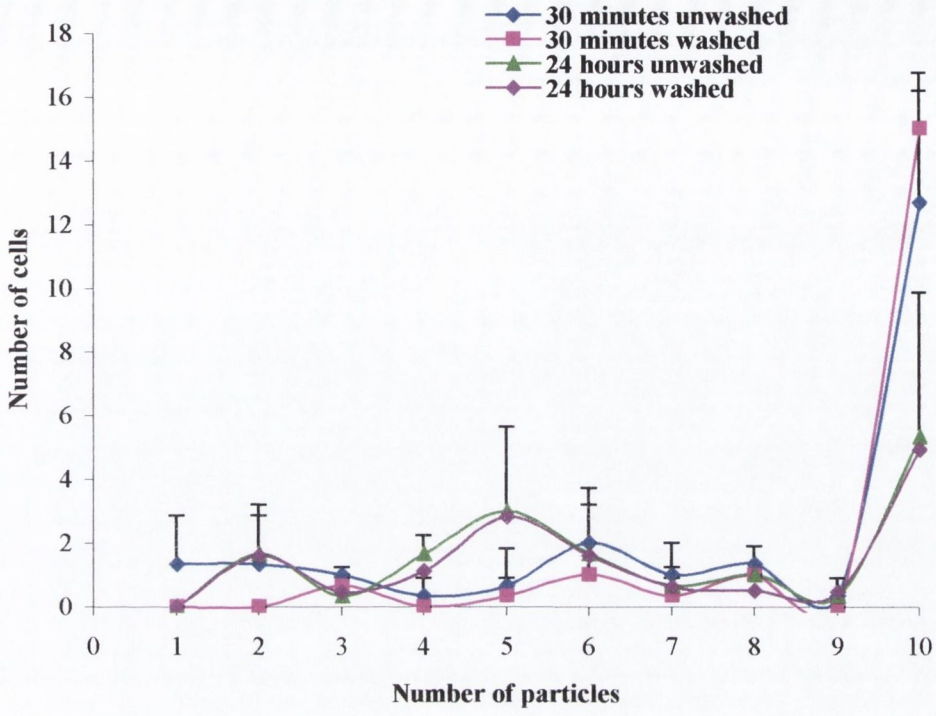


Figure 7- 20 Number of RG 752 spray-dried DNA-loaded particles taken up into macrophage cells
(samples represent the mean of three determinations)

Figure 7-21 illustrates the effect of incubation time on particle uptake. In this study the total number of associated particles were divided by total number of cells irrespective of whether or not they contained particles. From Figure 7-21 there is no significant difference in the number of protein-loaded particles taken up over the period of the study ($p > 0.05$) irrespective of polymer type. There is also no significant uptake in RG 752 spray-dried (SD) microspheres over the time of the study. However there is a significant difference in uptake over 24 hours of solvent-evaporated (SE) DNA-loaded RG 752 particles ($p<0.05$). The number of solvent-evaporated DNA-loaded RG 752 particles taken up per cell was also significantly different from numbers of particles from the remaining three systems taken up over the same period ($p<0.5$). Solvent-evaporated and spray-dried DNA-loaded RG 752 particles exhibited a lower zeta potential reading (0.9 ± 0.4 mV and -5.2 ± 3.2 respectively) when compared with protein-loaded RG 502 (-23.7 ± 3.9 mV) and protein-loaded RG 752 ($-40\ 0 \pm 2.3$ mV). However the same particles did show a lower particle size (4.48 ± 0.51 μm and 3.16 ± 0.36 μm) when compared with protein-loaded RG 502 (4.92 ± 0.15 μm) and protein-loaded RG 752 particles (4.55 ± 0.02 μm) which may be attributed to the greater particle uptake associated with microspheres belonging to these

systems. Spray-dried DNA-loaded RG 752 particles showed the highest initial uptake over the first 30 minutes of the study which also might be attributed to smaller particle sizes.

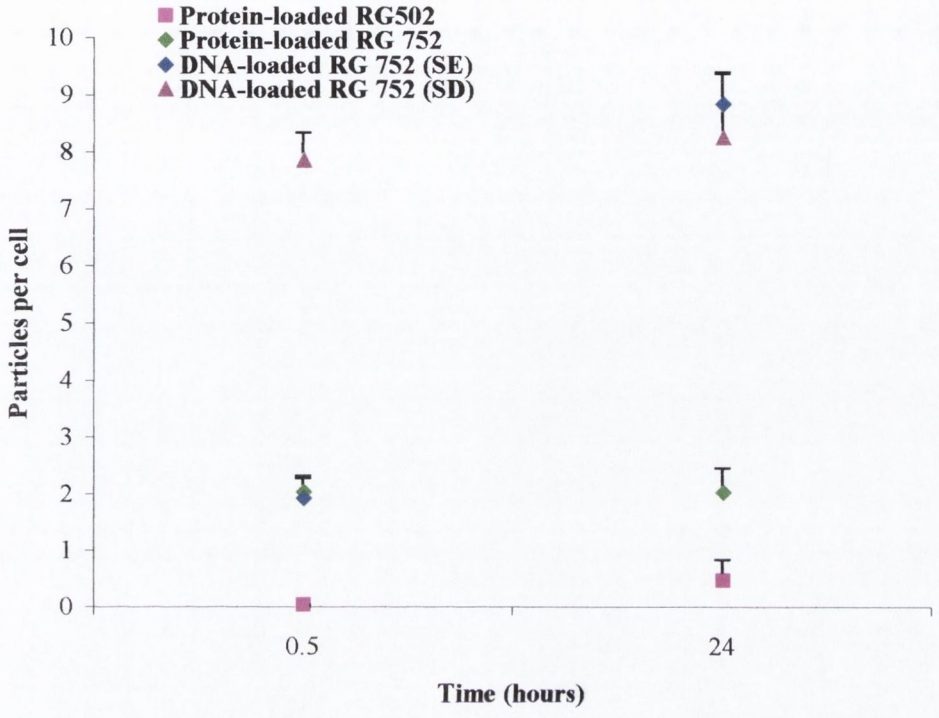


Figure 7- 21 Particle uptake by macrophages over 24 hours

(samples represent the mean of three determinations)

This work suggests that DNA-loaded RG 752 particles are more efficient at targeting immune system cells than protein-loaded particles manufactured with either RG 752 and RG 502. From Figure 7-20 it can be seen that unlike the case for protein-loaded microspheres the number of solvent-evaporated DNA-loaded particles taken up by cells increases over the 24 hours of the study. Initially the number of cells with one or two particles associated with them is high when compared with those cells which had 4 or more particles. However following 24 hours incubation with the particles the number of cells with 7 particles or more associated with them rose significantly.

Phase contrast microscopy failed to distinguish clearly the microspheres merely attached to the macrophage surface from those internalised within the cell. It was important to examine the internal structure of the cells to see whether or not the particles had been internalised by the process of phagocytosis. This was carried out using transmission microscopy of the cells examined by phase contrast microscopy. In this study J774 MΦ cells treated with microspheres were dehydrated with alcohol and embedded in resin prior to closer examination with a transmission microscope.

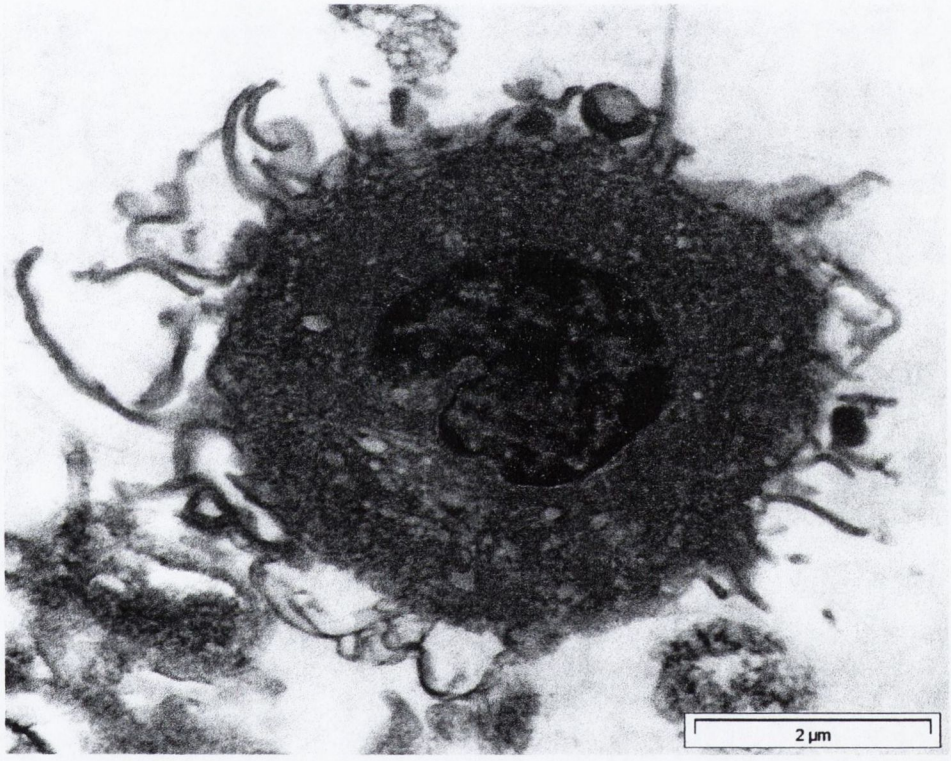


Figure 7- 22 Transmission electron microscopy image of a control J774 MΦ cell

Figure 7-22 illustrates the micrograph of a control cell J774 MΦ cell. These cells were treated in an identical manner to cells used in the phagocytosis study but were incubated in Dulbecco's Modified Eagles Medium in the absence of microparticles. Macrophages are relatively large immune system cells surrounded by pseudopodia or ruffles, which assist in the engulfing of foreign particles. These can clearly be seen in Figure 7-22 as can the large nucleus, which is found in macrophage cells. Absent from these negative control cells is the intracellular vacuoles which were seen in treated cells. The key at the bottom right corner of the image indicates that this cell was approximately 6 µm in diameter.

The following figures illustrate the phagocytosis of microspheres containing both protein and DNA by the J774 MΦ cells. TEM shows that the particles are indeed located within the cells surrounded by a phagosomal membrane. Support for the hypothesis that these are indeed the particles is confirmed by two observations. Firstly the structures are confined to samples which have been incubated with the microspheres and are not present in any of the controls and secondly the shape and size of these vacuoles closely resembles that of the microspheres and since it is known that during phagocytosis of foreign material the phagosomal membrane of macrophage cells become closely apposed to the surface of ingested foreign material it is highly likely that these vacuoles contain the spherical microspheres.

7.3.3.1 Phagocytosis of protein-loaded microspheres

In order to examine the effect of polymer composition and surface charge on particulate uptake, protein–loaded RG 502 and RG 752 particle were incubated with J774 macrophage cells. Figure 7-23 illustrates the phagocytosis of protein-loaded RG 502 microspheres by MΦ cells. As can be seen there are several cells with large spherical vacuoles, which are believed to contain the ingested microsphere. Also present in this sample are several cells, which do not contain these vacuoles. Clearly visible inside the cells is the darkly stained cell nucleus which accounts for a large percentage of the cell volume. This in conjunction with light microscope studies provided further support that there appeared to be a lower particle uptake with the RG 502 microparticles when compared with RG 752 particles. The key at the bottom right hand corner indicates that these cells are approximately 10-15 μm in size with the microspheres being approximately 5-10 μm in size. Micrographs of J774 macrophage cells incubated with RG 502 particles indicated that these particles were taken up by macrophage cells although not at the same density as RG 752 particles.

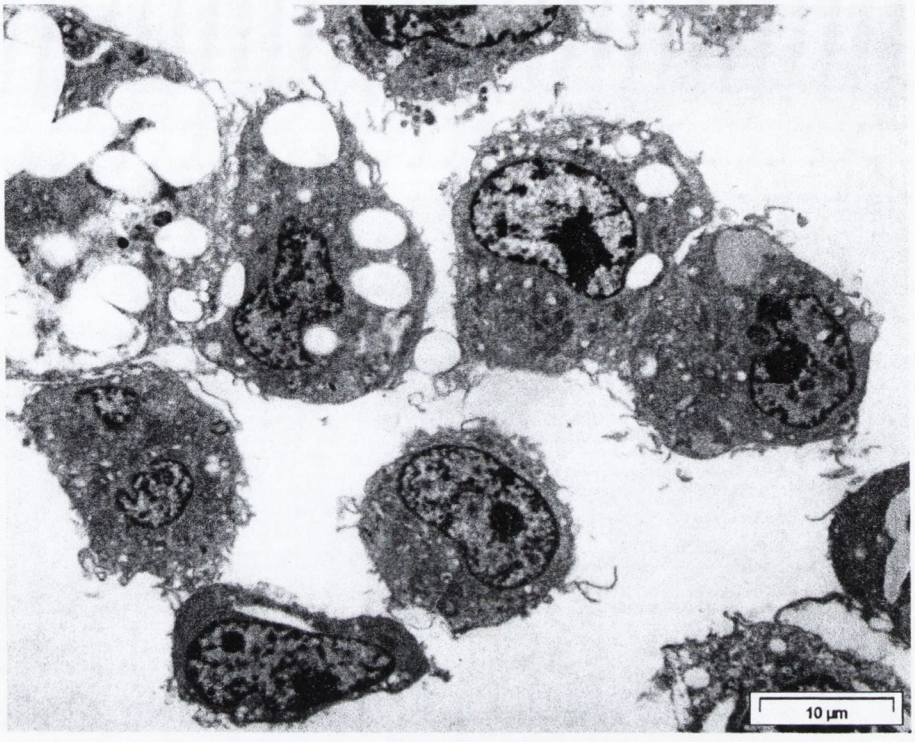


Figure 7- 23 Phagocytosis by MΦ cells of protein-loaded RG 502 microspheres

Closer examination of the cells (Figure 7-24) clearly shows the particles inside the cell with as many as five particles inside one cell with surrounding cells containing none. This mirrored the view under phase contrast microscopy where a large proportion of cells were seen not to be associated with any particles. The ruffles on the outside of the J774 MΦ cells, which appear as filament type structures on the outer cell wall, are observed which are used to sweep the foreign particle into the cell cytoplasm. Also visible is a particle outside the cell surrounded by these ruffles of the macrophage cell as it begins to phagocytose the microsphere.

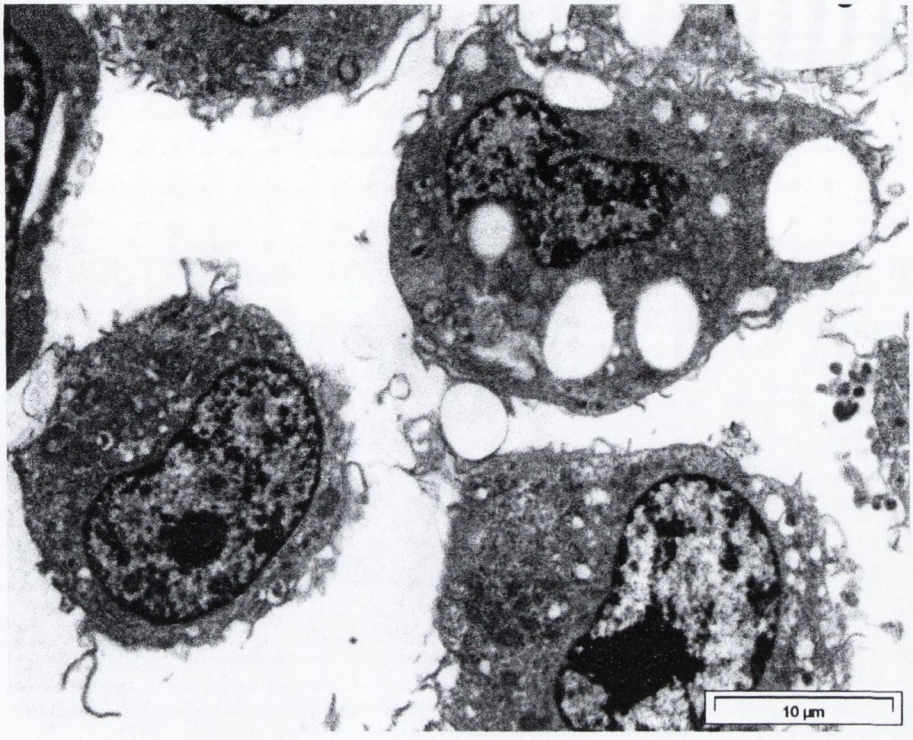


Figure 7- 24 Phagocytosis of protein-loaded RG 502 particles by MΦ cells

Figure 7-25 illustrates the phagocytosis of protein-loaded RG 752 microspheres by macrophage cells. Micrographs examined, unlike those examined following incubation with protein-loaded RG 502 particles, did not display any cells, which lacked vacuoles. This supported observations from the light microscopy images where there appeared to be a higher particulate uptake with protein-loaded RG 752 microspheres when compared with RG 502 microspheres. The difference in density of particulate uptake between RG 752 particles and RG 502 particles might be attributed to the difference in zeta potential between the two batches of microspheres (RG 502: - 23.7 $\pm$ 3.9 mV, RG 752: - 40 $\pm$ 2.3 mV) as there was no statistical difference between particle sizes.

Figure 7- 25 Transmission micrograph of the phagocytosis of protein-loaded RG 752 particles

7.3.3.2 Phagocytosis of DNA-loaded microspheres

Scanning electron micrographs of DNA-loaded microspheres had shown that microparticles manufactured via solvent evaporation and spray drying displayed different morphologies. Those manufactured following solvent evaporation were spherical and had smooth surfaces while those produced following spray drying predominantly doughnut shaped with their outer surface appearing to have collapsed inwards. Macrophage studies were undertaken to examine the effect of the morphology and the surface charges of these microspheres on phagocytosis. Mean particle sizes ranged between 3 and 5 µm in diameter. Zeta potential values for these microspheres were determined to be 0.9 ± 0.4 mV in the case of solvent-evaporated microspheres and -5.2 ± 3.2 mV for spray-dried particles.

Figure 7-26 illustrates the phagocytosis of a solvent-evaporated DNA-loaded RG 752 microspheres by a single macrophage cell. Several large vacuoles and the cell nucleus can be seen in this image (Figure 7-26).

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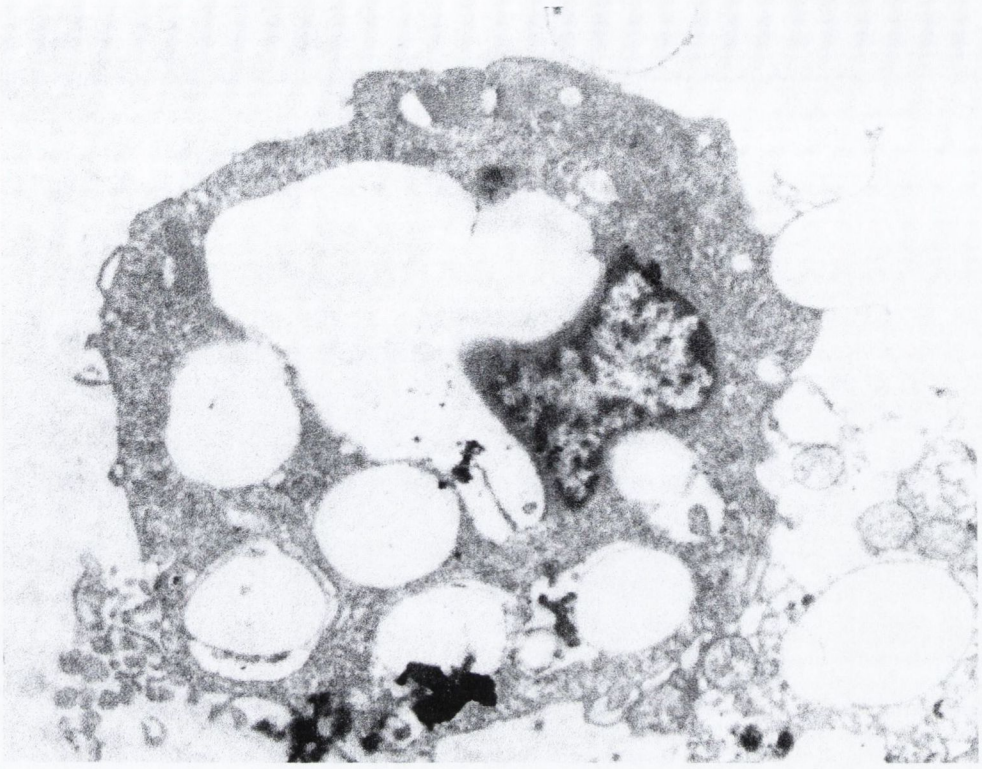

Figure 7- 26 Phagocytosis of solvent-evaporated DNA-loaded RG 752 microsphere

Light microscopy images of phagocytosed DNA-loaded RG 752 spray-dried particles indicated a high particle uptake (Figure 7-14 J and 7-15 J). These were examined under a transmission microscope and were found to contain several particle-containing vacuoles. Figure 7-27 illustrates what is believed to be several spray-dried particles outside the cell membrane. The darkly stained structure to the left of the image is the cell nucleus.

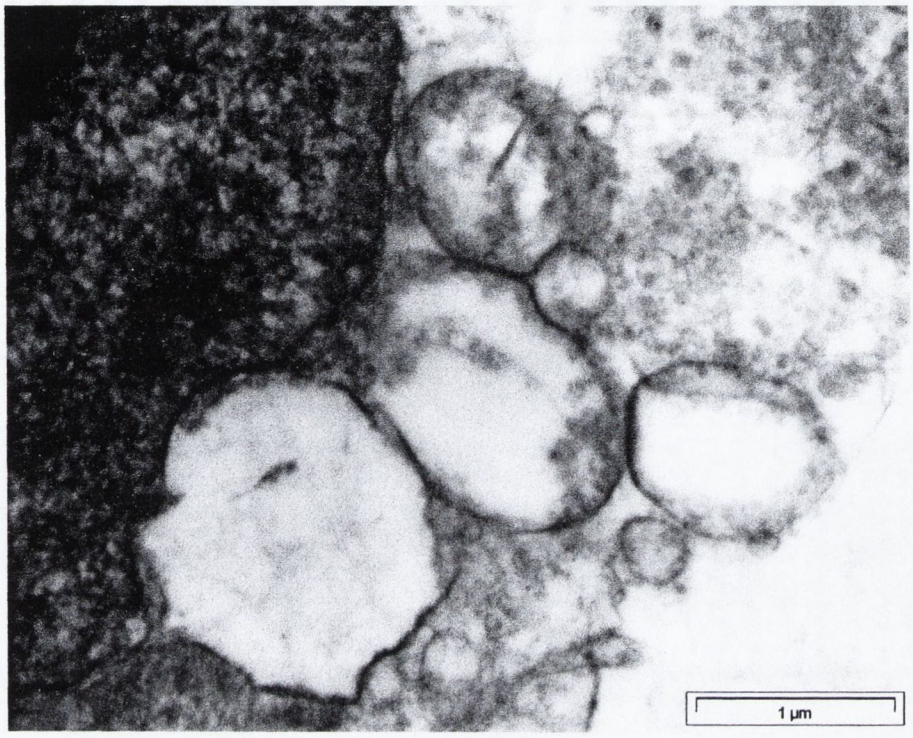

Figure 7- 27 Spray-dried particles outside the cell membrane

Further images of phagocytosis of DNA-loaded RG 752 spray-dried particles clearly show several particles inside the J774 MΦ cells (Figure 7-28). Unlike protein-loaded RG 502 particles there were no cells without these vacuoles, which supported the observation from the light microscope images that there was high particle uptake with these microspheres (Figure7-14 H and 7-15 H). Cells were approximately 10 to 15 µm in diameter with ingested particles approximately 2 to 5 µm in size, which is confirmed by particle size data.

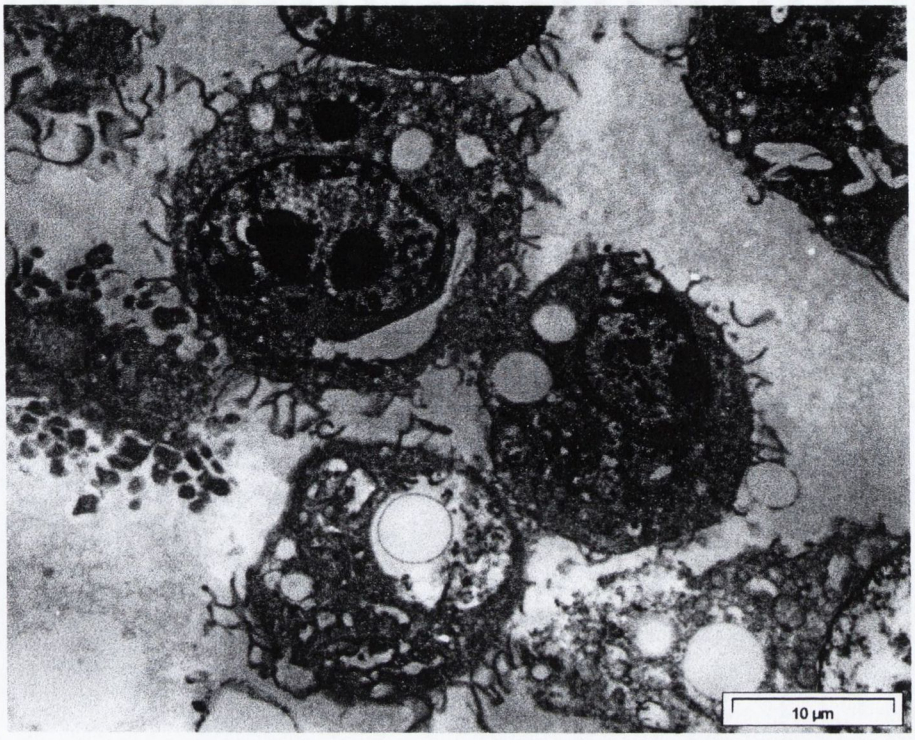

Figure 7- 28 Phagocytosis of DNA-loaded RG 752 microparticles by MΦ cells

Closer examination of the cells showed the uptake of a single microsphere (Figure 7-29). As can be seen the outer ruffles of the MΦ cell surrounds the particle forming a phagosomal membrane around it. This will eventually sweep the particle into the cellular cytoplasm.

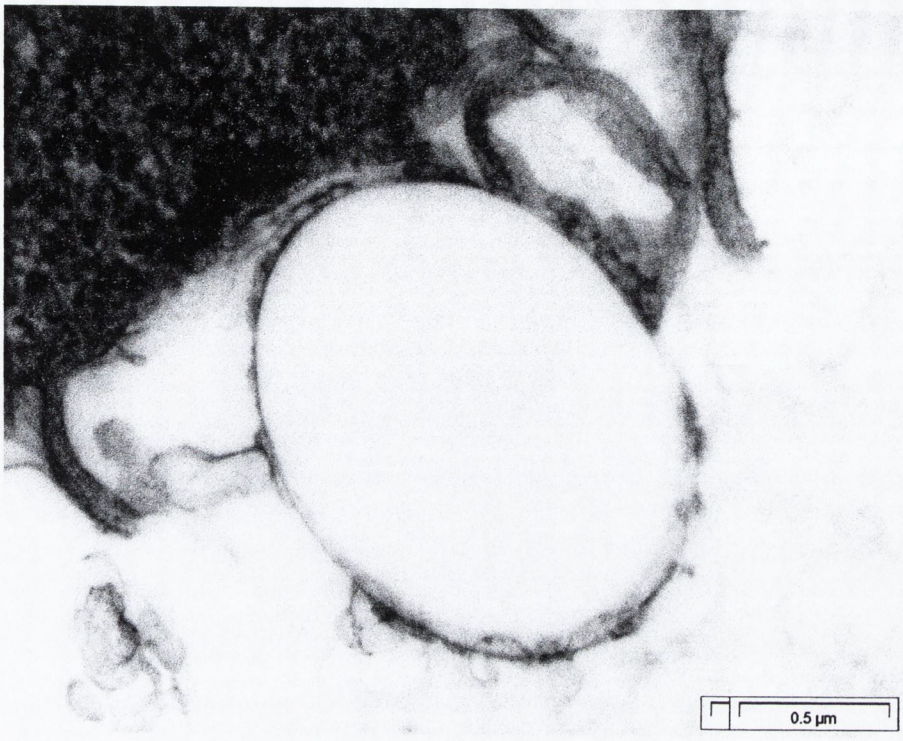

Figure 7- 29 Uptake of single RG 752 spray-dried microsphere

7. 4 Discussion

In the previous chapters we evaluated the physicochemical characteristics of PLGA microspheres containing both protein and DNA to assess the influence of manufacturing variables on these particulate systems. It was observed that while solvent evaporated protein and DNA-loaded systems resulted in encapsulation efficiencies which were in line with other researchers, spray-dried systems produced higher encapsulation efficiencies.

Biodegradable PLGA microspheres have been shown to be effective in the delivery of proteins and nucleic acids and have been suggested to be ideal carrier systems for oral vaccination. However in order for these systems to be effective as vaccines they must contain biologically potent DNA and be able to target immune system cells. In the current chapter we evaluated the distribution and the potency of DNA encapsulated in PLGA microspheres via solvent-evaporation and spray-drying and assessed relative benefits of one system over the other. The last section of the chapter examined the phagocytosis of the microspheres by immune system cells. Table 7-2 summaries the main advantages and disadvantages associated with each of the methods used in this chapter.

In the current study confocal microscopy was used to determine the distribution of ethidium bromide intercalated plasmid DNA within PLGA microspheres. Plasmid DNA was found to be encapsulated primarily in the core of the microsphere as determined by z-sections of a single microsphere. Confocal microscopy has previously been used to determine the location of a labelled agent with PLGA microspheres (Lacasse et al., 1998). It has also been used to determine the intracellular location of labelled nanospheres (Lutsiak et al., 2002). However DNA intercalated with ethidium bromide did not allow us to quantify the concentration of DNA within the microsphere due to the limited amount of ethidium bromide which could physically intercalate with the DNA.

Method	Advantages	Disadvantages
Confocal microscopy	Non- destructive. Allows for the distribution of the plasmid to be visualised.	There is a limit to ethidium bromide binding to DNA, which does not allow DNA to be quantified.
Bacterial cell transformation	Allows for the determination of DNA isoform.	Destructive – DNA has to be extracted from microsphere. Colony counting is subjective.
Cell transfection	Allows a qualitative estimate of DNA.	Again it is destructive, as the DNA must first be extracted from the microspheres.
Macrophage model	Non-destructive. May allow specific targeting to immune system cells.	The counting of the phagocytosed particles is subjective. Does not allow for estimate of DNA potency

Table 7- 2 Advantages and Disadvantages associated with the main methods used

Transformation of bacterial cells has been used to good effect in the determination of DNA isoform. Linear DNA has been reported not to confer antibiotic resistance of bacterial cells while circular and supercoiled isoforms do. In this study it was observed that colonies were found in relation to DNA extracted from the three systems of microspheres. This allows for the assumption that DNA structure was conserved to a certain degree following encapsulation. However it does not allow us to quantify the degree of conservation or the percentage of plasmid DNA which retained its structure. This is due to the fact that the exact concentration of bacterial cells is not constant from sample to sample hence the amount of cells initially may vary which would affect the concentration of cells to be plated out. Also colony counting is subjective and caution must be taken when determining cell numbers. However in the current study it did show that microencapsulation of plasmid DNA by Silverson or Ultra Turrax homogenisation followed by solvent evaporation and microencapsulation of plasmid DNA by Silverson followed by spray drying allowed the maintenance of circular or supercoiled DNA.

Biological indicator assays of DNA extracted from microspheres indicated that all samples retained the ability to transfect COS-7 mammalian cells. This would suggest that the structure of plasmid DNA was not significantly affected by the encapsulation procedures studied here.

The macrophage cell phagocytosis assay was based on observations by Walter et al. (2001) who examined the effect of varying polymer microspheres on the phagocytosis of DNA-loaded microspheres into macrophage and dendritic cells. These researchers found that the efficiency of particulate uptake was not altered by the physicochemical properties of the polymer. They examined both PLGA 75:25 and PLGA 50:50 microspheres and reported similar numbers of particles phagocytosed in both despite the hydrophilic nature of PLGA 50:50.

Macrophage studies in the current work examined the uptake of two different polymers, encapsulating two different agents and manufactured using three different methods. Results illustrated that particles manufactured with different polymer compositions displayed different uptake. This has been reported previously and has been attributed to surface hydrophobicity and zeta potential surface charge difference between the polymers (Takada and Ikada, 1988). In the current studies it was found that protein-loaded particles composed of PLGA 75:25 were more readily phagocytosed than protein-loaded PLGA 50:50 particles with PLGA 75:25 and PLGA 50:50 particles bearing surface charges of – 40.0 $\pm$ 2.3 mV and – 23.0 $\pm$ 3.9 mV respectively. There was no significant difference in particle size values between both systems. In relation to manufacturing techniques it was found that both spray-dried and solvent-evaporated DNA-loaded RG 752 microspheres were readily taken up by microspheres with zeta potential readings of –5.2 $\pm$ 3.2 and 0.9 $\pm$ 0.4 mV respectively. This observation has been reported previously by Walter et al., (2001) who reported that PLGA microparticles were readily phagocytosed despite differences in surface charge.

While we have shown that macrophages do indeed phagocytose microspheres several researchers have investigated the enhancement of microsphere uptake using precoated particles. Tabata and Ikada (1989) reported that microsphere phagocytosis was suppressed

by precoating with BSA, PVA, dextran, CMC, PVP and PAAm while it was enhanced by precoating with gelatin, FN, tuftsin and IgG. These authors also reported that an increased hydrophobicity led to a decrease in particle uptake. This was not found in this study. In fact RG 752 was more readily phagocytosed than the more hydrophilic RG 502. This observation may have been attributed to the lower zeta potential values associated with RG 752 microspheres which has also been reported to enhance phagocytosis.

In conclusion we have shown that DNA encapsulated in PLGA microspheres retains the ability to produce luciferase enzyme and to confer antibiotic resistance to transformed bacterial cells. We have also shown through confocal microscopy that the DNA is encapsulated within the microsphere and is held predominately in the microsphere core. We have also shown that phagocytosis of microspheres appears to be dependent on polymer type and appears to be independent of microsphere zeta potential. It has also been observed that, with regard to polymer type, phagocytosis of microspheres appears to occur within the first 30 minutes of incubation. From Chapter 6 it was known that spray-dried particle exhibited the highest encapsulation efficiency and displayed diffusion based release. From this chapter it was demonstrated that spray-dried particles had higher uptake into macrophage cells. DNA extracted from spray-dried systems also displayed higher transfection results than solvent-evaporated microspheres and transformed bacterial cells successfully. The current work predicts that spray drying is the most efficient system examined in terms of encapsulation efficiency, macrophage uptake and transfection efficiency.

Chapter 8

General Discussion

8. 1 Introduction

The potential role of gene delivery in medicine was highlighted originally in the 1950's when injection of crude preparations of DNA from tumours led to tumour formation in experimental animals (Stasney et al., 1950; Ito et al., 1961). Studies investigating the effects of parenteral administration of purified and recombinant viral DNA in animals confirmed the likely potential of gene transfer or delivery in therapeutics and disease modulation (Orth et al., 1964; Israel et al., 1979).

The concept of nucleic acid vaccination, where antigen-encoding plasmid DNA is taken up by cells and translated in the cell is a specialized form of gene therapy. A succession of publications reporting on cell transfection after injection by plasmid DNA encoding for proteins as diverse as the hepatitis B family of proteins, insulin and reporter molecules culminated in the exposition of the concept of de novo production of a required vaccine antigen by the host's cells (Will et al., 1982; Nicolau et al., 1983; Wolf et al., 1990). From 1992 onwards, a succession of publications reported firstly, on the ability of plasmid DNA to induce an immune response to an encoded foreign protein and secondly, on the cellular and humoral nature of the immune response with consequent protection for host animals challenged with the antigens under study (Tang et al., 1992, Ulmer et al., 1993; Fynan et al., 1993). The concept of DNA immunization has now been adopted by DNA vaccinologists worldwide using an ever increasing number of plasmids encoding immunogens from bacterial, viral and parasitic pathogens and a variety of tumours (Bright et al., 1996; Baspar et al., 1997; Fu et al., 1997).

With the advent of the Human Genome project it would thus appear that there are limitless opportunities for widespread applications of gene therapy in developing treatments and cures for a range of diseases. The project has already helped in positional cloning of 30 disease-related genes that may provide useful targets for therapeutic intervention (Anonymous, 2001). However our ability to capitalize on these opportunities is frustrated by inadequate systems currently available for delivery of therapeutic genes and nucleic acids to target cells.

Viral vectors can often achieve efficient transgene expression particularly in ex vivo or local application strategies. However, the occurrence of inflammatory reactions against viral proteins presented by infected cells together with concerns over the safety of virus application, particularly for non life-threatening disorders, is likely to hinder their adoption for routine therapeutic procedures. This will be particularly evident where the treatment must be administered repeatedly, or where the target cells are widespread or disseminated around the body. Non-viral vectors, in contrast are fully defined and have a superior safety profile. Although their transgene expression efficiency is relatively low, at least compared to viruses, they provide a platform for "bottom-up" development of efficient and fully defined vectors, with the feasibility of receptor-mediated targeting to specific cells. In addition, scale-up production of non-viral vectors is usually much cheaper and easier than using vectors.

Polymeric drug delivery systems have successfully been used to encapsulate and deliver plasmid DNA in vivo (Lunsford et al., 2000). These vehicles have been able to deliver genes in close proximity to the target site, which aids in the efficiency of transfection over naked DNA injections. Additionally, polymeric systems function in the protection of plasmid DNA from attack by both extracellular and intracellular nucleases (Lechardeur et al., 1999). A convenient and safe DNA vector would pose no threat to the gene therapy recipient, biodegrade into safe products once it has released its plasmid load, and release DNA over time in a sustained manner so as to increase both the chance of transfection and duration.

While a number of studies have examined the microencapsulation of DNA (Jones et al., 1997; Mathowitz et al., 1997; Walter et al., 1999; Hao et al., 2000; Liu et al., 2002), there is a lack of information about the influence of the manufacturing process on the loading efficiency and release characteristics of the encapsulated DNA. This study is a comprehensive investigation of how the manufacturing process underlying these systems critically impacts on the characteristics of the release systems.

PLGA polymers have been used in the encapsulation of a variety of different agents including proteins (O'Hagan et al., 1994; Cho et al., 2001), peptides (Hutchinson et al., 1985) and nucleic acids (Jong et al., 1997; Wang et al., 1999) in an attempt to prolong their duration of action and to improve their therapeutic efficacy. PLGA polymers and their

related analogues have been used for alternative biomedical applications for many years. PLGA polymers were initially developed by the pharmaceutical industry for use as degradable sutures (Frazza and Schmitt, 1971). Subsequently characteristics such as biocompatibility, controlled degradation kinetics, ease of fabrication and established regulatory approval for human applications have attracted interest in these polymers for alternative biomedical applications (O'Hagan et al., 1998). Particulate systems based on lactic and glycolic acids have been extensively studied particularly in the area of drug delivery and have been used as controlled release systems for a variety of pharmaceutical agents. PLGA polymers have also been used in the preparation of several commercially available drugs including Zoladex™ (Zeneca) and Decapeptyl™ (Ipsen Biotech), which are licensed for use in humans in Europe and the USA.

One of the most commonly employed methods in the development of particulates for drug delivery is solvent evaporation (Uchida et al., 1994; McGee et al., 1997; Singh and O'Hagan, 1998; Wang et al., 2002). The classical solvent evaporation method, in which the drug is dissolved/dispersed in an organic polymer solution followed by emulsification into an external aqueous phase to form an oil/water emulsion, does not result in acceptable encapsulation efficiencies for peptides (Bodmeier, 1995). Because of high water solubility peptide partitions into the external aqueous phase during microsphere preparation, thus resulting in microspheres with low drug loadings. The modified solvent evaporation method based on an external aqueous phase is a more suitable method for the encapsulation of water-soluble drugs. The method is based on the formation of a multiple emulsion (w/o/w) from which the oil phase (generally an organic solvent such as dichloromethane or ethyl acetate) is evaporated to form free flowing particles. The key parameter for the successful entrapment of the drug is its insolubility in the organic polymer solution; the polymer solution separates the dispersed drug crystals or the internal aqueous phase from the external aqueous phase and thus reduces drug loss into the external aqueous phase. Various other encapsulation techniques are also commonly employed including solvent extraction (Chen et al., 2001; Deng et al., 2001; Jiang et al., 2002), phase inversion (Li et al., 2000; Sandor et al., 2001) and spray drying (Pavanetto et al., 1992; Baras et al., 2000; Fu et al., 2001).

There are three important factors which should be examined before the development of a polymer controlled release system. These are the polymer dependent factors, the drug

dependent factors and the carrier system dependent factors (Washington, 1990). The parameters which dictate the characteristics of polymeric drug delivery systems are summarised in Table 8-1.

Systems	Parameters influencing release
Polymer dependent	Molecular weight
	Molecular distribution
	Co-polymer ratio
	Crystallinity
Drug dependent	Solubility of drug
	Molecular weight of drug
	Polymer-drug interactions
Carrier system dependent	Drug loading
	Physical state of drug in polymer matrix (i.e. dissolved or dispersed)
	Particle size and particle size distribution
	Porosity
	Internal structure of microsphere

Table 8- 1 Factors influencing polymer controlled release systems

The agent is generally encapsulated inside the polymer matrix from which it is released, either through the process of diffusion or through the degradation of the surrounding polymer. The rate and release of the encapsulated agent can be controlled following the manipulation of variables such as the co-polymer ratio and the polymer molecular weight. The kinetics of release of peptides from biodegradable polymers has been reported to be bi-phasic, characterised by an initial burst release followed by a slower second release phase which has been attributed to the degradation of the encapsulating polymer (Fitzgerald and Corrigan, 1993).

The primary aim of the present work was to investigate the impact of a range of processing parameters on the physicochemical properties of particulate dosage forms of protein and DNA. These parameters which included polymer type and molecular weight, microsphere

manufacturing processes and temperature, emulsification time and speed, plasmid molecular weight, loading and outer to inner aqueous volume ratio were evaluated in terms of their effect on the size, shape and release characteristics of the protein and DNA loaded microspheres. A secondary aim was to assess the impact different processes might have on the interaction of such systems by cells representative of the immune system.

8.2 Characterisation of polymers

Two PLGA polymers, RG 502 and RG 752 were selected for manufacture of model systems. Both polymers have previously been used in the encapsulation of a wide range of agents and had demonstrated suitable properties for this study. Previous studies employing RG 502 and RG 752 with similar molecular weights to those used in this study included work carried out by Sah et al., (1994), Mehta et al., (1996), and McGee et al., (1997).

8.3 Microencapsulation of Human Serum Albumin

Human serum albumin loaded microspheres were prepared from PLGA RG 502 and RG 752 following a modified double emulsion technique in which the emulsion was homogenised for varying time periods. These time periods were based on typical emulsion times reported in several studies of between 80 seconds and 30 minutes (Sah et al., 1994; Uchida et al., 1994; Coombes et al., 1998; Wang et al., 2002). Table 8-1 outlines the different protein systems manufactured with regard to particle size, morphology and encapsulation efficiency.

Polymer	Homogenisation time (seconds)	Particle size D[v,0.5] (μm) (n=3)	Scanning electron microscopy	EE (%) (n=9)
RG 502	180 Ultra Turrax (T25) Solvent-evaporated	6.33 ± 0.41	Smooth outer surface Some aggregation of particles	12.61
	240 Ultra Turrax (T25) Solvent-evaporated	4.92 ± 0.15	Smooth outer surface Less aggregation than at 180 seconds	10.95
RG 752	180 Ultra Turrax (T25) Solvent-evaporated	4.79 ± 0.20	Smooth outer surfaces Spherical Some particle aggregation	8.22
	240 Ultra Turrax (T25) Solvent-evaporated	4.55 ± 0.02	Smooth outer surfaces Spherical No aggregation	8.81
RG 752	240 Silverson (L4R) Solvent-evaporated	6.65 ± 0.01	Smooth outer surfaces No aggregation	8.79
RG 752	240 Silverson (L4R) Spray-dried	48.12 ± 0.24	Doughnut shaped particles Some aggregation	41.11
RG 752	240 Ultra Turrax (T25) Spray-dried	8.30 ± 0.51	Doughnut shaped particles No aggregation	6.13

Table 8- 2 PLGA protein systems (EE – encapsulation efficiency)

Particle size and scanning electron microscopy

In Chapter 5 the relationship between emulsification time and particle size was examined. The emulsification time has a significant effect on particle size as has been noted by other workers (Ferdous et al., 1998; Sturesson et al., 1999). In the current study it was found that in the case of both polymers emulsification time had an inverse relationship to particle size (Figure 8-1). As the emulsification time increased from 180 seconds to 240 seconds the mean particle size of the microspheres decreased. With regards to particle size it was also found that particles formulated with RG 752, the more hydrophobic of the two polymers,

were significantly smaller than those formulated with RG 502. Jung et al (2002) reported higher particle dimensions for the more hydrophobic 75:25 PLGA versus 50:50 PLGA but did not indicate whether these particle sizes were statistically different. Further studies by Prior et al. (2002) also reported smaller particle sizes for PLGA 75:25 gentamicin loaded particles versus PLGA 50:50.

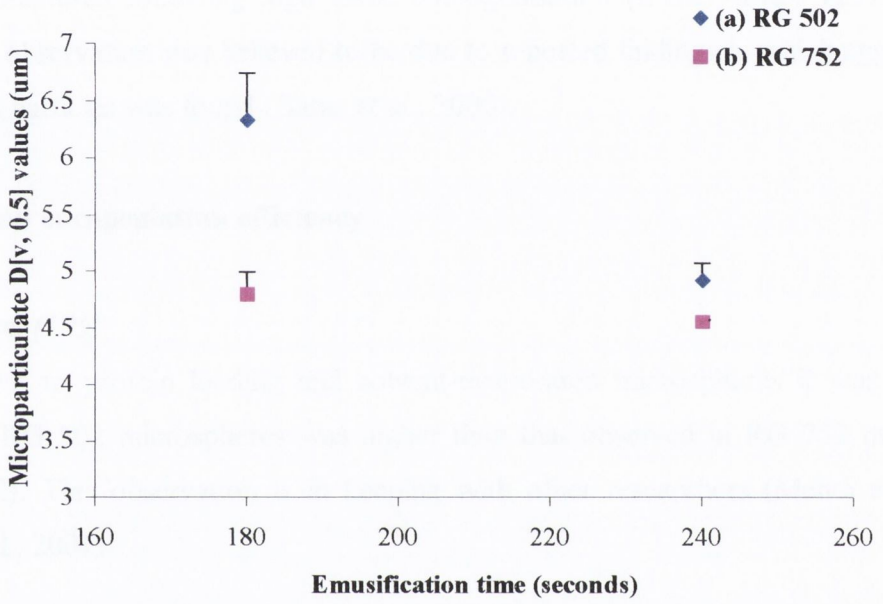

Figure 8- 1 Particle size of microspheres manufactured with RG 502 and RG 752, homogenised for 180 and 240 seconds

(samples represent the mean of three determinations)

Mehta et al. (1996) compared the particle size of RG 752 (15,577 Da) microspheres and RG 502 (10, 754 Da) microspheres and reported that RG 752 had a longer solidification time and a greater shrinkage rate which resulted in smaller particle size for RG 752 microspheres when compared with RG 502 microspheres

Scanning electron micrographs of microspheres formulated with a Silverson mixer showed microspheres with smooth surfaces and free from surface defects, which were present in some of the previous batches formulated following high-speed homogenisation. Emulsification with a Silverson homogeniser was carried out at 2,000 rpm compared with a speed of 8,000 rpm, which was used with the high-speed homogeniser (Ultra Turrax T25). This resulted in an increase in particle size, which was observed in microspheres formulated with the Silverson homogeniser when compared with those formulated with the

high-speed homogeniser (Ultra Turrax T25). This correlated with McGee et al. (1997) and Uchida et al. (1994) who reported that microparticulate size was dependent on emulsification speed, which resulted in a decrease in particle size with increasing homogenisation speed. The influence of spray drying on protein encapsulation was also evaluated and it was found that with regards to particle size, those microspheres homogenised with a Silverson mixer (L4R) and spray-dried were significantly larger than those manufactured following high-speed homogenisation (Ultra Turrax T25) and spray-dried. This observation was believed to be due to reported findings in which aggregation of spray-dried particles was found (Baras et al., 2000).

Loading and encapsulation efficiency

Ultra Turrax (T25)

With respect to protein loading and solvent-evaporated microspheres it was found that loading in RG 502 microspheres was higher than that observed in RG 752 microspheres (Figure 8-2). This observation is in keeping with other researchers (Mehta et al., 1996; Walter et al., 2001).

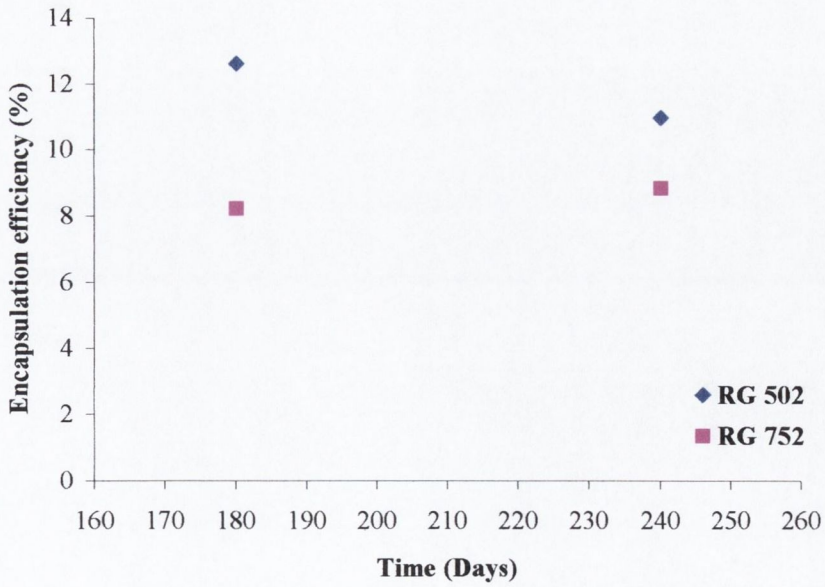

Figure 8- 2 Encapsulation efficiency according to polymer type

(samples represent the mean of nine determinations)

Silverson (L4R) and spray drier

Microspheres homogenised with a Silverson mixer (L4R) and spray-dried showed the highest encapsulation efficiencies of any protein-loaded systems examined. This observation was similar to other researchers (Pavanetto et al., 1992).

Protein release and modelling

The effect of polymer type and emulsification time on the release of HSA from PLGA microspheres was studied. Fractional release of human serum albumin from RG 502 was fitted to a mathematical model (Equation 1.15), which combined both the burst release of protein as seen from the release data and the sustained release as the polymer was seen to degrade (Gallagher and Corrigan, 2000). However due to the lack of polymer degradation seen in RG 752 microspheres the release from these systems was modelled to Equation 1-14. This equation was initially developed by Higuchi to describe diffusion from a slab and was then developed further by Peppas to describe diffusion from a sphere. While the encapsulation of proteins has been well documented there is limited information regarding modelling of protein release from PLGA systems. In the current study it was observed that the release from RG 502 microspheres was dependent on the initial burst release followed by a sustained release governed by the degradation of the polymer with almost 100% of total protein being released after one hundred days. Release from RG 752 microspheres was shown to fit an equation which describes diffusion from a polymer. This was due to the lack of degradation seen in these microspheres following one hundred days of incubation. This suggests that RG 752 would be suitable for use in longer sustained release systems with approximately 40% of total protein unreleased after one hundred days.

It was observed that fractional release of Human Serum Albumin fits well to both equations depending on the polymer used and that all batches exhibited a burst release in which, between 20 and 30% of the protein was released, followed by a secondary and in some cases a tertiary phase of drug release. The burst release from polymers has been attributed to the rapid release of the drug from the microsphere surface, while the depletion of the drug at the surface causes the cessation of the initial burst. The lag period then starts and lasts until extensive degradation of the polymer occurs (Wang et al. 2002). The burst fraction of protein released was found to be significantly higher for microspheres formulated with the RG 502 polymer when compared with the release from RG 752

microspheres. This is reflective of the degradation properties of the two polymers which indicated that RG 502 has a substantially shorter half-life when compared with RG 752 and which would result in greater protein release over the same time period. This would give rise to a longer lag release from microspheres formulated with RG 752 when compared with the more hydrophilic RG 502 and would allow RG 752 to be used in longer sustained release systems when compared with RG 502. The absorption of the protein back onto the surface of the microspheres was examined and it was found that when empty microspheres were incubated with protein for a pre-determined time the levels of protein detected were not significantly different from samples of protein alone (see Appendix).

Integrity of released protein

The integrity of HSA following encapsulation was examined following SDS-PAGE and silver staining of the gel. In the case of microspheres manufactured following homogenisation with an Ultra Turrax (T25) or a Silverson mixer (L4R) there was no denaturation of the protein when compared with control protein. However spray-dried microspheres homogenised with an Ultra Turrax (T25) and spray-dried at an inlet temperature of 80°C exhibited signs of denaturation with the presence of extra bands in acrylamide gel. This was not observed in microspheres which were homogenised with a Silverson mixer (L4R) and spray-dried at the same temperature. These results suggest that even at high inlet temperatures there are manufacturing techniques which allow for the spray drying of heat sensitive agents.

From the protein study it was observed that microspheres manufactured with RG 752 homogenised for 240 seconds with the Silverson mixer (L4R) displayed favourable surface morphologies, particle sizes and release characteristics. Importantly SDS-PAGE analysis of the extracted protein showed that this manufacturing technique did not appear to denature the protein.

8.4 Microencapsulation of plasmid DNA

Since the tertiary structure of DNA or topology has been shown to influence gene expression in vitro and in vivo, it is useful to think about DNA as a potential drug

substance in comparison to proteins. In terms of stability however, DNA has some very distinct advantages over proteins which can be inactivated under mild conditions simply due to small changes in their tertiary or quaternary structure. DNA usually requires chemical modification to produce an irreversible loss of biological activity (Middaugh et al., 1998). The encapsulation of proteins in PLGA microspheres following high-speed homogenisation and sonication has been well documented (Uchida et al., 1994; Yang et al., 2000 a). However these techniques have been shown to be unsuitable for the encapsulation of agents such as DNA and other nucleic acids, which have been shown to be susceptible to the high shear rate of such techniques (Adami et al., 1998; Middaugh et al., 1998). It was for these reasons that the integrity and physiochemical properties of HSA, encapsulated in RG 752, were studied following alternative encapsulation techniques. It was decided to investigate formulations using a Silverson mixer rather than the high-speed homogeniser (Ultra Turrax T25). Silverson homogenisation is a commonly used technique where the stability of the encapsulated agent is an issue. It is used in place of high-speed homogenisation and has been used with agents susceptible to denaturation or shearing (McKeever et al., 2002).

Processing parameters were shown in Chapter 5 to be influential in the physicochemical and release characteristics of a model protein. Several techniques have been documented in the encapsulation of DNA including solvent evaporation (Jones et al., 1997; Wang et al., 1999; Perez et al., 2001), heat-extrusion (Jong et al., 1997), spray drying (Walter et al., 2001) and phase inversion nanoencapsulation (Mathiowitz et al., 1997).

In the current study several manufacturing parameters were evaluated for their effects on the physicochemical properties of encapsulated DNA. These parameters included manufacturing temperatures, plasmid molecular weight, plasmid loading, outer to inner aqueous volume ratio and manufacturing technique. The effects of these processing parameters can be seen in Table 8-3.

Parameters	Variable parameter	Particle size D[v,0.5] (μm) (n=3)	SEM	EE (%) (n=3)
Temperature	4°C	6.92 ± 0.63	Some wrinkling of outer surface	43.513
	20°C	4.48 ± 0.51	Some wrinkling of outer surface	28.513
	37°C	3.32 ± 0.14	Defined wrinkling of outer surface with surface pores	29.344
Plasmid molecular weight (kDa)	2.65	4.49 ± 0.10	Smooth outer surfaces	29.465
	3.26	4.07 ± 0.28	Smooth outer surfaces	28.513
	4.43	2.89 ± 0.00	Wrinkled surface	15.491
Outer:inner aqueous volume	10:1	3.49 ± 0.20	Aggregation of particles	13.954
	15:1	3.50 ± 0.05	Wrinkled surfaces	10.094
	20:1	4.48 ± 0.51	Smooth outer surfaces	28.513
Theoretical loading μg plasmid DNA/mg PLGA	2.4	4.48 ± 0.51	Smooth outer surface	28.513
	4.7	3.53 ± 0.01	Some visible wrinkling	9.461
	11.8	3.10 ± 0.02	Wrinkled outer surface	9.021
Manufacture	Spray-drying	2.92 ± 0.00	Doughnut shaped particles	91.111
Manufacture process with different theoretical loading	Silverson (L4R) 0.47%	4.48 ± 0.51	Slightly wrinkled surfaces	9.021
	Silverson (L4R) 1.18%	3.10 ± 0.02	Slightly wrinkled surfaces	9.462
	Ultra Turrax (T25) 0.47%	4.55 ± 0.02	Smooth outer surfaces	4.427
	Ultra Turrax (T25) 1.18%	7.16 ± 0.50	Smooth outer surfaces	3.011

Table 8- 3 RG 752 DNA microparticulate systems

SEM –DNA systems

Microsphere morphology was found to be influenced by all techniques and observed changes included the production of pores in the outer layer and an increase in wrinkles in the surface layer. All batches of microspheres produced showed spherical particles with similar particle sizes. Outer pore formation was seen to increase as manufacturing temperatures increased. This was in good agreement with other researchers who reported similar observations (Jeyanthi et al., 1996; Yang et al., 2000 b). Microsphere morphology was also seen to be influenced by plasmid molecular weight and loading concentrations where the increase in molecular weight resulted in a more wrinkled outer surface. This correlated well with studies carried out by Sandor et al., (2001) where the morphology of microsphere encapsulated with different molecular weight agents was reported to have different external morphologies. Walter et al., (2001) reported that increased DNA loading resulted in a more wrinkled appearance. The aqueous phase ratio used during the formulation of DNA loaded microspheres was found to be an important factor in the external morphology of resultant microspheres. It was observed that microspheres formulated with low ratios of external to internal aqueous phases were not free flowing and exhibited particle aggregation. Spray-dried DNA-loaded microspheres were found to exhibit particle shapes unlike any other batches. These microspheres were found to be "doughnut" shaped, an observation which might be attributed to the hollow nature of spray-dried particles as reported by Broadhead et al. (1992). Several other researchers have reported particles of a non-spherical nature produced following spray drying (Pavanetto et al., 1993; Stahl et al., 2002).

Particle size –DNA systems

Median particle size data for all batches were found to be below 5 μm except for microspheres with 11.8 μg plasmid DNA/mg PLGA manufactured following Ultra Turrax homogenisation (T25), which were slightly larger in size. Results showed an inverse relationship between particle size and manufacturing temperature. This observation is in good agreement with published data (Yang et al., 2000 b). Interestingly there appeared to be no direct correlation between plasmid molecular weight and particle size. The largest particles observed were those containing the pGL3 plasmid (3.26 kDa) with those containing the pRL-CMV and pSV-B-galactosidase plasmid (2.65 and 4.43kDa

respectively) exhibiting smaller particle sizes. To our knowledge although other researchers have studied the effect of plasmid molecular weight no studies have been carried out on the influence of plasmid molecular weight on particle size. Results reported here were similar to observations reported by Sandor et al. (2001) who reported no relationship between the molecular weight of various encapsulated proteins and resultant particle sizes. There also appeared to be no correlation between outer aqueous volume ratios and particle sizes.

Loading and encapsulation efficiencies – DNA systems

The effect of different processing parameters had been studied with respect to protein encapsulation however, to our knowledge, the effect of processing parameters such as temperature has not yet been studied extensively with respect to DNA encapsulation.

Temperature

With respect to DNA loading it was found that temperature and plasmid molecular weight had a significant influence on DNA loading. As the temperature increased DNA loading was seen to decrease. Encapsulation efficiency indicated that DNA loading was greatest for microspheres manufactured at 4°C (43.51%) while those manufactured at 20°C (28.51%) exhibited the lowest loading values. Wang et al. (1999) reported similar encapsulation efficiencies following encapsulation of plasmid DNA in PLGA 50:50. They reported values of between 22.5 % and 53.3 % encapsulation efficiencies depending on the molecular weight of the PLGA used.

Molecular weight

In the case of the molecular weight of the DNA decreased loadings were reported for microspheres loaded with DNA of increasing molecular weight. In the case of plasmid molecular weight there appeared to be a direct inverse relationship between the size of the encapsulated plasmid and DNA loading with 29.47% in the case of the smallest plasmid studied, pRL-CMV, 28.51% in the case of pGL3 with the largest plasmid, pSV-β-Galactosidase exhibiting an encapsulation efficiency value of 15.49%.

Outer:inner aqueous volume ratio

Variations in the ratio of outer to inner aqueous volumes did not appear to have a direct correlation with loading of DNA. However loading with respect to aqueous volume ratio mirrored the particle size data for the same formulation and it would appear that there is a relationship between particle size and aqueous volume. This correlated well with Jeffery et al. (1993) who reported that there was a linear relationship between the volume of external aqueous phase and the amount of protein entrapped. DNA encapsulation efficiency levels were found to be highest for those microspheres which had been spray-dried with encapsulation efficiency levels of 91.11%. Encapsulation efficiencies reported here are in line with other researchers (Liu et al. 2002; Wang et al. 1999)

DNA release and modelling

Release of DNA from a microsphere delivery system depends on both erosion (subsequent to hydrolysis) and diffusion. A variety of factors can theoretically influence DNA release from microspheres, including chemical properties of the polymer, molecular weight, particle size and morphology, DNA loading and DNA solubility. This suggests that there are potentially very many approaches to controlling DNA release from microspheres. Therefore it is of interest that the models selected in this work in most cases seem to predict or quantify DNA release rates with good statistical fit.

Also, to our knowledge, this is one of the first approaches towards modelling of fractional DNA release from RG 752 microspheres. Luo et al. (1999) reported fitting the rate of release of DNA from implantable polymer matrices (EVAc: poly(ethylene-co-vinyl acetate) at early time points to evaluate the diffusion coefficient for several different DNA matrices. They used a model which quantified the rate of release from a disc. The model accounted for the cumulative mass of released DNA, the initial mass of the disc, the apparent diffusion coefficient and the thickness of the disc. In the current study data obtained from fitting the release results to Equation 1-14 demonstrated n and MSC values, which indicated good fits between the actual and theoretical data. Equation 1-14 as previously explained describes diffusion from a polymer.

Temperature and molecular weight

From Chapter 6 it can be seen that the microspheres formulated at high temperatures resulted in an increased rate of release when compared with those formulated at lower temperatures. It is believed that microspheres formulated at higher temperatures solidify more quickly which would promote the entrapment of DNA in the outer surface of the microsphere (Yang et al., 2000 a). This in turn is believed to increase the percentage of entrapped DNA available for initial release, which in this case results in the higher release of DNA over a given time period. Differences in plasmid molecular weight result in different release rates. Microspheres loaded with the smallest molecular weight plasmids exhibited the greatest release over the time period of the study. Release from microspheres loaded with the other two plasmid showed no statistical significance between each other. This was in good agreement with Sandor et al. (2001) who reported that the rate of release of protein from PLGA 50:50 microspheres was fastest for smaller molecular weight proteins.

Outer:inner aqueous volume ratio and loading

The pattern of plasmid release from microspheres formulated with varying outer :inner ratios of 15:1 and 20:1 appears to be biphasic with an initial burst release followed by a second phase of slower release (lag phase). However in the case of microspheres formulated with an outer:inner ratio of 10:1 release appears to follow a more linear pattern of release with no burst release. This lack of burst release would indicate a more complete encapsulation of the DNA within the polymer matrix in the case of microspheres formulated with a 10:1 outer:inner aqueous volume. DNA loading resulted in the highest release for microspheres loaded with 1.18% w/w DNA however when this was converted into fractional release it can be seen that over one hundred days the release from these microspheres was lower than that from other batches. Again this could be attributed to the more complete encapsulation of the DNA when present in higher concentrations and due to the increased particle sizes of these microspheres the diffusion of the DNA through the polymer matrix would be slower when compared with smaller particles.

Manufactuing process

DNA microspheres were also formulated following high-speed homogenisation (Ultra Turrax T25) and the release of DNA compared with that from those emulsified using a Silverson homogeniser. It was found that release from microspheres emulsified with a

Silverson homogeniser was higher than that from high-speed homogenised microspheres. This could be attributed to the shear rates associated with high-speed homogenisation and to the resulting denaturation and possible destruction of the DNA during the formulation process. Microspheres formulated following spray drying also had a lower rate of release when compared with those formulated following freeze drying, a result which might be due to the high temperatures of spray drying when compared with freeze drying. A DNA agarose gel of DNA extracted from the three batches of microspheres indicated a process related denaturation of the plasmid DNA from supercoiled to open circular and linear forms of DNA. This indicated that denaturation and shearing of the DNA is a potential problem with several well-used techniques. This has been studied by other researchers in the field and several studies aimed at protecting the DNA in plasmid form during microencapsulation have been attempted (Walter et al., 1999; Hao et al., 2000).

In summary several variables were introduced during the encapsulation of DNA in PLGA microspheres, which resulted in different physicochemical properties. These results show that variation in such factors should allow for the development of sustained released formulations with the desired characteristics.

8.5 Biological potency of extracted DNA and macrophage phagocytosis

Plasmids are extremely sensitive to several of the commonly used techniques involved in encapsulation such as temperature, sonication and high speed homogenisation. Plasmid DNA exists in three different conformational forms: supercoiled, open circular and linear. Recent reports have shown that conversion of plasmid DNA from the supercoiled form to the open circular form caused a 10% reduction in the gene transfer efficiency and a 90% reduction in the case of a linear DNA when compared with supercoiled DNA was observed (Adami et al., 1998). Shear stresses including sonication, vortexing, freeze-thaw cycles, exposure to UV light and heat can convert plasmid DNA from its supercoiled isoform to open circular, linear or fragmented nucleic acid (Capan et al., 1999). Due to the requirements of regulatory bodies such as the FDA nucleic acid vaccines must be fully characterised. It is of interest to investigate how in vitro systems can be studied to elucidate the interaction of such systems with cells of the immune system, their ultimate target.

In recent years a number of recombinant proteins, synthetic peptides and nucleic acids have been developed as potential therapeutic agents. The most commonly employed systems for the delivery of such agents comprises of microparticles developed from the poly(lactide-co-glycolide) polymers. A critical issue in the development of such systems is the determination of the levels and the structure of the released agent. There are several methods of quantitatively and qualitatively examining an encapsulated agent. In the current study two techniques were used in the quantitative examination of encapsulated DNA. These were mammalian cell transfection and bacterial cell transformation.

Bacterial cell transformation

Bacterial cell transformation is based on the principle of antibiotic resistance. Within the plasmid DNA used in this study is an ampicillin resistant gene which allows successfully transformed bacterial cells to grow on agar plates containing ampicillin. DNA was extracted from microspheres and was shown to transform *E. Coli* cells successfully. Tomley (1996) reported that the number of colonies obtained from each transformation is variable. Colony growth is dependent on several factors, such as the amount of DNA added and the efficiency of transformation. As a result there is no direct relationship between amount of DNA used and the number of colonies observed. However it has been reported that this type of assay method is more sensitive to DNA isoform than cell transfections (Hao et al., 2000) and as such allows for the assumption that plasmid DNA structure was conserved to a certain degree following encapsulation independent of homogenisation speeds and temperature of manufacture.

COS-7 cell transfections

COS-7 cells were transfected with extracted DNA from microspheres homogenised with a Silverson mixer (L4R) and an Ultra Turrax (T25) and solvent-evaporated or spray-dried. In all cases extracted DNA transfected cells successfully resulting in the production of luciferase enzyme as determined by luminometer results. This result confirms the transformation results and demonstrates that the manufacturing procedures examined would allow for the encapsulation of biologically active and potent DNA. This method may be better at predicting the biological potency of extracted DNA than bacterial transformation. This is due to the sensitivity of transformation experiments which may

underestimate the potency of DNA due to the inability of linear DNA to successful transform bacteria while still being able to transfect cells.

Macrophage cell model

Macrophage cells have been shown to play a major role in host defence against many infectious agents including bacteria, viruses, protozoa and parasites. Macrophages migrate to an infected focus following the attraction by a variety of substances including bacterial components and endotoxins. Once they reach the infected focus they may phagocytose and kill infectious agents (Chapter 2). By taking protein antigens and generating immunogenic fragments from them, macrophage cells also play a significant role in inducing and regulating the immune response (Ahsan et al. 2002). Macrophages are also important components of the host's frontline defence against tumour cells. However macrophages can be used as safe havens for some microorganisms resulting in macrophages acting as a reservoir for these microorganisms. Therefore targeting of drugs to macrophages could be used in the treatment of infectious diseases such as schistosmiasis and brucellosis. Drug targeting to macrophage cells may also be useful in the treatment of cancer, HIV and tuberculosis. Because of macrophages possible role in the pathogenesis of some diseases such as rheumatoid arthritis, systemic lupus and multiple sclerosis targeting macrophage cells could be useful in treatment of such diseases. To our knowledge there is only one product in the US market Ambisome® (Gilead, Boulder, CO) used in the treatment of visceral leishmaniasis, which is used to target amphotericin B to macrophage cells. Other studies, which target macrophage cells currently in clinical trials, include liposomal doxorubicin for the treatment of cancer and HIV (Hengge et al. 2001). These studies show that the employment of the phagocytic activity of macrophage cells offers an attractive opportunity to target antigen in microparticles directly to these potent stimulators of the immune system.

Particulate uptake by a macrophage model was studied because the feasibility of biodegradable microspheres for DNA and protein delivery has been confirmed under in vivo conditions but detailed information about the interaction of the delivery system and target cells is still lacking.

In the current work we examined the uptake by macrophage cells of RG 502 and RG 752 protein-encapsulated microspheres. Results illustrated that particles manufactured with different polymer compositions displayed different uptake patterns. This has been reported previously and has been attributed to surface hydrophobicity and zeta potential surface charge difference between the polymers (Takada and Ikada, 1988). In the current studies it was found that protein-loaded particles composed of PLGA 75:25 were more readily phagocytosed than protein-loaded PLGA 50:50 particles with PLGA 75:25 and PLGA 50:50 particles bearing surface charges of –40.0 $\pm$ 2.3 mV and – 23.0 $\pm$ 3.9 mV respectively. There was no significant difference in particle size values between both systems.

The uptake of DNA-loaded RG 752 microspheres manufactured using solvent-evaporation and spray-drying techniques were also examined. In relation to manufacturing techniques it was found that both spray-dried and solvent-evaporated DNA-loaded RG 752 microspheres were readily taken up by microspheres with zeta potential readings of –5.2 $\pm$ 3.2 and 0.9 $\pm$ 0.4 mV respectively. Higher numbers of the spray-dried microspheres were phagocytosed over 24 hours. This observation has been reported previously by Walter et al., (2001) who reported that spray-dried DNA-loaded PLGA microparticles of less than 10 μm were readily phagocytosed despite differences in surface charge.

Conclusions

In this study we explored microencapsulation of DNA based on manufacturing conditions originally developed from investigations using human serum albumin as a model protein. A range of processing conditions were studied as well as the effect of increasing plasmid molecular weight on the characteristics of the microparticulate systems. Qualitative and quantitative analysis of the plasmid prior to and after encapsulation was carried out. Assays of both plasmid functionality and particulate uptake were carried out in appropriate cell culture models.

We conclude that like any therapeutic agent, effective DNA formulations must allow for administration of the DNA in a manner which optimises its stability. We suggest that microencapsulation of DNA by spray-drying would seem to be the optimal method to

prepare delivery systems for DNA vaccination. Direct targeting of phagocytic cells is a potential mechanism of action. Gene expression in target cells will be necessary to confirm that such dosage forms offer a realistic option as novel, efficacious vaccine systems.

Future work

Development of more sophisticated cell models e.g. M-cell models for oral PLGA systems which would allow assessment of the transfection ability of the systems. In the current study it was not possible to evaluate the production of protein from the macrophage cell model due to the possible effect that the polymer degradation products would have on the macrophage cell cycle. This area needs to be developed further before this level of examination could be achieved.

Identification of suitable dyes/reagents which would allow more sensitive discrimination of different DNA loadings in microparticles. Ethidium bromide has been shown to be useful in the labelling of DNA however it does not allow for the quantification of DNA samples due to binding restrictions. Commercially available dyes such as PicoGreen might lend themselves to such an application since it is selective for double stranded DNA however this dye has only been validated for DNA detection for in vitro release studies presumably due to the likely interference by the various reagents utilised in microencapsulation.

Finally longer release studies need to carried out in particular on RG 752 microspheres to elucidate and model the behaviour of the DNA over a longer time period. In the current study a period of one-hundred days was used, this needs to be prolonged in order to fully characterise the release of DNA from this polymer which was shown to be the most efficient polymer examined in terms of immune cell targeting.

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Appendices

Appendix I

Protein release calibration curves

Calibration curves used during protein release studies were carried out according to the guidelines provided with the MicroBCA protein assay kit (Pierce). The curve was calculated from the absorbances recorded at 562 nm of a series of dilutions. It involved a series of seven dilutions of Bovine Serum Albumin. Concentrations of unknown samples were determined in mg/ml. An example of this is shown in Table 1 and the resulting calibration curve in Figure 1.

Concentration mg/ml	Abs 562 nm
40	0.872
20	0.57547
10	0.36234
5	0.20732
2.5	0.14043
1	0.11969

Table 1 calibration curve results

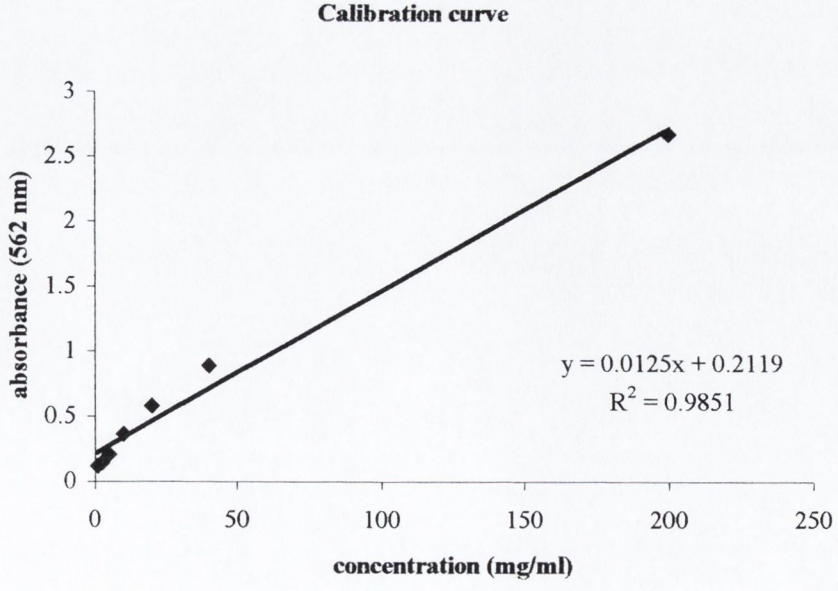

Figure 1 Example of protein calibration curve

Appendix 2

Composition of buffers used during DNA preparation and purification

Buffers listed in Table 2 were used in Chapter 4 Section during the preparation and purification of DNA from *E. Coli* cells and belong to the Qiagen Plasmid Purification kit.

Buffer	Composition	Storage
Buffer P1 (Resuspension Buffer)	50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A	2-8°C after addition of RNase A
Buffer P2 (Lysis Buffer)	200 mM NaOH, 1% SDS (w/v)	Room temp
Buffer P3 (Neutralisation Buffer)	3.0 M potassium acetate, pH 5.5	Room temp
Buffer FWB (QIAfilter Wash Buffer)	750 mM NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v)	Room temp or 2-8°C
Buffer QBT (Equilibration Buffer)	750 mM NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v) 15% Triton®X-100 (v/v)	Room temp
Buffer QC (Wash Buffer)	750 mM NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v)	Room temp
Buffer QF (Elution Buffer)	1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol (v/v)	Room temp
Buffer QN (Elution Buffer)	1.6 M NaCl 50mM MOPS, pH 7.0; 15% isopropanol (v/v)	Room temp
TE	10 mM NaCl; 10 mM Tris-Cl, pH 8.0 1 mM EDTA	Room temp

Table 2 List of buffer components used in Qiagen plasmid preparation kit

Appendix 3

DNA release calibration curves

DNA release was determined using a PicoGreen® dsDNA Quantitation Reagent kit which determines double stranded DNA on the basis of fluorescence intensity. Calibration curves were created using a five-point calibration curve the protocol of which is listed in Table 3. Table 4 lists a typical calibration curve used during the study and Figure 2 illustrates a typical calibration curve.

Volume (µl) of 2 µg/ml DNA stock	Volume (µl) of TE	Volume (µl) of diluted PicoGreen Reagent	Final DNA concentration in PicoGreen assay
1000	0	1000	1 µg/ml
100	900	1000	100 ng/ml
10	990	1000	10 ng/ml
1	999	1000	1 ng/ml
0	1000	1000	blank

Table 3 protocol for standard curve

Volume (µl) of 2 µg/ml DNA stock	Volume (µl) of TE
1000	0
100	900
10	990
1	999
0	1000

Table 4 Typical calibration curve

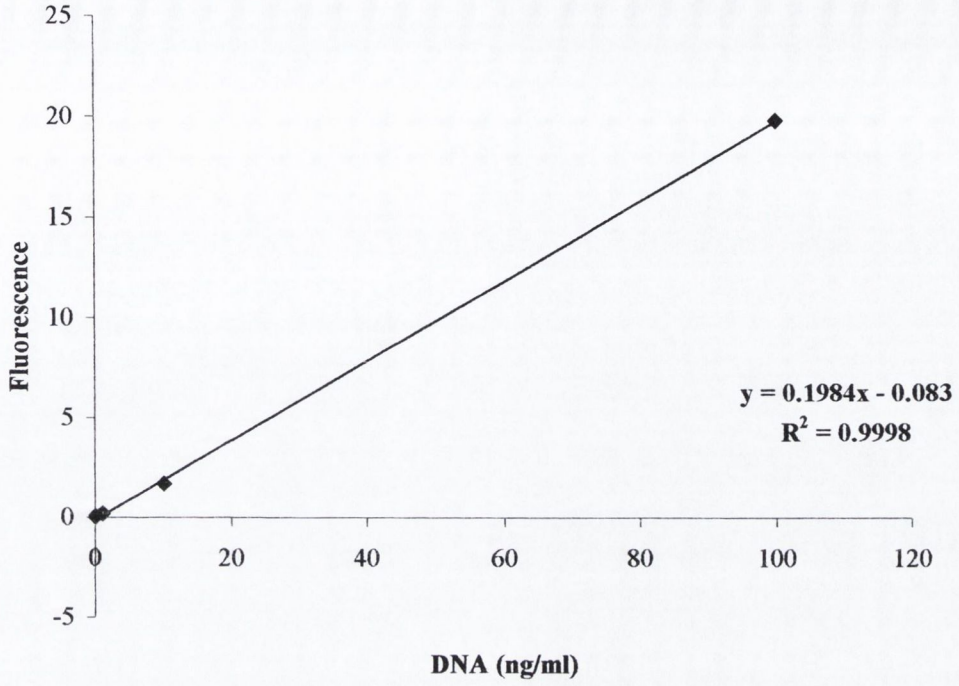

Figure 2 Typical calibration curve used in the determination of DNA concentrations

Appendix 4

Particle size analysis of protein and DNA-loaded microspheres

The particle size of microspheres was determined by laser diffraction with a Malvern 2600. A 63 mm lens was used which typically accommodates particles in the size range 0.5 μm - 118 μm. The samples were measured in triplicate. 10 mg of microspheres were blended with 5 ml of a 0.2% w/v solution of Tween 20 and sonicated for 10 minutes prior to measurement. The particles were plotted as cumulative (%) undersize with a histogram of the distribution. Figures 3 and 4 below illustrate typical data and histograms reported for the particle sizes of protein and DNA-loaded microspheres.

High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	Span 1.55
118	100	53.3	100	24.0	99.8	10.8	94.1	4.84	63.6	2.18	4.8	D[4,3] 5.00µm
110	100	49.5	100	22.3	99.8	10.0	93.0	4.50	59.3	2.03	3.5	
102	100	46.1	100	20.7	99.6	9.31	91.7	4.19	54.8	1.88	2.6	D[3,2] 3.69µm
95.2	100	42.8	100	19.3	99.3	8.66	90.1	3.89	49.7	1.75	2.1	
88.6	100	39.8	100	17.9	98.9	8.05	88.2	3.62	43.9	1.63	1.9	D[v,0.9] 8.63µm
82.4	100	37.0	100	16.7	98.4	7.49	85.8	3.37	37.4	1.52	1.7	
76.6	100	34.4	100	15.5	97.8	6.97	83.1	3.13	30.3	1.41	1.6	D[v,0.1] 2.55µm
71.2	100	32.0	100	14.4	97.2	6.48	79.8	2.91	22.4	1.31	1.4	
66.2	100	29.8	100	13.4	96.6	6.02	76.2	2.71	14.4	1.22	1.1	
61.6	100	27.7	99.9	12.5	95.9	5.60	72.1	2.52	5.4			D[v,0.5] 3.91µm
57.3	100	25.8	99.9	11.6	95.1	5.21	67.9	2.34	6.8			

Source = Res.:fiona
Record No. = 56
Focal length = 63 mm
Presentation = pil

Beam length = 14.3 mm
Log. Diff. = 2.456
Obscuration = 0.1516
Volume distribution

Model indp
Volume Conc. = 0.0014%
Sp.S.A 1.6291 m²/cc.

Figure 3a Particle size data for RG 752 microspheres homogenised for 180 seconds

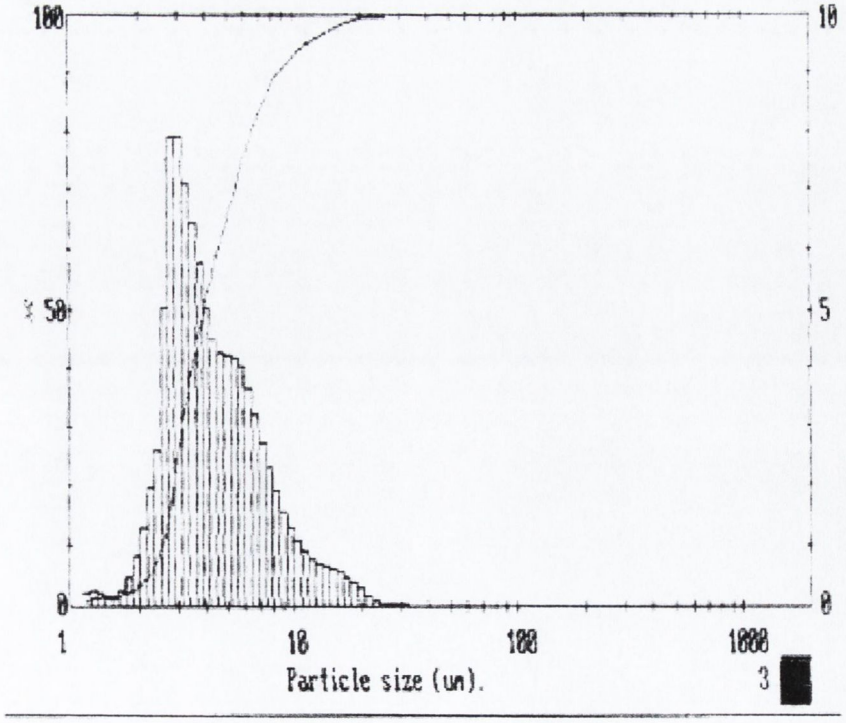

Figure 3b Particle size distribution for RG 752 microspheres homogenised for 180 seconds

2615 pil 1a66294

High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	High Size	Under %	Span 1.00
118	100	53.3	100	24.6	100	10.8	100	4.84	87.9	2.18	9.4	D[4,3] 3.27µm
110	100	49.5	100	22.3	100	10.0	100	4.50	84.9	2.03	3.9	
102	100	46.1	100	20.7	100	9.31	99.9	4.19	81.8	1.88	1.3	
95.2	100	42.8	100	19.3	100	8.66	99.9	3.89	78.3	1.75	0.4	D[3,2] 2.96µm
88.6	100	39.8	100	17.9	100	8.05	99.8	3.62	73.5	1.63	0.0	
82.4	100	37.0	100	16.7	100	7.49	99.7	3.37	68.5	1.51	0.0	
76.6	100	34.4	100	15.5	100	6.97	99.3	3.13	61.0	1.41	0.0	D[v,0.9] 5.10µm
71.2	100	32.0	100	14.4	100	6.48	98.4	2.91	51.6	1.31	0.0	
66.2	100	29.8	100	13.4	100	6.02	96.5	2.71	39.2	1.22	0.0	
61.6	100	27.7	100	12.5	100	5.60	93.9	2.52	27.5			D[v,0.1] 2.15µm
57.3	100	25.8	100	11.6	100	5.21	90.9	2.34	17.4			

Source • Res.:fiona	Beam length • 14.3 mm	Model indp
Record No. • 67	Log. Diff. • 2.567	
Focal length • 63 mm	Obscuration • 0.1797	Volume Conc. • 0.0014%
Presentation • pil	Volume distribution	Sp.S.A 2.0268 m²/cc.

D[v,0.5] 2.85µm

Figure 4a Particle size data for pSV-β-Galactosidase

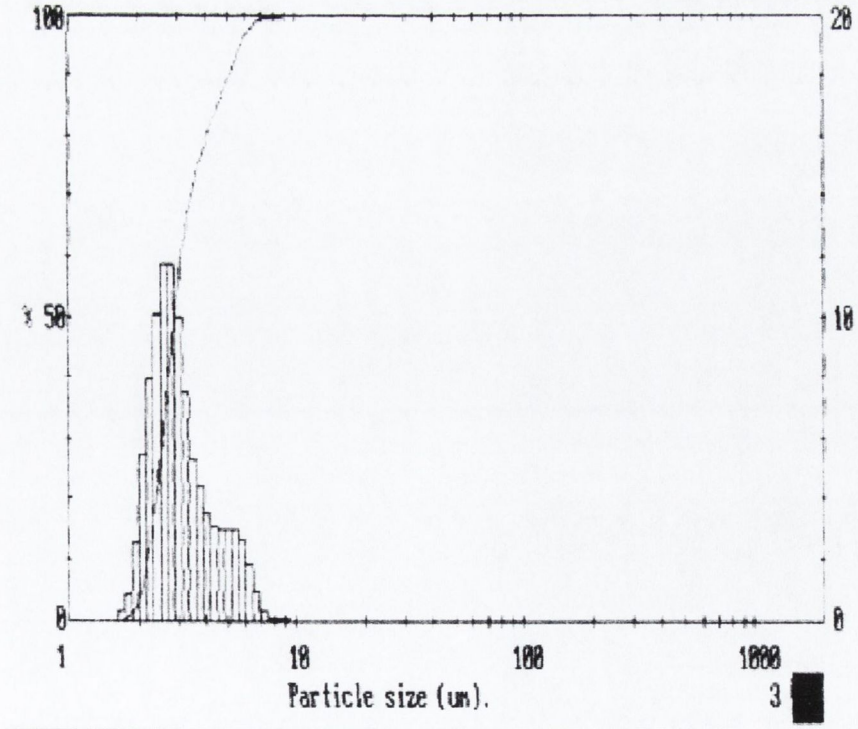

Figure 4b Particle size distribution of pSV-β-Galactosidase

Appendix 5

Protein adsorption onto microspheres during release studies

Figure 5 illustrates the concentration of HSA in the presence and absence of empty microspheres at 37°C. It shows the change in concentration of human serum albumin in the presence of empty microspheres compared with the change in concentration of the same protein without microspheres. The experiment was carried out in order to assess whether or not the protein release data was being affected by re-adsorption of protein back onto the surface of the microspheres prior to long term release studies.

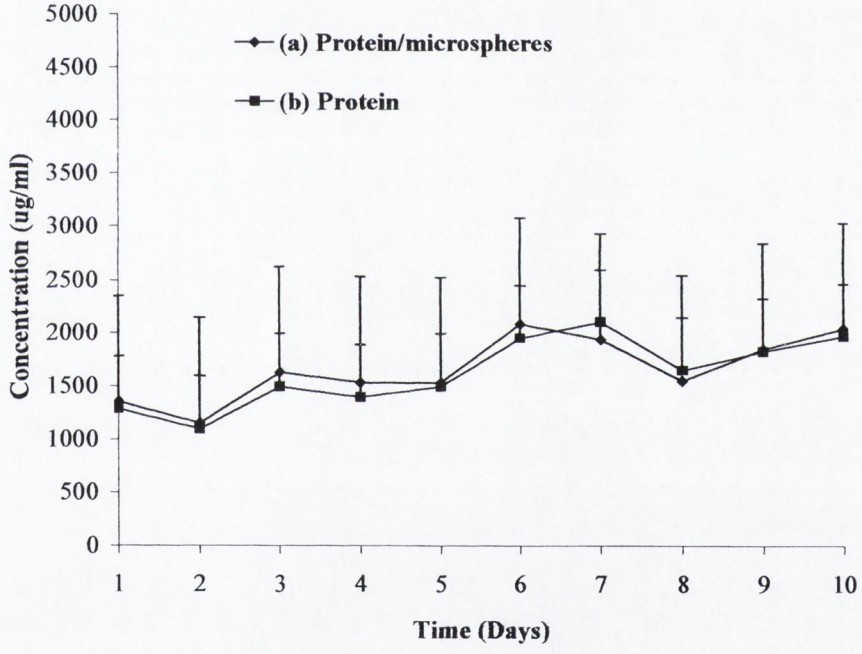

Figure 5Adsorption of HSA (a) protein and empty microspheres (b) control protein

The profiles illustrated indicate no variation in the concentration of protein in the presence of empty microspheres relative to protein solution alone.

Appendix 6

Mathematical modelling of release profiles

Equation 1-14

// MicroMath Scientist Model File

IndVars: T

DepVars: W

Params: K, n

W=K*T^n

***

Equation 1-15

// MicroMath Scientist Model File

IndVars: T

DepVars: FTOT

Params: KB, TMAX, FB, K

FTOT = FB*(1-EXP(-KB*T)) + (1- FB)*((EXP (K*T-K*TMAX))/ (1+(EXP(K*T-K*TMAX)))

Appendix 7

Genetic sequence of pRL-CMV, pGL3 and pSV-β-Galactosidase

Listed below are the genetic sequences and the sequence reference points of each of the three plasmid vectors used in the study. The sequences are listed in order of plasmid molecular weight; pRL-CMV, pGL3 and pSV-β-Galactosidase.

pRL-CMV Vector sequence map

pRL-CMV Vector* sequence reference points:

base pairs	4079 bases
CMV Enhancer and Immediate early promoter	7-803
Chimeric intron	860-996
T7 promoter (-17 to +2)	1040-1058
T7 promoter transcription start site	1057
Rluc reporter gene	1068-2003
SV40 late polyadenylation signal	2045-2246
Beta-lactamase (AmpR) coding region	2393-3253

..

1 AGATCTTCAA TATTGGCCAT TAGCCATATT ATTCATTGGT TATATAGCAT

51 AAATCAATAT TGGCTATTGG CCATTGCATA CGTTGTATCT ATATCATAAT

101 ATGTACATTT ATATTGGCTC ATGTCCAATA TGACCGCCAT GTTGGCATTG

151 ATTATTGACT AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA

201 GCCCATATAT GGAGTTCCGC GTTACATAAC TTACGGTAAA TGGCCCGCCT

251 GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA TGACGTATGT

301 TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA TGGGTGGAGT

351 ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA

401 AGTCCGCCCC CTATTGACGT CAATGACGGT AAATGGCCCG CCTGGCATTA

451 TGCCCAGTAC ATGACCTTAC GGGACTTTCC TACTTGGCAG TACATCTACG

501 TATTAGTCAT CGCTATTACC ATGGTGATGC GGTTTTGGCA GTACACCAAT

551 GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC
TCCACCCCAT

601 TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG
GACTTTCCAA
651 AATGTCGTAA TAACCCCGCC CCGTTGACGC AAATGGGCGG
TAGGCGTGTA

701 CGGTGGGAGG TCTATATAAG CAGAGCTCGT TTAGTGAACC
GTCAGATCAC

751 TAGAAGCTTT ATTGCGGTAG TTTATCACAG TTAAATTGCT AACGCAGTCA

801 GTGCTTCTGA CACAACAGTC TCGAACTTAA GCTGCAGAAG
TTGGTCGTGA

851 GGCACTGGGC AGGTAAGTAT CAAGGTTACA AGACAGGTTT
AAGGAGACCA

901 ATAGAAACTG GGCTTGTCGA GACAGAGAAG ACTCTTGCGT
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951 CACCTATTGG TCTTACTGAC ATCCACTTTG CCTTTCTCTC CACAGGTGTC

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AATACGACTC

1051 ACTATAGGCT AGCCACCATG ACTTCGAAAG TTTATGATCC
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1101 AAACGGATGA TAACTGGTCC GCAGTGGTGG GCCAGATGTA
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TATAGGTTAC

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1451 TTATAGCTAT GAGCATCAAG ATAAGATCAA AGCAATAGTT CACGCTGAAA

1501 GTGTAGTAGA TGTGATTGAA TCATGGGATG AATGGCCTGA TATTGAAGAA

1551 GATATTGCGT TGATCAAATC TGAAGAAGGA GAAAAAATGG TTTTGGAGAA

1601 TAACTTCTTC GTGGAAACCA TGTTGCCATC AAAAATCATG AGAAAGTTAG

1651 AACCAGAAGA ATTTGCAGCA TATCTTGAAC CATTCAAAGA GAAAGGTGAA

1701 GTTCGTCGTC CAACATTATC ATGGCCTCGT GAAATCCCGT TAGTAAAAGG

1751 TGGTAAACCT GACGTTGTAC AAATTGTTAG GAATTATAAT GCTTATCTAC

1801 GTGCAAGTGA TGATTTACCA AAAATGTTTA TTGAATCGGA CCCAGGATTC

1851 TTTTCCAATG CTATTGTTGA AGGTGCCAAG AAGTTTCCTA ATACTGAATT

1901 TGTCAAAGTA AAAGGTCTTC ATTTTTCGCA AGAAGATGCA CCTGATGAAA

1951 TGGGAAAATA TATCAAATCG TTCGTTGAGC GAGTTCTCAA AAATGAACAA

2001 TAATTCTAGA GCGGCCGCTT CGAGCAGACA TGATAAGATA CATTGATGAG

2051 TTTGGACAAA CCACAACTAG AATGCAGTGA AAAAAATGCT TTATTTGTGA

2101 AATTTGTGAT GCTATTGCTT TATTTGTAAC CATTATAAGC TGCAATAAAC

2151 AAGTTAACAA CAACAATTGC ATTCATTTTA TGTTTCAGGT TCAGGGGGAG

2201 GTGTGGGAGG TTTTTTAAAG CAAGTAAAAC CTCTACAAAT
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3051 GCTCGGCCCT TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT

3101 GAGCGTGGGT CTCGCGGTAT CATTGCAGCA CTGGGGCCAG ATGGTAAGCC

3151 CTCCCGTATC GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG

3201 AACGAAATAG ACAGATCGCT GAGATAGGTG CCTCACTGAT TAAGCATTGG

3251 TAACTGTCAG ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAAACT

3301 TCATTTTTAA TTTAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA

3351 TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC

3401 GTAGAAAAGA TCAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT

3451 CTGCTGCTTG CAAACAAAAA AACCACCGCT ACCAGCGGTG GTTTGTTTGC

3501 CGGATCAAGA GCTACCAACT CTTTTTCCGA AGGTAACTGG CTTCAGCAGA

3551 GCGCAGATAC CAAATACTGT TCTTCTAGTG TAGCCGTAGT TAGGCCACCA

3601 CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT

3651 TACCAGTGGC TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC

3701 TCAAGACGAT AGTTACCGGA TAAGGCGCAG CGGTCGGGCT GAACGGGGGG

3751 TTCGTGCACA CAGCCCAGCT TGGAGCGAAC GACCTACACC GAACTGAGAT

3801 ACCTACAGCG TGAGCTATGA GAAAGCGCCA CGCTTCCCGA AGGGAGAAAG

3851 GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG AGCGCACGAG

3901 GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC

3951 GCCACCTCTG ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG

4001 AGCCTATGGA AAAACGCCAG CAACGCGGCC TTTTTACGGT TCCTGGCCTT

4051 TTGCTGGCCT TTTGCTCACA TGGCTCGAC

pGL3-Promoter Vector sequence map

pGL3-Promoter Vector sequence reference points:

Base pairs	5010
Enhancer	(none)
Multiple cloning region	1-41
Promoter	48-250
Luciferase gene (luc+)	280-1932
GLprimer2 binding region	281-303
SV40 late poly (A) signal	1964-2185
RVprimer4 binding site	2253-2272
ColE1-derived plasmid replication origin	2510
Beta-lactamase gene (Ampr)	3272-4132
f1 origin	4264-4719
Synthetic (upstream) poly (A)signal	4850-5003
RVprimer3 binding region	4952-4971

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151 TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC

201 AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAAGCTT

251 GGCATTCCGG TACTGTTGGT AAAGCCACCA TGGAAGACGC CAAAAACATA

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AGGGACAAGA

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951 TCCTATTTTT GGCAATCAAA TCATTCCGGA TACTGCGATT TTAAGTGTTG

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1351 AAACCGGGCG CGGTCGGTAA AGTTGTTCCA TTTTTTGAAG CGAAGGTTGT

1401 GGATCTGGAT ACCGGGAAAA CGCTGGGCGT TAATCAAAGA GGCGAACTGT

1451 GTGTGAGAGG TCCTATGATT ATGTCCGGTT ATGTAAACAA TCCGGAAGCG

1501 ACCAACGCCT TGATTGACAA GGATGGATGG CTACATTCTG GAGACATAGC

1551 TTACTGGGAC GAAGACGAAC ACTTCTTCAT CGTTGACCGC CTGAAGTCTC

1601 TGATTAAGTA CAAAGGCTAT CAGGTGGCTC CCGCTGAATT GGAATCCATC

1651 TTGCTCCAAC ACCCCAACAT CTTCGACGCA GGTGTCGCAG GTCTTCCCGA

1701 CGATGACGCC GGTGAACTTC CCGCCGCCGT TGTTGTTTTG GAGCACGGAA

1751 AGACGATGAC GGAAAAAGAG ATCGTGGATT ACGTCGCCAG TCAAGTAACA

1801 ACCGCGAAAA AGTTGCGCGG AGGAGTTGTG TTTGTGGACG AAGTACCGAA

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2201 CGTCGACCGA TGCCCTTGAG AGCCTTCAAC CCAGTCAGCT CCTTCCGGTG

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2651 GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC

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2751 TCCAAGCTGG GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC

2801 CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT

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ACCTTCGGAA

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3051 GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA
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TGGAACGAAA

3151 ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG
GATCTTCACC

3201 TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT
AAAGTATATA

3251 TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAATCAGT
GAGGCACCTA

3301 TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG ACTCCCCGTC

3351 GTGTAGATAA CTACGATACG GGAGGGCTTA CCATCTGGCC
CCAGTGCTGC

3401 AATGATACCG CGAGACCCAC GCTCACCGGC TCCAGATTTA
TCAGCAATAA

3451 ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC
AACTTTATCC

3501 GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG
TAAGTAGTTC

3551 GCCAGTTAAT AGTTTGCGCA ACGTTGTTGC CATTGCTACA
GGCATCGTGG

3601 TGTCACGCTC GTCGTTTGGT ATGGCTTCAT TCAGCTCCGG
TTCCCAACGA

3651 TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG
CGGTTAGCTC

3701 CTTCGGTCCT CCGATCGTTG TCAGAAGTAA GTTGGCCGCA
GTGTTATCAC

3751 TCATGGTTAT GGCAGCACTG CATAATTCTC TTACTGTCAT
GCCATCCGTA

3801 AGATGCTTTT CTGTGACTGG TGAGTACTCA ACCAAGTCAT
TCTGAGAATA

3851 GTGTATGCGG CGACCGAGTT GCTCTTGCCC GGCGTCAATA
CGGGATAATA

3901 CCGCGCCACA TAGCAGAACT TTAAAAGTGC TCATCATTGG
AAAACGTTCT

3951 TCGGGGCGAA AACTCTCAAG GATCTTACCG CTGTTGAGAT
CCAGTTCGAT

4001 GTAACCCACT CGTGCACCCA ACTGATCTTC AGCATCTTTT
ACTTTCACCA

4051 GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC AAAATGCCGC
AAAAAAGGGA

4101 ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC
TTTTTCAATA

4151 TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA
TACATATTTG

4201 AATGTATTTA GAAAAATAAA CAAATAGGGG TTCCGCGCAC
ATTTCCCCGA

4251 AAAGTGCCAC CTGACGCGCC CTGTAGCGGC GCATTAAGCG
CGGCGGGTGT

4301 GGTGGTTACG CGCAGCGTGA CCGCTACACT TGCCAGCGCC
CTAGCGCCCG

4351 CTCCTTTCGC TTTCTTCCCT TCCTTTCTCG CCACGTTCGC CGGCTTTCCC

4401 CGTCAAGCTC TAAATCGGGG GCTCCCTTTA GGGTTCCGAT
TTAGTGCTTT

4451 ACGGCACCTC GACCCCAAAA AACTTGATTA GGGTGATGGT
TCACGTAGTG

4501 GGCCATCGCC CTGATAGACG GTTTTTCGCC CTTTGACGTT
GGAGTCCACG

4551 TTCTTTAATA GTGGACTCTT GTTCCAAACT GGAACAACAC
TCAACCCTAT

4601 CTCGGTCTAT TCTTTTGATT TATAAGGGAT TTTGCCGATT TCGGCCTATT

4651 GGTTAAAAAA TGAGCTGATT TAACAAAAAT TTAACGCGAA TTTTAACAAA

4701 ATATTAACGC TTACAATTTG CCATTCGCCA TTCAGGCTGC GCAACTGTTG

4751 GGAAGGGCGA TCGGTGCGGG CCTCTTCGCT ATTACGCCAG CCCAAGCTAC

4801 CATGATAAGT AAGTAATATT AAGGTACGGG AGGTACTTGG AGCGGCCGCA

4851 ATAAAATATC TTTATTTTCA TTACATCTGT GTGTTGGTTT TTTGTGTGAA

4901 TCGATAGTAC TAACATACGC TCTCCATCAA AACAAAACGA AACAAAACAA

4951 ACTAGCAAAA TAGGCTGTCC CCAGTGCAAG TGCAGGTGCC AGAACATTTC

5001 TCTATCGATA

pSV-beta-Galactosidase Vector Sequence

pSV-beta-Galactosidase Vector Sequence Reference Points:

Base pairs	6820
SV40 Early promoter and enhancer seqment	1-419
Transcription start sites	354, 360, 365
Possible start codons (ATG)	500, 530, 569
gpt promoter (-10 region)	428-433
lacZ start site	710
lacZ stop site (TAA)	3755
lac operon sequences	709-4020
lacY	3809-4011
SV40 small T antigen	4021-4156
beta-lactamase (Ampr) coding region	4784-5644

1 GCGCAGCACC ATGGCCTGAA ATAACCTCTG AAAGAGGAAC TTGGTTAGGT

51 ACCTTCTGAG GCGGAAAGAA CCAGCTGTGG AATGTGTGTC
AGTTAGGGTG

101 TGGAAAGTCC CCAGGCTCCC CAGCAGGCAG AAGTATGCAA
AGCATGCATC

151 TCAATTAGTC AGCAACCAGG TGTGGAAAGT CCCCAGGCTC
CCCAGCAGGC

201 AGAAGTATGC AAAGCATGCA TCTCAATTAG TCAGCAACCA
TAGTCCCGCC

251 CCTAACTCCG CCCATCCCGC CCCTAACTCC GCCCAGTTCC GCCCATTCTC

301 CGCCCCATGG CTGACTAATT TTTTTTATTT ATGCAGAGGC
CGAGGCCGCC

351 TCGGCCTCTG AGCTATTCCA GAAGTAGTGA GGAGGCTTTT
TTGGAGGCCT

401 AGGCTTTTGC AAAAAGCTTG GGATCTCTAT AATCTCGCGC AACCTATTTT

451 CCCCTCGAAC ACTTTTTAAG CCGTAGATAA ACAGGCTGGG
ACACTTCACA

501 TGAGCGAAAA ATACATCGTC ACCTGGGACA TGTTGCAGAT
CCATGCACGT

551 AAACTCGCAA GCCGACTGAT GCCTTCTGAA CAATGGAAAG
GCATTATTGC

601 CGTAAGCCGT GGCGGTCTGG TACCGGTGGG TGAAGACCAG
AAACAGCACC

651 TCGAACTGAG CCGCGATATT GCCCAGCGTT TCAACGCGCT
GTATGGCGAG

701 ATCGATCCCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC
CTGGCGTTAC

751 CCAACTTAAT CGCCTTGCAG CACATCCCCC TTTCGCCAGC
TGGCGTAATA

801 GCGAAGAGGC CCGCACCGAT CGCCCTTCCC AACAGTTGCG
CAGCCTGAAT

851 GGCGAATGGC GCTTTGCCTG GTTTCCGGCA CCAGAAGCGG
TGCCGGAAAG

901 CTGGCTGGAG TGCGATCTTC CTGAGGCCGA TACTGTCGTC GTCCCCTCAA

951 ACTGGCAGAT GCACGGTTAC GATGCGCCCA TCTACACCAA CGTGACCTAT

1001 CCCATTACGG TCAATCCGCC GTTTGTTCCC ACGGAGAATC CGACGGGTTG

1051 TTACTCGCTC ACATTTAATG TTGATGAAAG CTGGCTACAG GAAGGCCAGA

1101 CGCGAATTAT TTTTGATGGC GTTAACTCGG CGTTTCATCT GTGGTGCAAC

1151 GGGCGCTGGG TCGGTTACGG CCAGGACAGT CGTTTGCCGT CTGAATTTGA

1201 CCTGAGCGCA TTTTTACGCG CCGGAGAAAA CCGCCTCGCG GTGATGGTGC

1251 TGCGCTGGAG TGACGGCAGT TATCTGGAAG ATCAGGATAT GTGGCGGATG

1301 AGCGGCATTT TCCGTGACGT CTCGTTGCTG CATAAACCGA CTACACAAAT

1351 CAGCGATTTC CATGTTGCCA CTCGCTTTAA TGATGATTTC AGCCGCGCTG

1401 TACTGGAGGC TGAAGTTCAG ATGTGCGGCG AGTTGCGTGA CTACCTACGG

1451 GTAACAGTTT CTTTATGGCA GGGTGAAACG CAGGTCGCCA GCGGCACCGC

1501 GCCTTTCGGC GGTGAAATTA TCGATGAGCG TGGTGGTTAT GCCGATCGCG

1551 TCACACTACG TCTGAACGTC GAAAACCCGA AACTGTGGAG CGCCGAAATC

1601 CCGAATCTCT ATCGTGCGGT GGTTGAACTG CACACCGCCG ACGGCACGCT

1651 GATTGAAGCA GAAGCCTGCG ATGTCGGTTT CCGCGAGGTG CGGATTGAAA

1701 ATGGTCTGCT GCTGCTGAAC GGCAAGCCGT TGCTGATTCG AGGCGTTAAC

1751 CGTCACGAGC ATCATCCTCT GCATGGTCAG GTCATGGATG AGCAGACGAT

1801 GGTGCAGGAT ATCCTGCTGA TGAAGCAGAA CAACTTTAAC GCCGTGCGCT

1851 GTTCGCATTA TCCGAACCAT CCGCTGTGGT ACACGCTGTG CGACCGCTAC

1901 GGCCTGTATG TGGTGGATGA AGCCAATATT GAAACCCACG GCATGGTGCC

1951 AATGAATCGT CTGACCGATG ATCCGCGCTG GCTACCGGCG ATGAGCGAAC

2001 GCGTAACGCG AATGGTGCAG CGCGATCGTA ATCACCCGAG TGTGATCATC

2051 TGGTCGCTGG GGAATGAATC AGGCCACGGC GCTAATCACG ACGCGCTGTA

2101 TCGCTGGATC AAATCTGTCG ATCCTTCCCG CCCGGTGCAG TATGAAGGCG

2151 GCGGAGCCGA CACCACGGCC ACCGATATTA TTTGCCCGAT GTACGCGCGC

2201 GTGGATGAAG ACCAGCCCTT CCCGGCTGTG CCGAAATGGT CCATCAAAAA

2251 ATGGCTTTCG CTACCTGGAG AGACGCGCCC GCTGATCCTT TGCGAATACG

2301 CCCACGCGAT GGGTAACAGT CTTGGCGGTT TCGCTAAATA CTGGCAGGCG

2351 TTTCGTCAGT ATCCCCGTTT ACAGGGCGGC TTCGTCTGGG ACTGGGTGGA

2401 TCAGTCGCTG ATTAAATATG ATGAAAACGG CAACCCGTGG TCGGCTTACG

2451 GCGGTGATTT TGGCGATACG CCGAACGATC GCCAGTTCTG TATGAACGGT

2501 CTGGTCTTTG CCGACCGCAC GCCGCATCCA GCGCTGACGG AAGCAAAACA

2551 CCAGCAGCAG TTTTTCCAGT TCCGTTTATC CGGGCAAACC ATCGAAGTGA

2601 CCAGCGAATA CCTGTTCCGT CATAGCGATA ACGAGCTCCT GCACTGGATG

2651 GTGGCGCTGG ATGGTAAGCC GCTGGCAAGC GGTGAAGTGC CTCTGGATGT

2701 CGCTCCACAA GGTAAACAGT TGATTGAACT GCCTGAACTA CCGCAGCCGG

2751 AGAGCGCCGG GCAACTCTGG CTCACAGTAC GCGTAGTGCA ACCGAACGCG

2801 ACCGCATGGT CAGAAGCCGG GCACATCAGC GCCTGGCAGC AGTGGCGTCT

2851 GGCGGAAAAC CTCAGTGTGA CGCTCCCCGC CGCGTCCCAC GCCATCCCGC

2901 ATCTGACCAC CAGCGAAATG GATTTTTGCA TCGAGCTGGG TAATAAGCGT

2951 TGGCAATTTA ACCGCCAGTC AGGCTTTCTT TCACAGATGT GGATTGGCGA

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3101 TGGGTCGAAC GCTGGAAGGC GGCGGGCCAT TACCAGGCCG AAGCAGCGTT

3151 GTTGCAGTGC ACGGCAGATA CACTTGCTGA TGCGGTGCTG ATTACGACCG

3201 CTCACGCGTG GCAGCATCAG GGGAAAACCT TATTTATCAG CCGGAAAACC

3251 TACCGGATTG ATGGTAGTGG TCAAATGGCG ATTACCGTTG ATGTTGAAGT

3301 GGCGAGCGAT ACACCGCATC CGGCGCGGAT TGGCCTGAAC TGCCAGCTGG

3351 CGCAGGTAGC AGAGCGGGTA AACTGGCTCG GATTAGGGCC GCAAGAAAAC

3401 TATCCCGACC GCCTTACTGC CGCCTGTTTT GACCGCTGGG
ATCTGCCATT

3451 GTCAGACATG TATACCCCGT ACGTCTTCCC GAGCGAAAAC
GGTCTGCGCT

3501 GCGGGACGCG CGAATTGAAT TATGGCCCAC ACCAGTGGCG
CGGCGACTTC

3551 CAGTTCAACA TCAGCCGCTA CAGTCAACAG CAACTGATGG
AAACCAGCCA

3601 TCGCCATCTG CTGCACGCGG AAGAAGGCAC ATGGCTGAAT
ATCGACGGTT

3651 TCCATATGGG GATTGGTGGC GACGACTCCT GGAGCCCGTC
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3701 GAATTCCAGC TGAGCGCCGG TCGCTACCAT TACCAGTTGG
TCTGGTGTCA

3751 AAAATAATAA TAACCGGGCA GGCCATGTCT GCCCGTATTT
CGCGTAAGGA

3801 AATCCATTAT GTACTATTTA AAAAACACAA ACTTTTGGAT
GTTCGGTTTA

3851 TTCTTTTTCT TTTACTTTTT TATCATGGGA GCCTACTTCC CGTTTTTCCC

3901 GATTTGGCTA CATGACATCA ACCATATCAG CAAAAGTGAT
ACGGGTATTA

3951 TTTTTGCCGC TATTTCTCTG TTCTCGCTAT TATTCCAACC GCTGTTTGGT

4001 CTGCTTTCTG ACAAACTCGG AACTTGTTTA TTGCAGCTTA
TAATGGTTAC

4051 AAATAAAGCA ATAGCATCAC AAATTTCACA AATAAAGCAT
TTTTTTCACT

4101 GCATTCTAGT TGTGGTTTGT CCAAACTCAT CAATGTATCT TATCATGTCT

4151 GGATCCTCTA GAGTCGACCT GCAGGCATGC AAGCTGGCAC
TGGCCGTCGT

4201 TTTACAACGT CGTGACTGGG AAAACCCTGG CGTTACCCAA
CTTAATCGCC

4251 TTGCAGCACA TCCCCCTTTC GCCAGCTGGC GTAATAGCGA
AGAGGCCCGC

4301 ACCGATCGCC CTTCCCAACA GTTGCGCAGC CTGAATGGCG
AATGGCGCCT

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CACCGCATAT

4401 GGTGCACTCT CAGTACAATC TGCTCTGATG CCGCATAGTT
AAGCCAGCCC

4451 CGACACCCGC CAACACCCGC TGACGCGCCC TGACGGGCTT
GTCTGCTCCC

4501 GGCATCCGCT TACAGACAAG CTGTGACCGT CTCCGGGAGC
TGCATGTGTC

4551 AGAGGTTTTC ACCGTCATCA CCGAAACGCG CGAGACGAAA
GGGCCTCGTG

4601 ATACGCCTAT TTTTATAGGT TAATGTCATG ATAATAATGG
TTTCTTAGAC

4651 GTCAGGTGGC ACTTTTCGGG GAAATGTGCG CGGAACCCCT
ATTTGTTTAT

4701 TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACA
ATAACCCTGA

4751 TAAATGCTTC AATAATATTG AAAAAGGAAG AGTATGAGTA
TTCAACATTT

4801 CCGTGTCGCC CTTATTCCCT TTTTTGCGGC ATTTTGCCTT CCTGTTTTTG

4851 CTCACCCAGA AACGCTGGTG AAAGTAAAAG ATGCTGAAGA
TCAGTTGGGT

4901 GCACGAGTGG GTTACATCGA ACTGGATCTC AACAGCGGTA
AGATCCTTGA

4951 GAGTTTTCGC CCCGAAGAAC GTTTTCCAAT GATGAGCACT
TTTAAAGTTC

5001 TGCTATGTGG CGCGGTATTA TCCCGTATTG ACGCCGGGCA
AGAGCAACTC

5051 GGTCGCCGCA TACACTATTC TCAGAATGAC TTGGTTGAGT
ACTCACCAGT

5101 CACAGAAAAG CATCTTACGG ATGGCATGAC AGTAAGAGAA
TTATGCAGTG

5151 CTGCCATAAC CATGAGTGAT AACACTGCGG CCAACTTACT
TCTGACAACG

5201 ATCGGAGGAC CGAAGGAGCT AACCGCTTTT TTGCACAACA
TGGGGGATCA

5251 TGTAACTCGC CTTGATCGTT GGGAACCGGA GCTGAATGAA
GCCATACCAA

5301 ACGACGAGCG TGACACCACG ATGCCTGTAG CAATGGCAAC
AACGTTGCGC

5351 AAACTATTAA CTGGCGAACT ACTTACTCTA GCTTCCCGGC
AACAATTAAT

5401 AGACTGGATG GAGGCGGATA AAGTTGCAGG ACCACTTCTG
CGCTCGGCCC

5451 TTCCGGCTGG CTGGTTTATT GCTGATAAAT CTGGAGCCGG
TGAGCGTGGG

5501 TCTCGCGGTA TCATTGCAGC ACTGGGGCCA GATGGTAAGC
CCTCCCGTAT

5551 CGTAGTTATC TACACGACGG GGAGTCAGGC AACTATGGAT
GAACGAAATA

5601 GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG
GTAACTGTCA

5651 GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTTA

5701 ATTTAAAAGG ATCTAGGTGA AGATCCTTTT TGATAATCTC
ATGACCAAAA
5751 TCCCTTAACG TGAGTTTTCG TTCCACTGAG CGTCAGACCC
CGTAGAAAAG

5801 ATCAAAGGAT CTTCTTGAGA TCCTTTTTTT CTGCGCGTAA
TCTGCTGCTT

5851 GCAAACAAAA AAACCACCGC TACCAGCGGT GGTTTGTTTG
CCGGATCAAG

5901 AGCTACCAAC TCTTTTTCCG AAGGTAACTG GCTTCAGCAG
AGCGCAGATA

5951 CCAAATACTG TTCTTCTAGT GTAGCCGTAG TTAGGCCACC
ACTTCAAGAA

6001 CTCTGTAGCA CCGCCTACAT ACCTCGCTCT GCTAATCCTG
TTACCAGTGG

6051 CTGCTGCCAG TGGCGATAAG TCGTGTCTTA CCGGGTTGGA
CTCAAGACGA

6101 TAGTTACCGG ATAAGGCGCA GCGGTCGGGC TGAACGGGGG
GTTCGTGCAC

6151 ACAGCCCAGC TTGGAGCGAA CGACCTACAC CGAACTGAGA
TACCTACAGC

6201 GTGAGCTATG AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA
GGCGGACAGG

6251 TATCCGGTAA GCGGCAGGGT CGGAACAGGA GAGCGCACGA
GGGAGCTTCC

6301 AGGGGGAAAC GCCTGGTATC TTTATAGTCC TGTCGGGTTT
CGCCACCTCT

6351 GACTTGAGCG TCGATTTTTG TGATGCTCGT CAGGGGGGCG
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6401 AAAAACGCCA GCAACGCGGC CTTTTTACGG TTCCTGGCCT
TTTGCTGGCC

6451 TTTTGCTCAC ATGTTCTTTC CTGCGTTATC CCCTGATTCT GTGGATAACC

6501 GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCCGCAG
CCGAACGACC

6551 GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG GAAGAGCGCC
CAATACGCAA

6601 ACCGCCTCTC CCCGCGCGTT GGCCGATTCA TTAATGCAGC
TGGCACGACA

6651 GGTTTCCCGA CTGGAAAGCG GGCAGTGAGC GCAACGCAAT
TAATGTGAGT

6701 TAGCTCACTC ATTAGGCACC CCAGGCTTTA CACTTTATGC
TTCCGGCTCG

6751 TATGTTGTGT GGAATTGTGA GCGGATAACA ATTTCACACA
GGAAACAGCT

6801 ATGACATGAT TACGAATTCG