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FORMULATION AND CHARACTERISATION OF CATIONIC MICROPARTICLES FOR THE DELIVERY OF DNA VACCINES

SARAH FRANCES ATKINSON

Doctor of Philosophy

ASTON UNIVERSITY

March 2004

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FORMULATION AND CHARACTERISATION OF CATIONIC MICROPARTICLES FOR THE DELIVERY OF DNA VACCINES

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The aim of this research was to formulate a novel biodegradable, biocompatible cationic microparticle vector for the delivery of DNA vaccines. The work builds upon previous research by Singh *et al* which described the adsorption of DNA to the surface of poly (D,L-lactide-co-glycolide) (PLG) microparticles stabilised with the surfactant cetyltrimethyl ammonium bromide (CTAB). This work demonstrated the induction of antibody and cellular immune responses to HIV proteins encoded on plasmid DNA adsorbed to the particle surface in mice, guinea pigs and non-human primates (Singh *et al*, 2000; O'Hagan *et al*, 2001). However, the use of surfactants in microparticle formulations for human vaccination is undesirable due to long term safety issues. Therefore, the present research aim was to develop an adsorbed DNA vaccine with enhanced potency and increased safety compared to CTAB stabilised PLG microparticles (PLG/CTAB) by replacement of the surfactant CTAB with an alternative cationic agent. The cationic polymers chitosan and poly (N- vinylpyrrolidone/2-dimethylaminoethyl methacrylate), dimethyl sulfate quaternary (PVP-PDAEMA) were investigated as alternative stabilisers to CTAB.

From a variety of initial formulations, the most promising vector(s) for DNA vaccination were selected based on physicochemical data (chapter 3) and *in vitro* DNA loading and release characteristics (chapter 4). The chosen formulation(s) were analysed in greater depth (chapters 3 and 4), and gene expression was assessed by *in vitro* cell transfection studies using 293T kidney epithelial and C_2C_{12} myoblast non-phagocytic cell lines (chapter 5). The cytotoxicity of the microparticles and their constituents were also evaluated *in vitro* (chapter 5). Stability and suitability of the formulation(s) for commercial production were assessed by cryopreparation and lyophilisation studies (chapters 3 and 4). Gene expression levels in cells of the immune response were evaluated by microparticle transfection of the dendritic cell (DC) line 2.4 and primary bone marrow derived DCs (chapter 6). *In vivo,* mice were injected i.m. with the formulations deemed most promising on the basis of *in vitro* studies and humoral and cellular immune responses were evaluated (chapter 6).

Keywords: poly (D,L lactide-co-glycolide); chitosan; DNA delivery

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Abbreviations

AAV	adeno-associated virus
APC	antigen presenting cell
ASOR	asialoorosomucoid
BCA	bicinchoninic acid
BMDC	bone marrow derived dendritic cell
CMV	cytomegalovirus
CTAB	cetyltrimethyl ammonium bromide
CTL	cytotoxic T lymphocyte
DAEM	(2-dimethylaminoethyl) methacrylate, methylmethacrylate
DC	dendritic cell
DCM	dichloromethane
DDW	double distilled water
DMEM	dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA/MP	DNA loaded PLG/LMWC microparticles
DNase	deoxyribonuclease
dsDNA	double stranded DNA
EDTA	diamenoethanetetra-acetic acid
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked immunospot assay
F6	FuGENE 6 transfection reagent
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
GM-CSF	granulocyte/macrophage colony-stimulating factor
IC	isoamyl alcohol
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
i.v.	intravenous
kb	kilobase pairs
km	kanamycin
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
LD_{50}	lethal dose 50%
LMWC	low molecular weight chitosan
Luc	luciferase
lyo	lyophilised
MHC	major histocompatibility complex
MNC	mononuclear cells
MMWC	medium molecular weight chitosan
MTT	tetrazolium salt
MW	molecular weight

NLS	nuclear localisation sequence
nm	nanometers
NP	influenza nuclear protein
NPC	nuclear pore complex
OD_{260}	optical density at 260nm
o/w	oil in water
PBS	phosphate buffered saline
pDNA	plasmid DNA
PEI	polyethleneimine
PEG	poly(ethylene glycol)
pen/strep	penicillin/streptomycin
PIC	phenol/IC solution
PLA	poly-L-lactide
PLG	poly (D,L-lactide-co-glycolide)
PLG/CTAB	PLG microparticles stabilised with CTAB
PLG/LMWC	PLG microparticles stabilised with LMWC
PLG/MMWC	PLG microparticles stabilised with MMWC
PLG/PVA	PLG microparticles stabilised with PVA
PLG/PVP-PDAEMA	PLG microparticles stabilised with PVP-PDAEMA
PLL	poly-L-lysine
PVA	poly (vinyl alcohol)
PVP	polyvinyl pyrrolidone
PVP-PDAEMA	Poly (N- vinylpyrrolidone/2-dimethylaminoethyl methacrylate), dimethyl sulfate quaternary
RLU	relative light units
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
SF	serum free
SLM	solid lipid microparticles
SLN	solid lipid nanoparticles
TA	tibialis anterior
TE	TRIS/EDTA
TEM	transition electron microscope
T_H	T helper cell
TLR	toll-like receptor
TNF	tumour necrosis factor
TRIS	tris-(hydroxymethyl)-methylamine
UV	ultraviolet

1. Introduction

1.1 Background

The evolution of modern day vaccines has occurred over the last two centuries. In the early 1700s Lady Mary Wortley Montagu (Plotkin & Plotkin, 1999) introduced to England the practice of variolation performed in ancient China. This involved artificially inoculating a healthy individual with pus from a smallpox patient, usually resulting in reduced pathology and subsequent immunity to the disease. In the late 1700s it was observed that inoculation of healthy individuals with cowpox pus from cattle led to only mild symptoms and prevention of the human smallpox disease. In 1796 Edward Jenner founded the science of vaccinology based on these proofs of principle (Jenner, 1798). Manufacture and use of the smallpox vaccine subsequently spread throughout the world. In the latter half of the nineteenth century pioneers in the field of vaccinology included Louis Pasteur and Robert Koch who discovered a number of human microbial pathogens and prepared vaccines against them. During this period Paul Ehrlich produced the first therapeutic drug, salvarsan against syphilis and, when Emil Von Behring discovered antibodies, the field of passive immunotherapy was born (Hilleman, 2000; Hilleman, 2003).

In the twentieth century two influential technological breakthroughs in vaccinology occurred. Goodpasture discovered microbial propagation in embryonated eggs (Woodruf & Goodpasture, 1931) which led to the development of many new vaccines including influenza and, in1950, Enders developed the cell culture propagation of viruses (Enders *et al,* 1949). As a result the poliovirus and many other live vaccines were produced. Other important developments in this century included individual vaccines for measles, mumps and rubella, the combined measles-mumps-rubella (MMR) vaccine (Buynak *et al,* 1969), plasma derived and recombinant hepatitis B vaccines (Hilleman, 1979; Hilleman 1996), killed hepatitis A vaccines (Hilleman, 1996; Hilleman 1993). In addition, in the UK in 1992 the first protein-conjugated bacterial polysaccharide vaccine for haemophilus influenzae type b (Hib) was licensed for infants (McVemon *et al,* 2004).

The twenty first century is likely to be fruitful for new vaccine development. In part this will be due to the wide array of technologies recently available to vaccine researchers including recombinant expression systems, recombinant vectors, and delivery systems that elicit humoral and cellular immunity. In addition, new areas of science such as proteomics, genomics and informational technologies, coupled with rapid throughput assays can only accelerate the identification of novel antigens for vaccine development (Hilleman, 2002; Hilleman, 2003).

1.2 The immune response

Key considerations in the design and development of vaccines in the present era are safety and efficacy. Ideally an effective vaccine should stimulate both arms of the immune system; humoral and cellular immunity (fig 1.2A).

1.2.1 Humoral immunity

Primarily the humoral immune response defends the body against bacteria, bacterial toxins, and viruses that enter the body's various fluid systems. Humoral immunity relies on glycoproteins present in the bloodstream and tissue fluid of mammals, collectively called antibodies, or immunoglobulins (Ig). Serum glycoproteins can be split into five groups; albumin, alpha-I globulin, alpha-2 globuilin, beta globulin and gamma globulin. The latter group contains a heterogenous class of immunoglobulins including IgG, IgA, IgM and IgD. These immunoglobulins differ in size, chemical structure and surface charge. Most antibodies found in serum are of the IgG variety.

In conjunction with the antibody response humoral immunity also involves complement activation. Complement is one of the body's first defensive responses, and comprises a set of serum proteins that are activated to kill bacteria or foreign eukaryotic cells both nonspecifically and in conjunction with the antibody response described below.

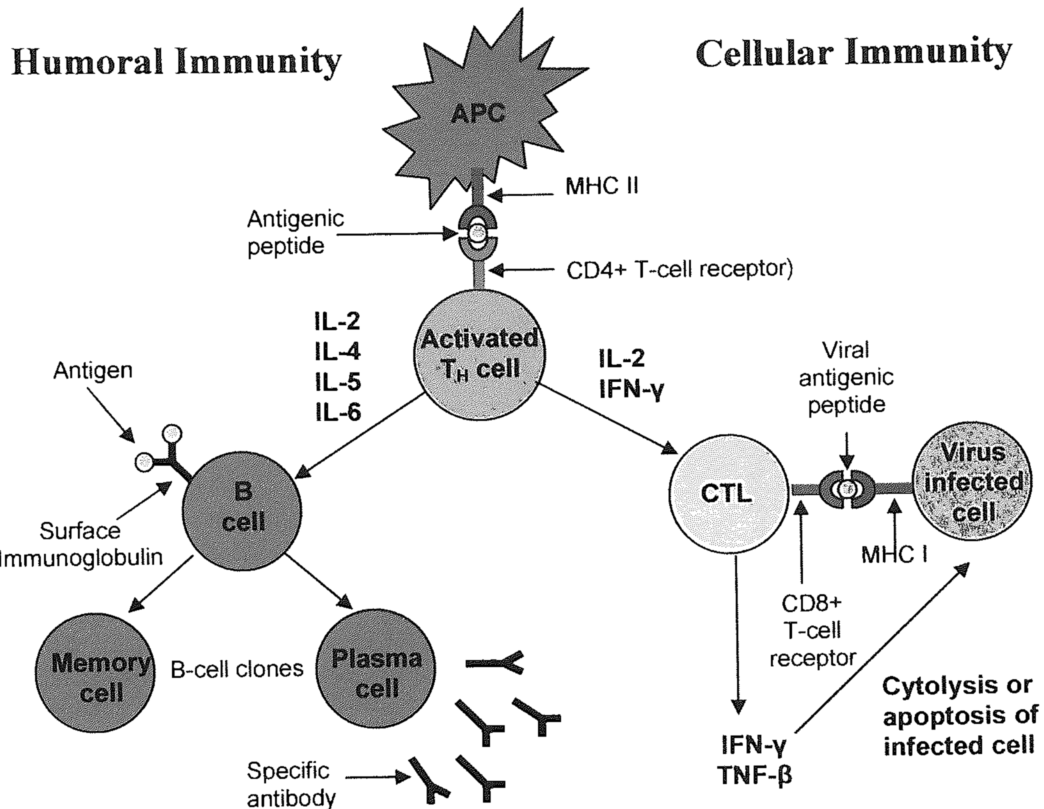


Figure 1.2A Simplified schematic diagram of the processes involved in humoral and cell mediated immunity. Professional APCs process and present antigenic peptide on their surface in conjunction with MHC class II molecules. T_H (CD4+) cells recognise the antigen and become activated. Once activated the T_H cells secrete a variety of cytokines which stimulate other cells of the immune system. Circulating B cells also recognize foreign antigen in the bodies' fluid systems through surface immunoglobulin receptors. In conjunction with a stimulatory signal from T_H cells (IL-2, IL-4, IL-5 and IL-6 secretion) B cells are triggered to proliferate and differentiate into plasma cells secreting antibody and memory cells. In cellular immunity CTLs become activated when they recognise viral antigenic peptide on the surface of infected cells in conjunction with MHC class I molecules. However, CTL activation is also reliant on IL-2 and IFN-γ secretion by activated T_H cells. Once activated CTLs grow and proliferate to produce clones of killer cells. These cells sectrete IFN-γ and TNF-β which can cause cytolysis or apoptosis of infected cells.

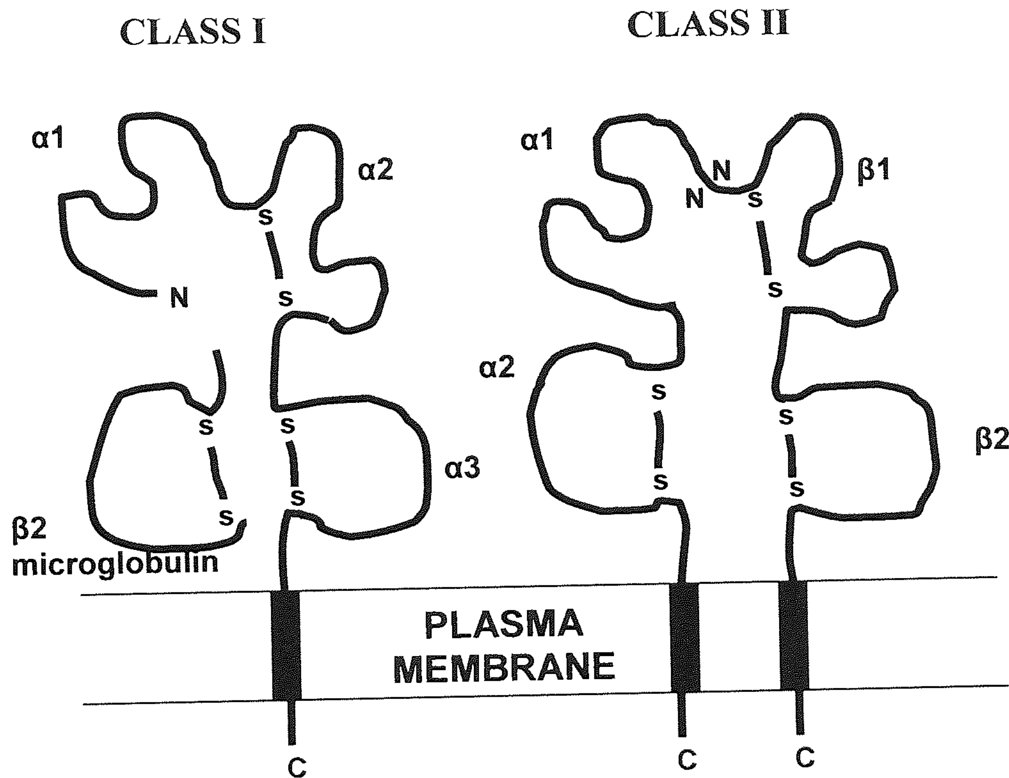


Figure 1.2B Schematic diagram of major histocompatibility complex (MHC) molecules. Both MHC class I and class II molecules belong to the immunoglobulin gene superfamily. MHC class I molecules are found on the surface of most cells in the vertebrate body and consist of a complex of two protein chains. The two chains contain four regions. The outer segment of the heavy chain can be divided into three functional domains designated α1, α2 and α3. The β2 microglobulin region makes up the light chain. MHC class II molecules are found only on the surface of professional antigen presenting cells and B cells. These molecules also contain two folded chains consisting of α and β regions.

Complement proteins make up much of the globulin fraction of serum. Complement is usually activated after the binding of antibodies to an antigen. Antigens are non-self foreign substances such as proteins, nucleoproteins, polysaccharides and some glycolipids. Complement can also be activated directly by some microbial products. Complement proteins are capable of lysing antibody-coated eukaryotic cells and bacteria. They can also mediate inflammation and attract and activate phagocytic cells. Complement proteins act in a cascade fashion with activation of one protein leading to activation of the next. Ultimately the membrane attack complex is activated and this complex creates a pore in the plasma membrane of the target cell. As a result, eukaryotic cells lyse due to osmotic pressure as Na^+ and H_2O enter through the pore. If the target cell is a Gram –ve bacteria, the pore allows lysozyme from the blood to enter and digest the peptidoglycan cell wall causing the bacterium to lyse osmotically. Although complement helps to enhance the antibody response it will not be discussed further here.

The antibody response is facilitated by a class of lymphocytes called B cells. Lymphocytes differentiate into B cells in the foetal liver and in adult bone marrow. B cells make up 20-30% of the circulating lymphocytes in the blood and also reside in lymphoid organs. Each B cell synthesizes a specific antibody and displays it on its cell surface. If an antigen specific to this antibody comes in to contact with the B cell, the antibody receptor will form a complex with the antigen. Internalization of the antibody leads to swelling of the B cell and the cell begins to divide rapidly. Activated B cell clones are produced which can differentiate into either plasma cells or memory cells (fig 1.2A). Plasma cells produce thousands of antibodies against the specific antibody that provoked their formation (primary response). Memory B cells persist in the body for many years and boost the immune systems readiness to eliminate the same antigen if it presents itself in the future (secondary response).

The primary antibody response which occurs upon first challenge with an antigen will occur only after a lag phase of several days. During the primary response IgM is the first antibody to be produced followed by IgG. The affinity of these antibodies for the antigen

is low to moderate during the initial response. The secondary antibody response is characterized by a much faster more powerful reaction to the same antigen. The lag phase is shorter, the concentration of IgG in the blood is higher and the affinity of the antibodies for the antigen is increased (fig 1.2.1).

Immunoglobulin molecules contain two binding regions. The first is the antigen-binding Fab region. The second is a crystalisable fragment (Fc region) which allows the antibody to bind host tissue, various cells of the immune system, some phagocytic cells, or the first component of the complement system. Destruction of the antigen or the microorganism, cell or agent to which the antigens attach does not occur upon binding to the antibody. Instead the target is 'tagged' for immunological attack or destruction by the nonspecific immune response. Phagocytosis by neutrophils or macrophages is enhanced if the target is covered by antibodies. This process is called opsonization. Antibody induced activation of the complement system can also occur.

There is a second group of lymphocytes that play an important role in humoral immunity. These cells are called T-lymphocytes and they differentiate in the thymus. Like B cells some T cells are found in the lymphoid organs, the rest are transported to the blood where they comprise 70 to 80% of the circulating lymphocytes. T-helper ($T_{H)}$ cells are a subset of T-lymphocytes. In the humoral immune response mature T_H cells secrete the lymphokines interleukin 2 (IL-2), IL-4, IL-5 and IL-6 which are B-cell growth and differentiation factors and stimulate maturation of B cells into plasma cells. T_H cells are important because most antigens have more than one epitope on each molecule. B cells specific for a given epitope on the antigen often cannot develop into plasma cells without the aid of lymphokines secreted by T_H cells. Likewise, T_H cells do not mature without the aid of antigen presenting cells (APCs), in particular macrophages. Macrophages are found in the blood and in all tissues. These cells and other APCs such as dendritic cells (DCs) are capable of nonspecifically engulfing foreign materials and degrading them. The resulting peptides are then carried to the cell surface where they are displayed in conjunction with the cells own major histocompatibility (MHC) class II molecule. MHC

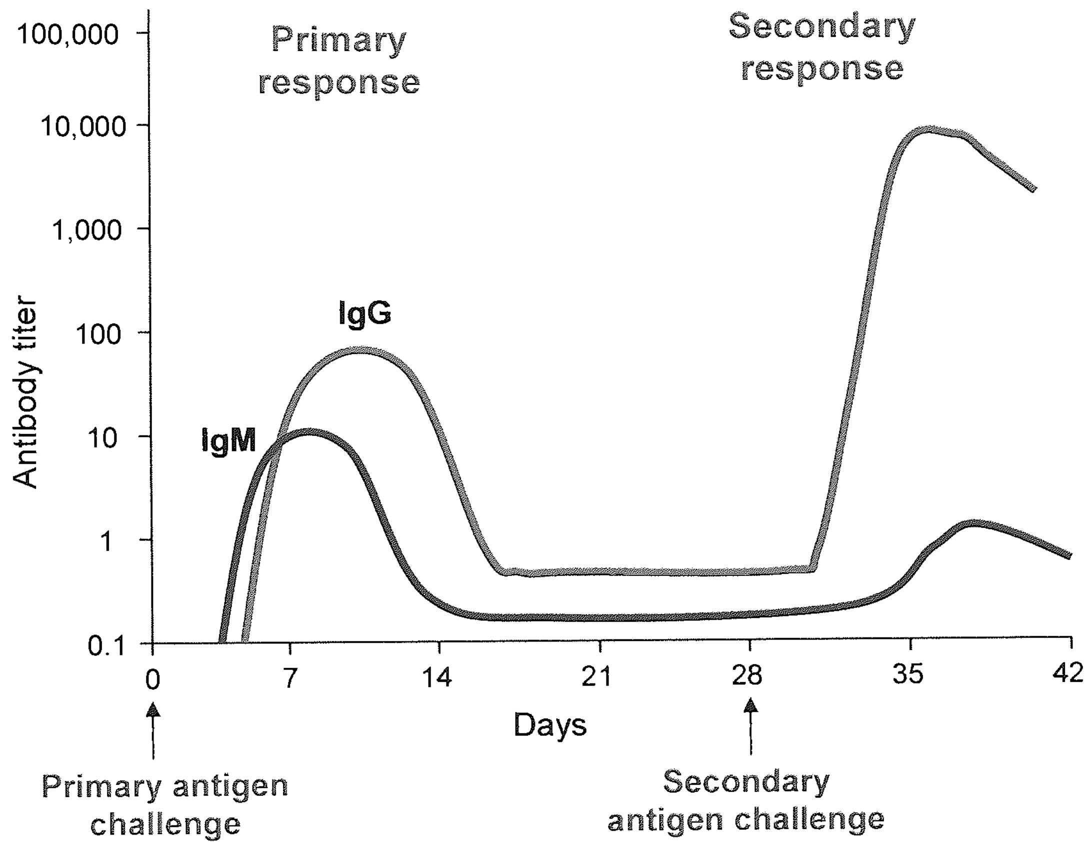


Figure 1.2.1 Primary and secondary antibody responses. Following a primary challenge with antigen there is an initial lag phase of several days. During the primary response, IgM appears first, then IgG. The affinity for the antigen's determinants is low to moderate during this primary antibody response. Upon secondary antigen challenge, the B cells mount a heightened response to the same antigen. The secondary antibody response has a shorter lag phase, attains a higher IgG titer and produces antibodies with a higher affinity for the antigen.

molecules are two-chain proteins of the immunoglobulin gene superfamily (fig 1.2B). MHC class II molecules are found only on the surface of professional APCs and B cells. Precursors to T_H cells recognize and become activated by interaction with peptide presented in conjunction with MHC class II on the surface of APCs. This leads to maturation of the T_H cell (fig 1.2A). B cells are also capable of endocytosing foreign material and displaying peptide on their surface in conjunction with MHC class II molecules. T_H cells previously stimulated by macrophage-bound carrier peptides are capable of recognizing the peptides on the B cell surface and becoming further stimulated.

There are two subsets of T_H cells; T_H 1 and T_H 2. T_H 2 cells are important in humoral immunity as described above and secrete lymphokines that promote B cell maturation and antibody production. By comparison T_H 1 cells secrete IL-2, IFN-γ, and other cytokines involved in cellular immunity. The role of T_H 1 cells in cellular immunity will be discussed below.

1.2.2 Cellular immunity

The cellular immune response principally protects the body against viruses, which can infect cells and replicate inside them. The effector cells of this response are another group of T lymphocytes called cytotoxic T cells (CTLs). T_H cells and CTLs can be distinguished from each other by the molecules they express on their cell surface. T_H cells express a CD4 glycoprotein which acts as a cell adhesion molecule with high affinity for MHC class II molecules. In contrast CTLs express the polypeptide CD8. CD8 is also a cell adhesion molecule and is involved in the interaction with MHC class I molecules found on target cells in the cellular immune response (fig 1.2B). Like MHC class II molecules, MHC class I molecules are two-chain proteins and belong to the immunoglobulin gene superfamily. However, unlike MHC class II molecules which are found only on the surface of professional APCs and B cells, MHC class I molecules are found on the surface of most vertebrate cells and identify the cell as 'self'.

Cellular immunity is stimulated when endogenous antigenic proteins (e.g. antigens from replicating viruses) are digested inside an infected cell as part of the natural process by which a cell continually renews its protein content. Once digested peptides are transported to the endoplasmic reticulum. Here MHC class I molecules that specifically bind peptides originating from the cytosol complex with the fragments. The complexes are transported to the cell surface. If the peptide is foreign, for example short pieces of viral protein, a passing $CD8^+$ CTL will recognise it and release cytokines such as IFN-γ and tumour necrosis factor that directly destroy the entire cell. For this to occur both the MHC class I molecule and the peptide must be jointly recognized on the same cell at the same time (fig 1.2A)

Once interaction has occurred between the $CD8^+$ CTL precursor and an infected cell expressing MHC class I with bound foreign peptide, growth and maturation of the CTL occurs. The cell is stimulated to divide and proliferate providing a clone of killer T cells able to destroy target cells displaying peptide. However, growth is also dependent on IL-2 secreted from a subset of mature T_H cells. This subset (T_H 1) will only mature upon interaction with endogenous foreign peptide expressed on the surface of macrophages or DCs in conjunction with MHC class II molecules. Without the involvement of professional APCs, the full immunological defense provided by CTLs would not occur highlighting once again how important these cells are in the immune response. Mature CTLs are capable of secreting toxic molecules that induce virus-infected cells to kill themselves by apoptosis or programmed cell death thus preventing the spread of further infection.

1.3 Current vaccines

Vaccines in current use fall into four main categories: *attenuated live vaccines* e.g. measles, mumps, rubella, oral polio, *killed whole viruses or bacteria* e.g. hepatitis A and injected polio, *subunit vaccines* e.g. hepatitis B and *conjugate vaccines* e.g. haemophilus influenzae type b (Hib) and meningococcal C. However, each of these groups of vaccines has advantages and limitations.

Attenuated live vaccines

Attenuated live vaccines do not produce disease in the inoculated individual but they are capable of infecting cells. As a result these vaccines stimulate both humoral and cellular immunity. Only a small amount of inoculum is required to elicit a sustained response making live vaccines relatively inexpensive. However, there is a small risk that a live vaccine may revert to its virulent form. In some cases, for instance if an HIV vaccine were developed, this risk would be unacceptable. Attenuated live vaccines are not safe for use in immuno-compromised groups, for example cancer sufferers, the elderly and people with HIV or AIDS. Live attenuated vaccines cannot be made for all pathogens as some organisms cannot be grown in culture and others cannot be rendered non-pathogenic (Wilkinson & Borysiewicz, 1995; Robinson, 1995).

Killed whole viruses and bacteria

Viruses and bacteria are usually killed using either heat or paraformaldehyde. However, these processes can lead to distortion of the antigenic properties of the pathogen resulting in a decrease in efficacy of the vaccine. Killed vaccines do not infect cells so will only stimulate humoral immunity. Protection often wears off and periodic booster shots may be required. Any whole organism vaccine whether live or dead may trigger allergic reactions (Robinson, 1995; Weiner & Kennedy, 1999).

Subunit vaccines

Subunit vaccines usually consist of a single protein, for example the surface coat of a virus or bacterium. These proteins may be produced in yeast, bacterial cultures or mammalian cells using recombinant DNA techniques. As with the killed vaccines, subunit vaccines usually only stimulate humoral immunity and protection may be short lived (Robinson, 1995; Weiner & Kennedy, 1999). In addition, these vaccines often require adjuvants to be effective.

Conjugate vaccines

Conjugate vaccines consist of a bacterial capsular polysaccharide conjugated to a carrier protein, usually diphtheria or tetanus toxoid. Vaccination using conjugates has been very successful. However, this type of vaccine elicits only humoral immunity and vaccine induced antibody levels have been shown to wane over time (Claesson, 1991) leading to increased susceptibility to disease and a need for booster injections.

1.4 DNA Vaccines

The problems associated with conventional vaccines such as transient protection, unsuitability for administration to immuno-compromised individuals and potential adverse reactions to the vaccine highlight the obvious need for the development of safer more effective therapies. Gene therapists working in the 1970s and early 1980s observed that an immune response was often triggered against the protein encoded by genes they were introducing into cells. However, it was not until the early 1990s that some laboratories began to research whether this unwanted phenomenon could be put to good use in vaccine research. Over the next few years independent studies revealed that DNA vaccines delivered into cells could stimulate both arms of the immune response in rodents, cattle and non-human primates (Davis *et al,* 1993; Robinson *et al,* 1993; Ulmer *et al,* 1993; Cox *et al,* 1993; Wang *et al,* 1993). In 1995 the first human clinical trial began. Plasmids containing HIV genes were delivered to patients already infected with the virus, and in 1996 healthy individuals were injected with genes encoding HIV or influenza proteins (Kim & Weiner, 1997). Unfortunately, early clinical trials of plasmid DNA vaccines have shown limited potency and as a result second generation DNA vaccines are being developed using various delivery systems to improve the magnitude and duration of the immune response.

1.4.1 Advantages of DNA vaccines over current vaccines

DNA vaccines offer many advantages over conventional vaccines. Unlike killed or subunit vaccines a single injection may impart a sustained response and both humoral and

cellular immunity can be stimulated. In comparison to live attenuated viral vaccines, DNA vaccines are potentially safe for use in immuno-compromised individuals. DNA vaccines are also commercially viable. They are relatively inexpensive to manufacture with no requirement for production and purification of proteins and, because DNA is not sensitive to temperature, it can be lyophilized facilitating storage and transportation.

1.5 Factors affecting the transfection efficiency and transgene expression of DNA vaccine vectors

In spite of the obvious advantages of DNA vaccines over conventional therapies, there are a number of factors which must be taken into consideration in designing an efficient vaccine delivery vector. Firstly, transfection must be efficient. This is the process by which DNA crosses the cell membrane and enters the cell. Secondly efficient transgene expression must occur. Transgene expression is the process by which the antigenic protein encoded on the plasmid is translated in the cell. Factors which can affect these processes are discussed below.

1.5.1 Plasmid and Cell Surface Charge

The surface of mammalian cells is negatively charged due to carboxylic groups on the sialic acid of glycoproteins, the sulphate groups of proteoglycans and the phosphate groups of glycerophosphates (Singh *et al*, 1992). Except in certain cell types, such as muscle and epidermal cells (Wolff *et al*, 1990), the passive transfer of naked DNA across the cell membrane does not occur. This is partly because under normal physiological conditions, DNA is highly negatively charged, resulting from its one negative charge per phosphate group, and is repulsed from the cell surface. It is therefore vital that this charge is neutralized to aid the passage of the DNA across the membrane (Zhdanov *et al*, 2002). This can be achieved using cationic polymers, and provided a net positive charge of the complex is maintained, contact of the carrier with the negatively charged cell membrane can enable efficient internalization into the target cell (Zuidam *et al,* 2000). The only disadvantage of this cationic state is that some target cell specificity may be lost. The

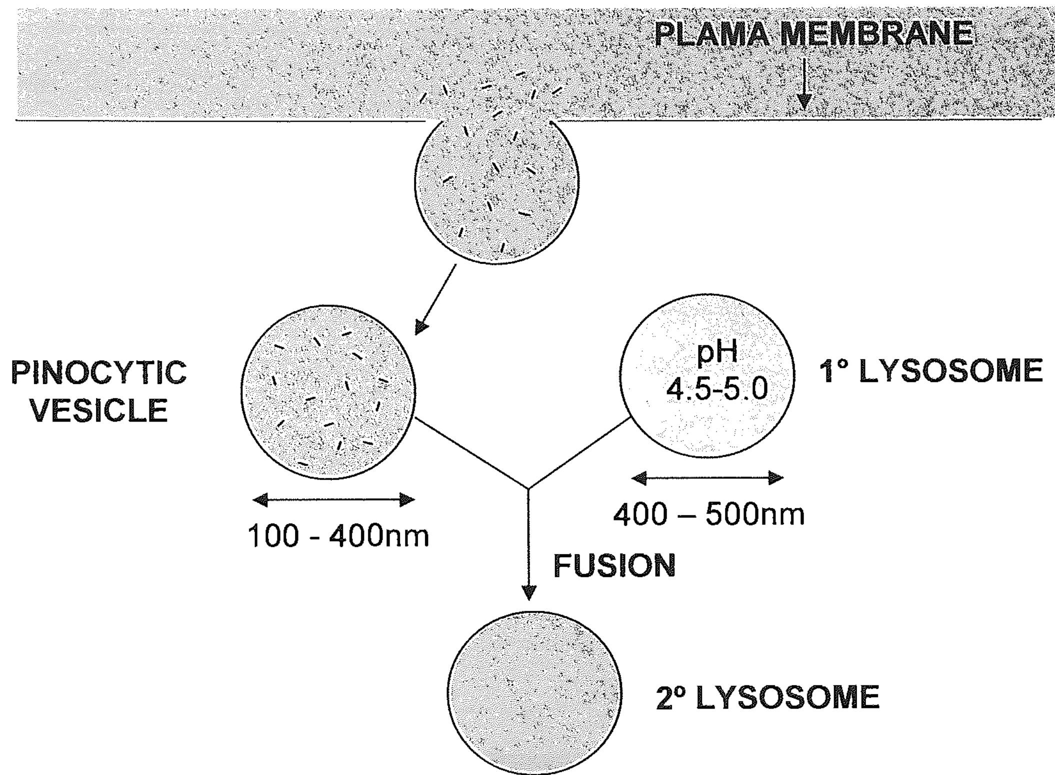
variable distribution of proteoglycans between cell types may explain why some cell types are capable of higher levels of transfection than others, as there is direct evidence that the negatively charged proteoglycans play a significant role in mediating uptake of positively charged complexes (Mislick & Baldeschwieler, 1996).

1.5.2 Crossing the cell membrane

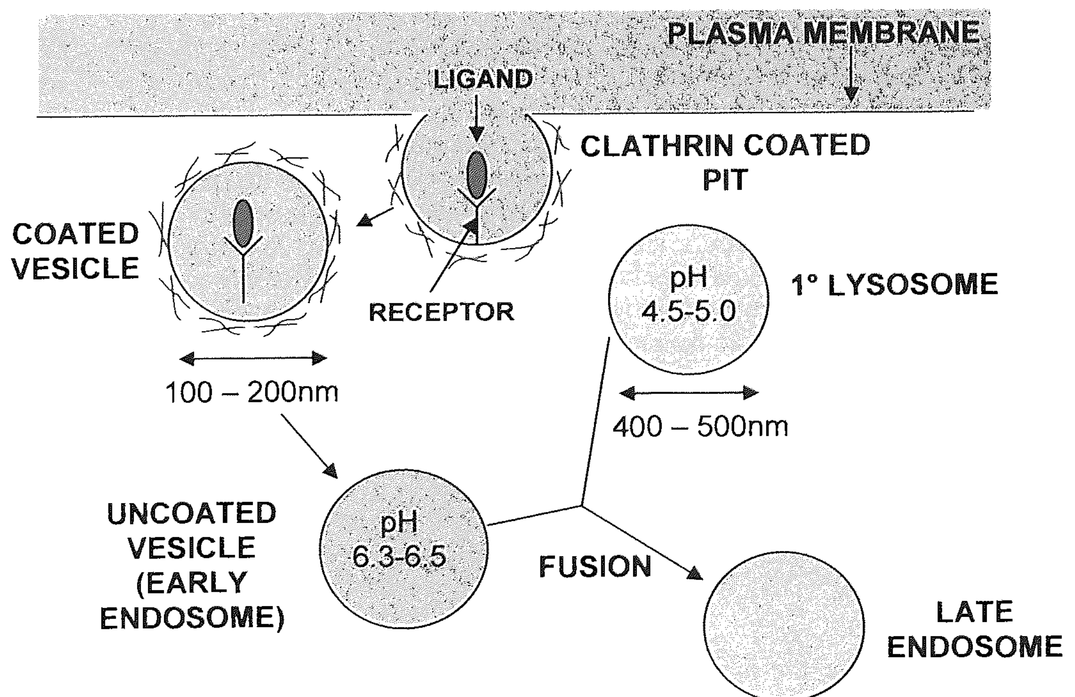
Plasmid DNA is a long flexible molecule which as well as being negatively charged is large in size (13-15 kilobase plasmid). These physicochemical characteristics restrict the unaided entry of DNA across cellular membranes. Within cells, constraint of the DNA double helix relies upon positively charged molecules such as histones and polyamines. These molecules condense DNA to within the restricted volume of the nucleus. Efficient transfection of exogenous plasmid DNA is closely related to effective condensation of the molecule (Zhdanov *et al*, 2002). Experiments on uptake of oligonucleotides into cells have shown that even methylphosphonate oligonucleotides up to 15 mer long do not cross membranes by passive diffusion at a significant rate, even when chemically modified to be more hydrophobic than the naturally occurring phosphodiester (Akhtar *et al*, 1991).

Once DNA is condensed, the size of the resulting complex will not only determine which cell type will take the complex up, but also which uptake mechanism the cell will adopt. There are a variety of mechanisms by which macromolecules may be taken up by cells. The first example is pinocytosis (fig 1.5.2A). Pinocytosis removes material either found suspended in the outside medium (fluid-phase pinocytosis) or attached to the cell membrane (adsorptive pinocytosis). Adsorptive pinocytosis although usually non-specific can include specific adsorption. When pinocytosis occurs the cell membrane invaginates and pinches off to form a vesicle inside the cell which can range from 100nm to several hundred nm in size. Once material is taken up by pinocytosis it is directed within the cell to the acidic lysosomal compartment (pH 4.5-5.0). The lysosomal compartment also contains a range of degradative enzymes at high concentrations (Pillay *et al*, 2002).

A. PINOCYTOSIS



B. RECEPTOR MEDIATED ENDOCYTOSIS



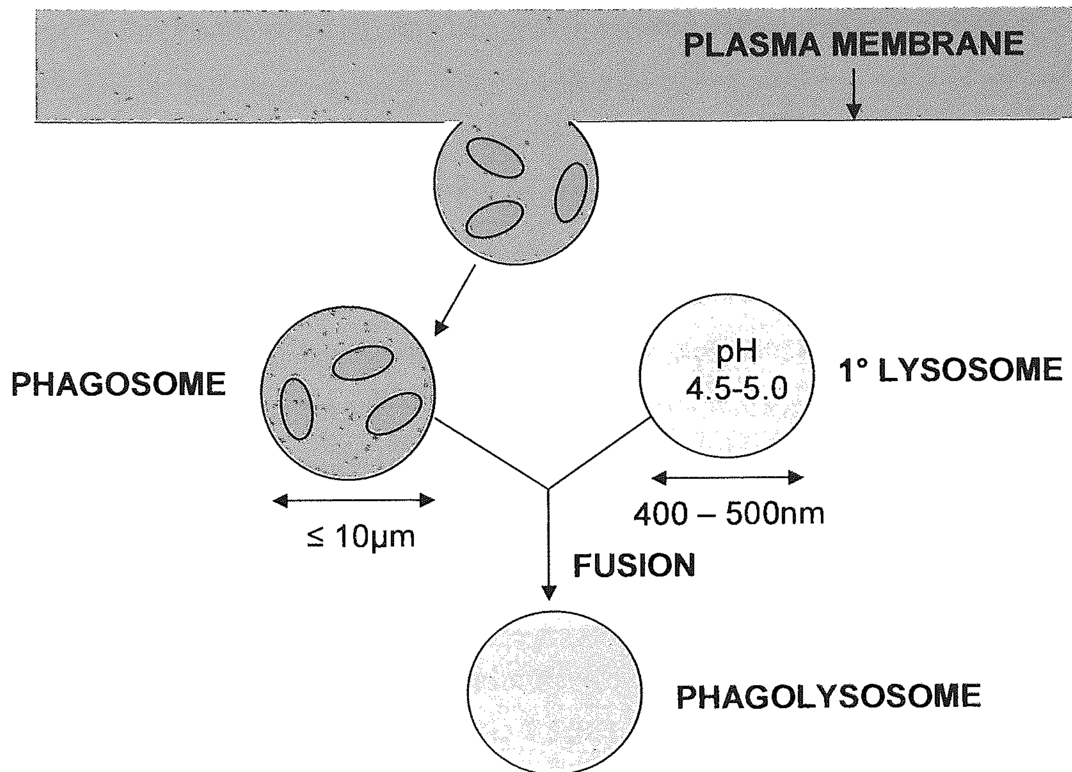


Figure 1.5.2 Schematic diagram of cellular uptake mechanisms. A. Pinocytosis: The cell membrane invaginates and pinches off to form a vesicle inside the cell (pinocytic vesicle). The pinocytic vesicle is directed within the cell to the acidic lysosomal compartment (pH4.5-5.0) which it fuses with. The lysosomal compartment contains a range of degradative enzymes at high concentrations. B. Receptor mediated endocytosis: A ligand becomes associated with a receptor on the cell surface. The membrane invaginates to form a clathrin coated pit which pinches off inside the cell to form a coated vesicle. Coated vesicles eventually become endosomal compartments which contain few degradative enzymes and have a pH of 6.3-6.5. The final stage of endocytosis occurs when the endosome fuses with an acidic lysosome to form a late endosome and the material is digested. C. Phagocytosis: This process is specific to APCs such as macrophages and dentritic cells (DCs). During phagocytosis exogenous foreign material is ingested by the cell into phagosomes. The phagosome then fuses with a lysosome and the foreign matter is digested.

Because of this harsh environment and the lack of cell specificity, pinocytosis is not generally exploited as an uptake mechanism for DNA delivery.

The second major uptake mechanism is receptor mediated endocytosis (fig 1.5.2B). Principally this occurs when a ligand becomes associated with a receptor on the cell surface. The membrane invaginates to form a clathrin coated pit which pinches off inside the cell to form a coated vesicle. Freeze fracture micrographs have shown clathrin coated pits to be between 100 and 200nm (Robenek *et al,* 1991) thus placing an upper limit on the size of the DNA complexes (Goldstein *et al,* 1985) that can be taken up by this route. Coated vesicles eventually become endosomal compartments which contain few degradative enzymes and have a pH of 6.3-6.5 (Pillay *et al,* 2002). The final stage of endocytosis occurs when the endosome fuses with an acidic lysosome and the material is digested. Because of the ability to target DNA to particular cell types using this uptake mechanism it has frequently been exploited to deliver nucleic acid to cells (Maruyama *et al,* 2004, Quian *et al,* 2002; Hussey & Peterson, 2002).

The third cellular uptake mechanism is phagocytosis and is specific to APCs such as macrophages and dentritic cells (DCs) (fig 1.5.2C). Because of the important role these cells play in the immune response, much research into the development of vaccines has focused on targeting this uptake mechanism. During phagocytosis exogenous foreign material is ingested by the cell into phagosomes. The phagosome then fuses with a lysosome and the foreign matter is digested. DNA/microparticle complexes in the size range of 1-10μm are too large to enter cells via pinocytosis or endocytosis and therefore target phagocytic cells by size exclusion (Tabata & Ikada,1990; Guy *et al,* 1995). It has been demonstrated that biodegradable microparticles made with poly (D,L-lactide-co-glycolide) (PLG) and complexed with DNA can also successfully deliver genetic material to APCs (Denis-Mize *et al,* 2000).

1.5.3 Endosome to cytoplasm transfer

One of the major problems in delivering DNA to cells is that nucleic acid becomes trapped in the endosomal compartment and is eventually degraded by lysosomal enzymes. This can severely reduce transgene expression. Naturally occurring molecules taken up by endocytosis contain characteristics that the cell recognizes so they are targeted intracellularly within the vesicles to the correct destination. Cellular trafficking is controlled by unique compartment specific proteins. Non-viral vectors are foreign to the cell and usually lack the characteristics necessary to direct their transport inside the cell.

To reduce degradation of DNA by lysosomal enzymes it is essential to enhance the release of DNA from the cellular compartments. Influenza virus, which is taken up by receptor mediated endocytosis has developed a way of escaping from the endosome before fusion with lysosomes. One of the virus's capsid proteins contains a peptide sequence which undergoes a conformational change when it enters the slightly acidic environment of the endosome. This destabilizes the lipid membrane and the contents of the internal vesicle are released into the cytoplasm.

To improve DNA delivery in non-viral systems many components of naturally occurring organisms have been exploited. In addition to influenza derived peptides other viral fusogenic peptides (Wagner *et al,* 1992), viral replication-defective or inactivated adenovirus or rhinovirus particles (Cotten *et al,* 1992; Zauner *et al,* 1995) and translocation sequences derived from protein toxins (Fisher & Wilson, 1997) have been incorporated in non-viral systems to enhance endosomal release. The lysosomotropic agent chloroquine has also been shown to enhance transfection efficiency in cells treated with agents such as calcium phosphate and DEAE dextran (Luthman & Magnusson, 1986). Chloroquine increases the endosomal or lysosomal pH (Maxfield, 1982). This may result in inhibition of pH-dependent enzymes (Wibo & Poole, 1974), enhanced dissociation of DNA from complexes (Erbacher, 1996) or a delay in transfer of DNA conjugates to lysosomes (Hedin & Thyberg, 1989; Stenseth & Thybert, 1989).

Furthermore, there is evidence that certain cationic compounds mediate improved access to the cytosol from endosomal compartments (Raychaudhuri & Rock, 1998). Cationic polymers that gain or lose protons freely at the pH of the endosome will act like a 'proton sponge'. A large number of ions will be attracted into the endosome which will eventually burst due to osmotic pressure. Endosomolysis may also be enhanced physically if, like PEI, the cationic polymer undergoes a pH-dependent configurational change (Haensler & Szoka, 1993; Boussif *et al,* 1995; Pollard *et al,* 1998).

1.5.4 Crossing the nuclear membrane

A major barrier to efficient transgene expression is the nuclear membrane. It has been observed that dividing cells are easier to transfect than non-dividing cells (Takeshita *et al,* 1994; Vitadello *et al,* 1994). This is because during mitosis the nuclear membrane ceases to exist. In non-dividing cells such as primary cell cultures, and most cells of the body which will be quiescent or post mitotic, the nuclear membrane is intact and intracellular and macromolecular transport into and out of the nucleus must occur through the nuclear pore complex (NPC) that has a functional diameter of 26nm (Dworetzky *et al,* 1988) (fig 1.5.4).

Two systems have been exploited to enhance DNA transfer into the nucleus. The first is a nuclear homing system. This utilizes nucleoproteins which contain short cationic motifs called "nuclear localization sequences" (NLS). The NLS bind the importing proteins and this leads to rapid entry through the NPC. In several reports NLS bound to DNA have been shown to enhance entry into the nucleus and subsequently transgene expression (Kaneda *et al,* 1989; Remy *et al,* 1995; Fritz *et al,* 1996; Collas *et al,*1996; Fominaya & Well, 1996; Dean, 1997; Langle-Rouault *et al,* 1998). Alternatively, adenoviral hexon proteins, which mediate efficient transfer of the virion to the nuclear pore complex, have

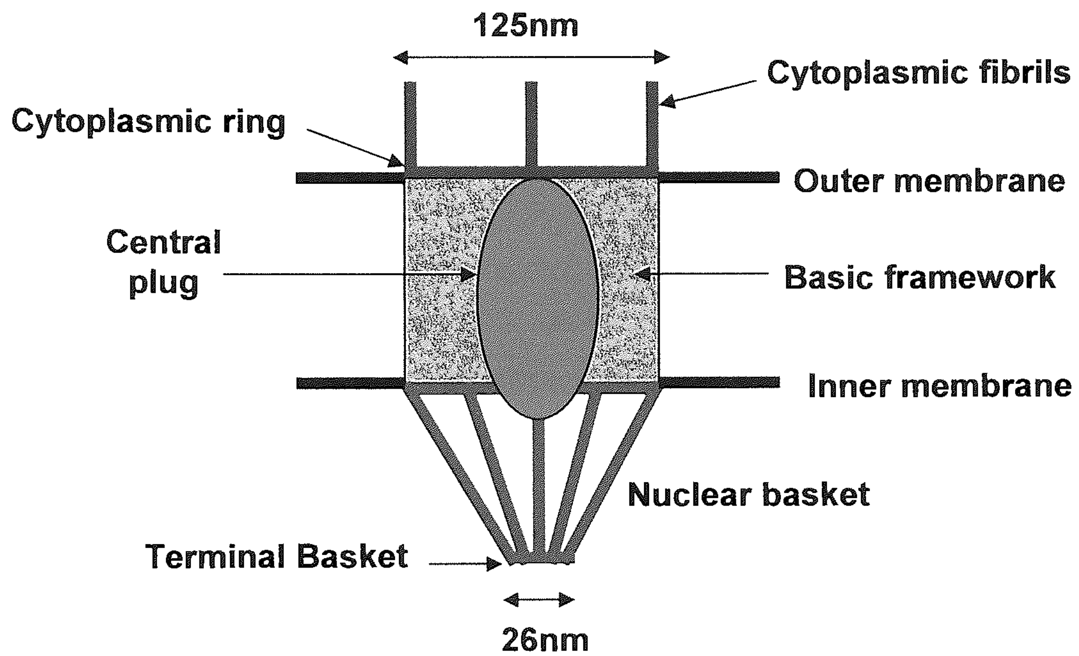


Figure 1.5.4 Schematic diagram of the membrane bound nuclear pore complex (NPC). The major structural components of the NPC include the basic framework, the central plug or gated channel, the cytoplasmic and nuclear rings, and the cytoplasmic fibrils and nuclear basket. The functional diameter of the complex is 26nm.

been exploited. These proteins have been used in polymer/DNA complex systems resulting in more efficient nuclear entry and higher levels of transgene expression.

1.5.5 Promoter activity

If large amounts of DNA remain in the cytoplasm following delivery into the cell, the problem of DNA transfer into the nucleus of non-dividing cells can be circumvented using cytoplasmic expression systems. In this system genes are introduced into cells under the control of a bacteriophage promoter, such as T7, with a source of T7 RNA polymerase expressed as an autogene. This promoter will drive the expression of the genes without the need for nuclear transcription machinery (Gao & Huang, 1993; Brisson *et al*, 1999). It may also be important to consider the choice of promoter if optimal transgene expression is to be achieved. Some promoters are only switched on in specific cell types, for example the albumin promoter in hepatocytes (Balague *et al*, 2000) whereas others, such as the cytomegalovirus immediate early promoter (CMV) will be switched on and drive expression in all cell types. If a DNA delivery vector is to be targeted to a specific cell type, then choosing a specific promoter may enhance protein expression.

As an awareness of the potential difficulties of delivering DNA efficiently to cells has grown, various delivery systems have been developed to try and minimise some of the problems described above.

1.6 Delivery of DNA vaccines

Delivery vectors for DNA vaccines fall broadly into two categories; viral and non-viral. The choice of vector depends on many factors including the administration route, the size of the gene(s) to be delivered and the health of the patient. The primary requirement of the delivery system is that it will enable functionally active DNA to enter the nucleus of target cells. In order for this to occur the DNA must cross the cell membrane, the nucleic acid must be protected from enzymes in the cellular compartments which will cause

degradation, and the DNA must cross the nuclear membrane and exist in the nucleus without integrating into the host genome. Ideally the delivery vector will be non immunogenic.

1.6.1 Viral delivery vectors

A virus can be attenuated to produce a vector for vaccine delivery using standard recombinant techniques. The plasmid DNA required for viral replication or pathogenicity is removed and replaced with the desired gene or genes. The characteristics of the virus determine the types of cells it can infect and the length of time that gene expression lasts. In 1989, Miller *et al* were the first to achieve gene expression using a mouse retrovirus (Miller & Rosman, 1989). In the proceeding years a number of other non-replicating viral vectors including adenovirus (Prevec *et al*,1991; Graham & Prevec, 1992), fowlpox virus (Baxby & Paoletti, 1992) and adeno-associated virus (AAV) (Muzycka, 1992) were successfully used to generate transgene expression. Alphavirus (Lundstrom, 2001), herpes simplex virus (Fink *et al,* 1996) and lentivirus (Chang & Gay, 2001), a subset of the retrovirus family which can deliver genetic material to non-dividing cells, have also been investigated for their vector potential. A major advantage of using viral vectors is the high levels of transgene expression achievable. Conversely, because the vectors still contain viral proteins an immune response may be launched against them. This makes repeat dosing problematic and delivery of other vaccines using the vector less viable. Additionally, if an individual has had previous exposure to, for example, adenovirus, then vaccination using an adenoviral vector may be limited in its effectiveness as the vector will be cleared quickly by the immune system. There are also problems associated with specific vectors. Retroviral vectors integrate DNA into the host genome. This occurs randomly and may cause mutations within the host cell that lead to oncogenicity (Su *et al,* 2000). AAV vectors have only a limited capacity to carry genes because of the small size of the virion (20 nm), which only permits the packaging of <5kb of exogenous DNA and gene expression using adenoviral vectors may be short lived because its DNA persists as an extra-chromosomal element (Flotte, 2000).

1.6.2 Non-viral delivery vectors

Non-viral delivery vectors effectively eliminate any pathological risks associated with the use of viral delivery vehicles. DNA does not integrate into the host genome and the vector will not trigger immunogenicity against itself (Wolff *et al,* 1992). In addition, industrial production and quality control of non-viral vectors is much less challenging.

1.6.2.1 Naked DNA

In the early 90s the results of three *in vivo* studies carried out by independent groups' triggered investigations into the use of naked DNA as a potential vaccine. These studies demonstrated gene expression after direct injection of plasmid DNA into skeletal muscle (Wolff *et al,* 1990) and cardiac muscle (Lin *et al,* 1990), and in 1991 a delivery device known as the gene gun was used to transfect epidermal cells. This device worked by bombarding the epidermal cells at high-velocity with DNA coated gold microparticles. (Williams *et al,* 1991). Early fears that plasmid DNA could integrate randomly into the host genome were discredited in 1992 in a study by Wolff which demonstrated that plasmid DNA taken up by cells did not replicate and persisted episomally with no evidence of integration (Wolff *et al,* 1992). Protective immunity in a mouse model following i.m. injection of a plasmid encoding a viral protein was first observed in 1993 by Ulmer *et al* (1993). Within the last 6 years nonhuman primates have been shown to exhibit antibody and cytotoxic T lymphocyte (CTL) responses after repeat doses of 1-2mg plasmid DNA on multiple occasions (Letvin *et al,* 1997), and in 1998 antibody and CTL responses were induced in human volunteers given high doses (>1mg) of DNA (Calarota *et al,* 1998; Wang *et al,* 1998; MacGregor *et al,* 1998). The studies in nonhuman primates and human volunteers highlight one of the problems of developing naked plasmid DNA vaccines for commercial use in humans. Because of the instability of naked DNA within the cellular compartments, transgene expression is relatively inefficient. As a result, in humans, high repeated dosing is required to elicit an immune response. This imposes serious limitations on the number of constructs that could be included in a vaccine and is less favourable economically.

CpG motifs within bacterial propagated DNA vectors

It has been demonstrated *in vitro* that plasmid DNA has a direct immunostimulatory effect on immune cells (Messina *et al,* 1991; Rankin *et al,* 2002). Plasmid DNA is bacterial in origin and contains frequently occurring unmethylated DNA sequences called CpG motifs. Not only are there fewer CpG motifs in vertebrate DNA, but when they do occur they are methylated and usually flanked by specific DNA sequences (two 5' purines and two 3' pyrimidines) (Krieg *et al,* 1995). As a result, cells of the innate immune system such as macrophages and dendritic cells can distinguish between bacterial and self CpG sequences. Toll-like receptors (TLRs) are a group of type I transmembrane proteins involved in innate immunity. Bacterial CpGs are thought to signal through a specific toll-like receptor, TLR9 found on the surface of innate immune cells (Hemmi *et al,* 2000; Bauer *et al,* 2001). Activation of these cells leads to cytokine production and enhanced expression of costimulatory molecules. However, recently the importance of CpG-DNA/TLR9 interactions in long term vaccination have been called in to question. Spies *et al* demonstrated *in vivo* that immune responses in TLR9 negative and TLR9 positive mice immunised with plasmid DNA were comparable (Spies *et al,* 2003).

1.6.2.2 Cationic lipid-based delivery vectors

Formation of DNA/lipid complexes occur spontaneously when plasmid DNA is mixed with preformed small cationic lipid vesicles called liposomes. The cationic lipid interacts electrostatically with the negatively charged phosphate backbone of the plasmid DNA. These complexes then interact with and are taken up by target cells. In most cases optimal cell transfection conditions are based on the proportion of plasmid DNA to cationic liposomes in the formulation. This will reflect the overall ratio of positive charges on the cationic lipid to the number of negative charges on the DNA, a critical determinant of transfection efficiency. In addition, cytotoxicity induced by cationic lipids at higher charge ratios is thought to be responsible for lower transfection efficiencies (Felgner *et al,* 1987; Gao & Huang, 1991; Farhood *et al,* 1992). There are two main pathways by which lipid/DNA complexes may enter the cell. The first pathway is by

endocytosis of the DNA/lipid complex. This is followed by destabilisation of the endosomal membrane by the cationic lipid and subsequent release into the cytoplasm. The second is by direct fusion with the cellular membrane although only a small proportion (approximately 2%) of the complexes are thought to enter the cell by this route (Zhou & Huang, 1994). Often neutral lipids such as dioleoyl phosphatidylethanolamine (DOPE) or cholesterol are incorporated in the formulation together with the cationic lipid. It is believed that the neutral lipids promote fusion between the lipid/DNA complex and the endosomal membrane improving the efficiency of gene transfer into the cytoplasm (Farhood *et al,* 1995). More recently some groups have been investigating the use of solid lipid nanoparticles (SLN) and solid lipid microparticles (SLM) formed from biocompatible lipids as drug carrier systems (Morel *et al,* 1996; Muller *et al,* 2000; Reithmeier *et al,* 2001; Mehnert & Mader 2001). Solid lipid nanoparticles, stabilised in the form of aqueous dispersions by suitable surfactants, generally posses a solid matrix composed of physiological and well-tolerated lipids (Muller *et al,* 2000). Erni *et al* recently described efficient *in vitro* transfection of non-phagocytic cells by SLM. The microparticles were formed by the introduction of cationic lipids into a tripalmitin matrix to allow subsequent complexation of DNA on the resulting cationic surface (Erni *et al,* 2002).

There have been many successful studies of gene delivery using cationic/lipid DNA complexes both *in vitro* (Solodin *et al,* 1995; Lewis *et al,* 1996; Vitiello *et al,* 1998) and *in vivo* (Wheeler *et al,* 1996,; Liu *et al,* 1997; Mahato *et al,* 1998). Liposomes have also been widely investigated for DNA vaccine delivery. Recently, mice immunised with liposome entrapped plasmid DNA encoding the hepatitis B surface antigen were shown to exhibit enhanced humoral and cell mediated responses in comparison to animals immunised with naked DNA (Perrie *et al,* 2002; Gregoriadis *et al,* 2002). Cationic liposomes are able to interact with DNA to promote its transfection in both replicating and non-replicating cells (Felgner *et al,* 1987; Gao & Huang, 1995), they protect DNA from enzymatic degradation (Crook *et al,* 1996), they enhance cellular internalisation and they allow passive or active targeting strategies (Gregoriadis, 1988). Liposome vectors can also be manufactured to drug standard, are biodegradable, and are non-immunogenic.

One of the drawbacks with using cationic lipids is that there are reports that the liposomes are toxic to cells *in vitro* and *in vivo* (Li & Huang, 1997; Brown *et al,* 2001). Additionally, because the liposomes associate with serum proteins, transgene efficiency may be inhibited by serum in the blood. This would be a particularly important consideration if a vaccine were to be administered intravenously (i.v). The *in vivo* circulation half-life of complexed DNA administered systemically is short lived because of the afore mentioned interaction with serum components (Mahato *et al,* 1995). Circulation half-life can be extended by coating liposomes with the hydrophilic polymer poly(ethylene glycol) (PEG) (Allen & Chonn, 1987; Klibanov *et al,* 1990; Blume & Cevc, 1990; Papahadjopoulos *et al,* 1991). Cationic liposomes also demonstrate a lack of tissue specificity and even though liposomal vectors are much safer than viral vectors the level of expression from transferred genes is comparatively low.

1.6.2.3 Soluble cationic polymer delivery vectors

Poly-L-lysine (PLL)

PLL is the most commonly used of a group of molecules capable of condensing DNA called polyaminoacids, which also include poly-L-arginine and poly-L-ornithine. Wu *et al* were the first group to report gene transfer using polycation conjugates of PLL (Wu & Wu, 1987; Wu & Wu, 1988). The positively charged PLL interacts electrostatically with the negatively charged DNA molecule (Laemmli, 1975) and condenses DNA to nanometer sized complexes that can be taken up by cells. Once complexed, it has been shown that DNA within the particle is protected against hydrolytic enzymes (Baeza *et al,* 1987; Chiou *et al,* 1994). *In vitro* PLL has excellent applications due to its flexibility regarding the size of DNA to be transferred, its amenability to ligand complexation and its relatively efficient transfection levels. A variety of ligands have been utilised to allow cell type-specific transfection with DNA/PLL particles including transferrin (Wagner *et al,* 1990), mannose (Nishikawa *et al,* 2000) and folic acid (Mislick *et al,* 1995). However, for *in vivo* applications PLL does not represent such an attractive prospect. One limitation is that PLL is toxic to living cells at nM concentrations (Han *et al,* 2000).

Additionally, there are varying reports as to the immunogenicity of the polyaminoacid. Some reports suggest PLL has low immunogenic potential (Maurer *et al,* 1959; Stakovics *et al,* 1994; Ferkol *et al,* 1996) but Vermeersch and Remon reported the production of antibodies after injection of PLL in the presence of Freunds adjuvant (Vermeersch & Remon, 1994). Furthermore, PLL is known to interact with serum components and has been shown to be rapidly removed from circulation of mice following systemic administration (Lollo *et al,* 1997). Although there are few reports describing the use of PLL for vaccination, recently, Rogers *et al* injected mice i.v. with plasmid DNA encoding a herpes simplex glycoprotein (gD-1) complexed with asialoorosomucoid (ASOR) conjugated to poly-L-lysine. This group demonstrated that the ASOR-poly-L-lysine DNA carrier system promoted hepatic expression of gD-1 and suggested this could result in an immune response to the protein, aiding vaccination against herpes simplex virus type-1 (Rogers *et al,* 2000).

Polyethylenimine (PEI)

PEI is a synthetic compound that was identified over 50 years ago. Its safety has been demonstrated by its varied and intensive uses, including water purification, ore extraction and shampoos. PEI is a cationic polymer with a high charge density that forms complexes with DNA. The size, charge and solubility of the complexes formed with PEI are strongly dependent on the charge ratio of the polycation and the DNA (Kabanov & Kabanov, 1995; Kabanov *et al,* 1998). PEI conjugates have been shown to be more efficient in transfection studies than PLL conjugates. Diebold's group demonstrated that transferrin conjugated complexes of PEI/DNA resulted in higher transgene expression in a number of cell types compared to conjugated PLL/DNA (Diebold *et al,* 1998). The enhanced levels of transfection observed with PEI/DNA when compared to PLL/DNA can probably be accredited to the buffering capacity of PEI in the lysosomal compartment. This may result in inhibition of nucleases that have an acid optimal pH and could also alter the osmolarity of the vesicle. PEI would in effect act as a 'proton sponge'. In the presence of the polycation a net increase in the ionic concentration within the endosome would occur. This would lead to swelling of the polymer by internal charge repulsion, and osmotic

swelling of the endosome, due to water entry resulting in membrane destabilization. This process may enhance release of DNA into the cytosol (Remy, *et al,* 1998).

PEI complexed with DNA can enhance levels of transfection not only *in vitro* but also *in vivo* (Boussif *et al,* 1995; Abdallah *et al,* 1996; Goula *et al,* 1998), and although serum has been shown to reduce transgene expression of DNA complexed with branched PEI it has no effect on the fully linear isomer of PEI (Boussif *et al,* 1995). One of the drawbacks in using PEI as a delivery vector *in vivo* is that transfection efficiency and cytotoxicity are closely related to the molecular weight (MW) of the compound. At higher MWs (>25kDa) transfection efficiency is high but so is cytotoxicity. By comparison, if a low molecular weight PEI is used, although little toxicity is observed, transfection is negligible (Godbey *et al,* 1999a). In order to alleviate this problem poly ethylene glycol (PEG) a positively charged fusogenic polymer was grafted onto the surface of PEI/DNA complexes resulting in increased transfection levels without an increase in cytotoxicity (Lee *et al,* 2002). Recently it has been described that mice injected with reporter plasmid/PEI complexes produced a mixed Th1/Th2 immune response resulting in T_H cell and CTL activation. However, despite these promising results, genes involved in other cellular responses such as apoptosis, stress responses and oncogenesis were also activated (Regnstrom *et al*, 2003).

Chitosan

Preparation of self-assembling polymeric and oligomeric chitosan/DNA complexes were first described in 1995 by Mumper *et al* (Mumper *et al,* 1995). More recently, promising results have been obtained using chitosan as a gene delivery vehicle (Lee *et al,* 1998; Leong *et al,* 1998; Köping-Höggard *et al,* 1998). The physicochemical and biological properties of chitosan are discussed in greater detail in section 1.8.2. Chitosan is a polycation that can spontaneously form complexes with negatively charged DNA. When mixed at a 1:1 ratio, or at an excess with chitosan, the complexes will completely incorporate the DNA. Bound nucleic acid is protected from nuclease degradation (Aral *et al,* 2000). Condensation of DNA by chitosan has been shown in TEM studies to form

toroids, the size of which are dependent on the molecular weight of the chitosan used (MacLaughlin *et al,* 1998; Thanou *et al,* 2001). Using a coacervation process chitosan has also been used to produce uniform, spherical nanospheres between 200 and 500nm in size (Mao *et al,* 1996). Erbachers' group was one of the first to demonstrate that chitosan/DNA complexes were capable of transfecting cells. They produced a stable, homogenous population of chitosan/DNA complexes with a diameter of approximately 50-100nm. They demonstrated that the complexes in the presence of serum could effectively transfect HeLa cells and, that by 96h transfection was 10-times more efficient than PEI mediated transfection (Erbacher *et al,* 1998) Since then, chitosan/DNA complexes have been reported to transfect a variety of cell types *in vitro* (MacLaughlin *et al,* 1998; Ishii *et al,* 2001; Mao *et al,* 2001; Sato *et al,* 2001) and chitosan/DNA complexes or chitosan based nanoparticles have resulted in successful transgene expression *in vivo* (MacLaughlin *et al,* 1998; Roy *et al,* 1999). In the field of DNA vaccines, chitosan nanoparticles have been used for topical genetic immunization in mice (Cui & Mumper, 2001) and chitosan complexes have been used as a nasal delivery system for vaccines (Illum *et al,* 2001).

Although the non-viral delivery vectors described above exhibit potential for use in DNA vaccination, they all have limitations. Naked DNA is only potent in humans at repeated high doses (Calarota *et al,* 1998; Wang *et al,* 1998; MacGregor *et al,* 1998) and cationic lipid based vectors are very effective *in vitro* but *in vivo* their use is precluded by toxicity and reduced efficacy. Of the soluble polymers PLL is ineffective *in vivo* and too toxic for use in vaccination. High molecular weight PEI is also toxic *in vivo* while the transfection efficiency of low molecular weight PEI is low, and although chitosan exhibits high transfection efficiency *in vitro* successful *in vivo* vaccination studies are limited (Cui & Mumper, 2001; Illum *et al,* 2001). There is therefore an obvious need to develop a non-viral vector which reduces the dose of DNA required to elicit an immune response, maintains vaccine potency and is non-toxic *in vivo*.

1.7 Cationic polymer microparticles as a delivery vector for DNA vaccines

Since 1990 cationic microparticles have been used for the successful delivery of protein vaccine antigens (Singh *et al,* 1998; Kim *et al,* 1999; Chattaraj *et al,* 1999). However, it is only more recently that this technology has been exploited for the delivery of DNA vaccines (Jones *et al,* 1997; Hedley *et al,* 1998). Cationic microparticles are usually produced by an emulsification and solvent evaporation process (section 1.8) and are typically composed of a neutral or negatively charged core polymer stabilised by a cationic component. Frequently used core polymers include poly-L-lactide (PLA) or poly (DL-lactide-co-glycolide) (PLG). DNA can be either encapsulated within the microparticle, or adsorbed to the cationic surface.

There are a number of advantages in using cationic microparticles for DNA vaccine delivery over other non-viral systems. The particles can predominantly be formulated from well-defined, biocompatable polymers, thus reducing the chances of the vector causing toxicity *in vivo.* In addition, because of the size of the particles direct targeting of professional APCs can be achieved. In 1998 Banchereau *et al* were the first group to demonstrate the efficient uptake of microparticles by dendritic cells *in vivo* and it is now well established that particles between 1 and 10μm target professional APCs by size exclusion (Tabata & Ikada, 1990). Furthermore, the cationic nature of the microparticles may assist the passage of nucleic acid across the cellular membrane and enhance the transfer of DNA from acidic cellular compartments into the cytoplasm.

1.7.1 Microencapsulation of DNA

Traditionally PLG microparticles have been used as delivery systems for entrapped vaccine antigens including encapsulation of *H.Pylori* (Kim *et al,* 1999), diphtheria toxoid (Singh *et al,* 1998) and influenza vaccine (Chattaraj *et al,* 1999). However, in 1997 induction of antibody and CTL responses to protein encoded on plasmid DNA microencapsulated within PLG microspheres was demonstrated for the first time (Jones *et al,* 1997) and the following year, Hedley *et al* (1998) used plasmid DNA entrapped in

PLG microparticles to elicit CTL responses in immunised mice. In principle this technique is advocated because it offers protection of the plasmid DNA from degradation and DNA release can be precisely controlled allowing for a sustained delivery formulation. However, there are significant drawbacks to microencapsulation. Often the efficiency of DNA encapsulation is very low (approximately 20%) (Ando *et al,* 1999; Wang *et al,* 1999) although Özbas-Turan *et al* demonstrated DNA incorporation efficiencies as high as 90% using chitosan microspheres produced by a complex coacervation technique (Özbas-Turan *et al,* 2003). Another disadvantage of using the traditional double emulsion process (section 1.8) is that in many instances entrapment has resulted in major degradation of the DNA during encapsulation and release (Ando *et al,* 1999; Walter *et al,* 1999; Tinsley-Brown, 2000). Plasmid DNA can be found in three conformations; supercoiled, nicked (open circle) and linear (fig 1.7.1), but it is well established that it is the supercoiled conformation that retains the highest level of bioactivity (Yamaizumi *et al,* 1983; Xu & Szoka, 1996; Middaugh *et al,* 1998). The high shear during homogenization and the energy required during the preparation process leads to nicking of the DNA and a significant reduction in the supercoiled conformation. Only approximately 10-20% of encapsulated material retains its supercoiled structure (Ando *et al,* 1999; Tinsley-Brown *et al,* 2000). Damage to the DNA not only occurs during the encapsulation process but also during the course of PLG degradation (Walter *et al,* 1999; Wang *et al,* 1999). Breakdown of the polymer generates an extreme acid environment (pH 2-3) which can destabilise DNA (Gander *et al,* 1993; Schwendeman *et al,* 1996). In some cases microencapsulation can also lead to the production of very large microparticles (100μm). These particles would be too large to be phagocytosed by APCs reducing their potential to target cells of the immune response (Luo *et al,* 1999). Finally, entrapped DNA is generally released very slowly from microparticles in small quantities (Walter *et al,* 1999). For use in vaccination this release profile may not be advantageous. If DNA is not immediately released from the particle, or only very small quantities of DNA are released within target cells, the speed and magnitude of the immune response may be adversely affected.

1.7.2 Surface adsorption of DNA

In 1999 Esposito *et al* described a novel carrier system for DNA involving surface adsorption of plasmid to a cationic microparticle (Eposito *et al*, 1999) and in 2000, Singh *et al* were the first to demonstrate antibody and CTL responses to plasmid DNA delivered on the surface of PLG/CTAB microparticles (Singh *et al*, 2000). The level of immune enhancement observed using this system was significantly greater than responses achieved by DNA entrapped in PLG microparticles (Singh *et al*, 2000). Although the idea of delivering DNA on the surface of microparticles was new, this technique had been used previously to successfully deliver surface adsorbed protein to immune cells (Coombes *et al*, 1999; Kazzaz *et al*, 2000). Surface adsorption of DNA offers numerous advantages over microencapsulation. DNA adsorbed to the surface of microparticles will not be exposed to the thermal, chemical or mechanical stresses often endured in the encapsulation process (Ando *et al*, 1999; Walter *et al*, 1999; Tinsley-Brown *et al*, 2000) and so its integrity will be maintained. Singh *et al.* demonstrated that not only did DNA associate efficiently with PLG but also that adsorbed DNA maintained its super-coiled conformation (Singh *et al*, 2000). Another advantage of DNA being adsorbed to the surface is that it provides a means of rapid delivery and release of the DNA in APCs. PLG/CTAB microparticles which stimulated immunity *in vivo* exhibited rapid release of a significant fraction of DNA between 1-24h under *in vitro* conditions (Singh *et al*, 2000). In addition, immediate release of encapsulated antigen from PLGA microspheres *in vitro* was shown to elicit a long lasting immunogenic response *in vivo* (Tobio *et al*, 2000). Long term stability problems could be eradicated by keeping sterile, lyophilized preparations of both DNA and microparticles separately. Reconstitution and mixing of the components need only occur prior to immunization.

Due to the potential of polymer microparticles to target the most potent cells of the immune response (APCs), and the obvious advantages associated with surface adsorption of DNA compared to microencapsulation, the research presented in this thesis focuses on the development of novel cationic microparticles with surface adsorbed DNA for the

delivery of vaccines by intramuscular (i.m.) injection, the optimal route for immunisation with naked DNA.

1.8 Composition and preparation of cationic polymer microparticles for DNA adsorption

An established method for producing biodegradable microparticles (from the lactide-glycolide polymers for example) uses an emulsification and solvent evaporation process (O'Donnell & McGinty, 1997). The core polymer is dissolved in an organic solvent and the polymer solution is emulsified in an immiscible non-organic solvent such as water to produce a suspension of droplets of polymer solution. Precipitation and hardening of the polymer occurs by movement of the solvent into the continuous phase and gradual solvent evaporation. Following solvent evaporation, the hardened microparticles are harvested (e.g. by centrifugation). Frequently a stabiliser such as poly (vinyl alcohol) is added to the aqueous phase of the formulation. The type of stabiliser and the concentration used can affect the size of the microparticles. During particle formation because the stabiliser will be present at the boundary layer between the water phase and the organic phase it can become incorporated at the particle surface affecting properties such as zeta potential and mucoadhesion (Scholes *et al,* 1993; Feng & Huang, 2001).

1.8.1 Core polymer

Poly (L-lactide)[PLA] and Poly (D,L-lactic-co-glycolide)[PLG]

Both PLA and PLG have been widely used in the preparation of microparticles. This is primarily because these compounds have been used in several biomedical products and are known to be safe, biodegradable and biocompatible (Anderson & Shive, 1997). PLA and PLG are degraded principally by hydrolysis in a non-enzymatic fashion and are cleaved to form the naturally occurring, toxicologically acceptable products lactic and glycolic acid (Visscher *et al,* 1985; Coombes *et al,* 1999). Particular interest has been shown in PLG as it degrades more quickly than PLA and has a long history of safe use in

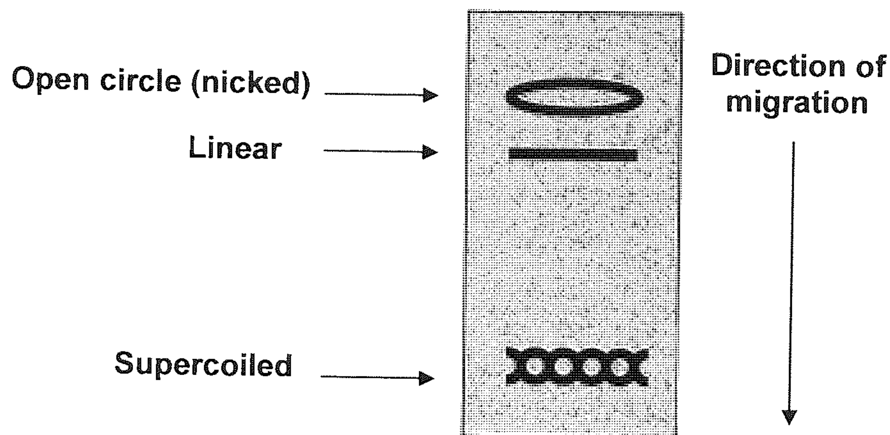


Figure 1.7.1 Schematic diagram of plasmid DNA: Structural configurations separated by agarose gel electrophoresis. The direction of migration is indicated by the arrow. A supercoiled configuration occurs when both strands of DNA are intact circles. Compact supercoiled DNA runs considerably faster than open circle or linear DNA upon electrophoresis in agarose gel. If one of the DNA strands is damaged (nicked) the molecules are described as open circles. Open circle DNA usually runs the slowest. If both DNA strands are damaged the molecule takes on a linear configuration.

Polymer	DL lactide/glycolide mole ratio	Degradation time (months)
L-PLA	N/A	>24
DL-PLA	N/A	12-16
DL-PLG 85:15	85/15	5-6
DL-PLG 75:25	75/25	4-5
DL-PLG 65:35	65/35	3-4
DL-PLG 50:50	50/50	1-2

Table 1.8.1. Degradation times of poly-lactide (PLA) and poly-lactide-co-glycolide polymers (PLG). Lactide exists as two optical isomers, D and L. L-lactide occurs naturally whereas DL-lactide is a synthetic blend of D-lactide and L-lactide.

veterinary and medical applications (table 1.8.1). PLG has been exploited for a range of biomedical purposes, including the preparation of controlled release drug delivery systems (Okada, 1997) and for the manufacture of implants and internal sutures (Visscher *et al,* 1985). PLG can be obtained in a range of molecular weights and with varying ratios of lactic to glycolic acid. The degradation rate is considerably affected by these parameters and it is therefore possible to tailor the PLG used to a particular application.

To formulate PLA or PLG microparticles with a cationic surface a positively charged stabiliser must be introduced into the aqueous phase of the emulsification and solvent evaporation process. Possible stabilisers are described below.

1.8.2 Cationic stabilisers

Cetyltrimethyl ammonium bromide (CTAB)

CTAB is a water soluble cationic surfactant which has previously been exploited for a range of biomedical applications, including use as an antibacterial in eye drops (Singh *et al,* 2000) (fig 1.8.2A). Early attempts utilising CTAB-oligonucleotide complexes bound to polyalkylcyanoacrylate nanoparticles to deliver antisense oligonucleotides to macrophage cell lines *in vitro* were unsuccessful (Chavany *et al,* 1994; Fattal *et al,* 1998). These vehicles were incapable of transfecting DCs with plasmids carrying recombinant genes. However, in 2000 Singh *et al.* used CTAB stabilised PLG microparticles to adsorb DNA and demonstrated successful transfection of DCs (Denis-Mize *et al,* 2000) as well as the generation of an immune response following i.m. injection in mice (Singh *et al,* 2000). More recently PLG/CTAB microparticles have been shown to elicit a potent immune response in other animals including non-human primates (O'Hagan *et al,* 2001). The use of CTAB as a cationic stabiliser may in part be responsible for the successful transfection efficiency of these microparticles. CTAB on the surface of the microparticle may act in a similar way to PEI leading to disruption of cellular compartments, and enhanced release of DNA into the cytoplasm. However, despite these promising results and the fact that the toxicity of CTAB itself is well defined, there are still important

considerations as to the long term safety of using surfactant-stabilised microparticles for human vaccines.

Chitosan

Chitosan is obtained by the alkaline deacetylation of chitin (fig 1.8.2B). Chitin is the second most abundant polysaccharide in nature and is found in the exoskeleton of crustacean, insects and some fungi. The main commercial sources of chitin are the shell wastes of shrimp, lobster, krill and crab (Rha *et al,* 1984; Struszczyk *et al,* 1991; Roberts, 1992a). Chitosan is a biodegradable (Struszczyk *et al,* 1991), biocompatible (Chandy & Sharma, 1990; Hirano *et al,* 1990) polycationic polymer with low immunogenicity and low toxicity (Arai *et al,* 1968; Brine *et al,* 1992; Carrero-Gomez & Duncan, 1997; Richardson *et al,* 1997). Oral administration of chitosan to rats demonstrated an LD_{50} as high as 16g/kg (Knapczk *et al,* 1984; Hirano *et al,* 1990). Metabolism of chitosan occurs in the lysosomal compartment of cells where chitosan is degraded into a common amino sugar, N-acetyl glucosamine, which is incorporated into the synthetic pathway of glycoproteins, and is subsequently secreted as carbon dioxide (Chandy & Sharma, 1990)

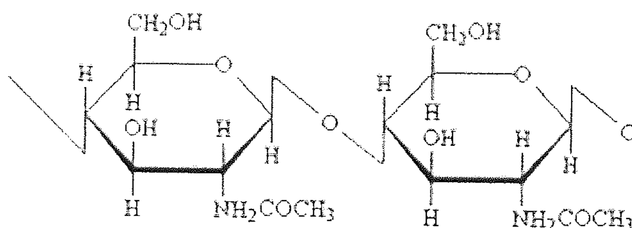
The chitosan molecule is a copolymer with a sugar backbone consisting of *N*-acetyl-D-glucosamine and D-glucosamine residues (Roberts, 1992b; Domard & Cartier, 1992; Hoppe-Seiler, 1994). However, the word chitosan does not just refer to a single polymer but to a large number of polymers which differ in their molecular weight (50,000-2,000,000 Da) and degree of N-deacetylation (40-98%). These parameters have a distinct effect on solubility and the viscosity of the polymer in solution. Chitosan is insoluble at neutral pH and alkaline pH because it is a weak base with a pKa value of the D-glucosamine residue of about 6.2-7.0. Protonation of the amine groups of the polymer occur in acidic medium resulting in a positively charged polysaccharide that has a high charge density (Fukuda & Kikuchi, 1978; Fukuda, 1980; Sandford, 1989; Knapczyk *et al,* 1989; Errington *et al,* 1993; Amiji & Patel, 1996). The polysaccharide has an average amino group density of 0.837 per disaccharide unit (Leong *et al,*1998).

A.

Cetyltrimethyl ammonium bromide
[CTAB]

B.

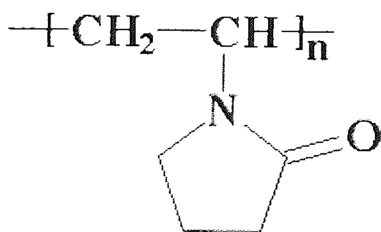
Chitosan



C.

Polyvinylpyrrolidone
[PVP]

Poly(2-(dimethylamino)ethyl methacrylate)
[PDAEMA]



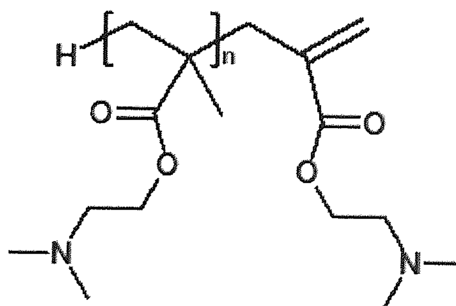


Figure 1.8.2. Chemical structures of potential cationic stabilisers for PLA/PLG microparticles. A. The cationic surfactant cetyltrymethylammonium bromide [CTAB]. B. The cationic polymer chitosan. C. The cationic copolymer Poly (N-vinylpyrrolidone/2-dimethylaminoethyl methacrylate), dimethyl sulfate quaternary made up of polyvinylpyrrolidone [PVP] and Poly(2-(dimethylamino)ethyl methacrylate) [PDAEMA].

Chitosan has been used frequently in gene delivery systems (section 1.4.2). However, it has not previously been used as a stabiliser for PLG microparticles to target professional APCs in vaccine development. Because of the long term safety issues regarding surfactant-stabilised microparticles, chitosan was considered in the present study as an attractive alternative to CTAB.

Poly (N- vinylpyrrolidone/2-dimethylaminoethyl methacrylate), dimethyl sulfate quaternary. (PVP-PDAEMA)

In this study PVP - PDAEMA was also considered as an alternative stabiliser to CTAB (fig 1.8.2C). The polymer is water soluble and cationic with a molecular weight of 100,000 Da. The rationale for investigating this cationic polymer is that it may behave in a similar way to poly (ethylene glycol) – dextran conjugates used by Coombes *et* al to stabilise PLG microparticles (Combes *et al,* 1997). Their approach was based on the concept that the first hydrophilic species (PEG) would dissolve in the polymer solution droplets formed in the emulsification/solvent evaporation technique and provide the 'anchor' to the particle surface. The second hydrophilic species (dextran) would extend from the particle surface. In the case of PVP - PDAEMA the solvent soluble PVP entity would act as the 'anchor' and the positively charged conjugate would extend from the surface and interact with the DNA.

There is no literature describing the use of this particular PVP-conjugate for gene or vaccine delivery, and as a result the cytotoxicity of the compound is not well defined. However, both components of the conjugate, PVP and Poly((2-Dimethylamino)Ethyl Methacrylate) PDAEMA, have been used in gene delivery experiments. Studies on mammalian cells showed that under short-term incubation conditions, the viability of cells as measured by FACS, was unaffected by PVP (Kabanov *et al,* 1993). In addition, PDAEMA has been shown to form complexes with DNA in growth medium and these complexes were found to be 8-fold more active than PLL-DNA complexes in the presence of chloroquine. Under optimal conditions for each agent it was also

demonstrated that PDAEMA/DNA complexes were more effective than the commercially available transfection agent lipofectin at transfecting COS-7 cells. The complexes were also found to be unaffected by serum and less cytotoxic than PLL (Cherng *et al,* 1996; van de Wetering *et al,* 1998).

1.9 Aims and objectives

The aim of this study was to develop a novel cationic microparticulate DNA vaccine delivery system. This system will build on technology developed by Singh *et* al (2000) whereby DNA was adsorbed to the surface of PLG microparticles stabilised with the cationic surfactant cetyltrimethyl ammonium bromide (CTAB). To minimise the long-term safety issues associated with the use of surfactants in human vaccine development the present study focused on the formation of cationic particles produced using the core polymer PLG stabilised by the cationic polymers chitosan or PVP-PDAEMA.

The particles were designed in such a way as to maximise DNA adsorption and facilitate passive targeting of professional APCs to stimulate potent immune responses following i.m. injection. Chosen formulations were fully characterised and their transfection efficiency and effect on cell viability assessed by *in vitro* cell assays. Stimulation of humoral and cell mediated immunity were evaluated in a mouse model.

2. Materials and methods

2.1 Materials

Poly (L-lactic acid) (PLA) MW 2000 and 100,000 was obtained from Boehringer Ingelheim, Germany. 50:50 (RG503) and 75:25 (Medisorb) Poly (DL-lactide co-glycolide) (PLG) was obtained from Laboratoire Pharma Biotech, France. Penta-sodium tripolyphosphate (PSTP) and paraformaldehyde were purchased from Aldrich Chemical Company, UK. Poly (N-vinylpyrrolidone/2-dimethylaminoethyl methacrylate), dimethyl sulfate quaternary was obtained from Polysciences, Inc, UK. Low molecular weight chitson (LMWC, MW 50-190kDa) and medium molecular weight chitosan (MMWC, MW 190-310kDa) were purchased from Sigma-Aldrich, UK. Lactic acid was obtained from May and Baker Ltd, UK. Acetone (HPLC grade) dichloromethane (HPLC grade) and absolute ethanol were provided by Fischer Scientific, UK. TE buffer (pH 8), DMEM cell culture medium, foetal calf serum (FCS), Trypsin/EDTA, agarose and 5% goat serum were purchased from Gibco BRL, UK. The restriction enzymes PvuII and EcoRI, and their buffers, were purchased from Helena Biosciences, UK. The *Label* IT Nucleic Acid Labelling Kit was obtained from Mirus, UK. 293T human kidney epithelial cells were supplied by Dr A. Vernalis, Aston University. The murine dendritic cell line (DC) 2.4 and primary bone marrow DCs were supplied by Dr E. Lavelle, Trinity College Dublin. Competent E. Coli JM109 cells, the Wizard *Plus* Maxiprep DNA Purification System , the Luciferase Assay System, cell lysis reagent and RQ1 DNase I were purchased from Promega, UK. pCMV-km-Luc and pCMVkm p55 gag plasmids were supplied by Chiron Corp., USA. The gWIZ luciferase expression plasmid was purchased from Aldevron, USA. Dimethyl sulfoxide (DMSO) was purchased from ACROS organics, UK. The PicoGreen dsDNA quantitation kit and Hoechst 33342 were obtained from Molecular Probes, UK. FuGENE 6 transfection reagent was obtained from Roche Molecular Biochemical, UK and gel mount was purchased from Biomeda Corp., USA. Goat anti-mouse IgG-horseradish peroxidase was supplied by Caltag, USA. Monoclonal rat anti-mouse anti-IFN-γ antibody, gag-derived p7g peptide, anti-CD3, anti-CD8 and biotynylated anti IFN-γ were purchased from Pharmingen, USA. Tetramethylbenzidine

was obtained from Kirkegaard and Perry Laboratories, USA. All other chemicals and reagents were obtained from Sigma, UK.

2.2 Formulations

2.2.1 Cetyltrimethyl ammonium bromide (CTAB) stabilised PLA and PLG microparticles

Following the method of Singh *et al.* (Singh *et al,* 2000) microparticles were prepared using either PLG 75:25 or PLA (MW 100,000) and the cationic surfactant cetyl trimethyl ammoniumbromide (CTAB) as a stabiliser. A solution of PLA (5% w/v) in DCM (10ml) was homogenized for 2 min at 7500 rpm using a Silverson L4RT homogenizer (Silverson Machines, Chesham, Bucks,UK). The polymer solution was added to a solution of CTAB (0.5% w/v) in water (50ml) and homogenized at 9500rpm for a further 4 min. The resulting suspension was stirred overnight to evaporate the solvent. Particles were washed three times in double distilled water (DDW) by centrifugation at 3500rpm in a MSE Mistral 2000 centrifuge (Alertville, Minnesota, USA) followed by resuspension.

2.2.2 Poly (N-vinylpyrrolidone/2-dimethylaminoethyl methacrylate), dimethyl sulfate quaternary (PVP-PDAEMA) stabilised PLG microparticles

Microparticles were prepared using PLG 75:25 by the solvent evaporation process described in section 2.2.1. However, varying concentrations of PVP-PDAEMA solution (5%-40% w/v) in distilled water were used as the stabiliser.

2.2.3 Chitosan microparticles

Chitosan microparticles were prepared using chitosan (0.1% w/v) dissolved in (1% v/v) lactic acid. Addition dropwise of 150-200μl of a 5% w/v solution of penta-sodium tripolyphosphate (PSTP) to chitosan solution (10ml) whilst stirring with a magnetic stirrer for 20-30 min, resulted in production of an emulsion.

2.2.4 Chitosan-stabilised PLA and PLG microparticles

Microparticles were prepared using PLA (MW 100,000) or PLG (50:50 or 75:25) by the solvent evaporation process described in section 2.2.1. However, varying concentrations of medium (190-319 kDa) or low (50-190 kDa) molecular weight chitosan in lactic acid (1% v/v), were used as a stabiliser.

2.2.5 PLA lamellar particles

PLA (MW 2000) lamellar particles were produced by non-solvent induced precipitation from a solution of PLA in acetone. Distilled water (10ml) was added dropwise to 5ml of a 2% w/v PLA solution. The solution was stirred overnight using a magnetic stirrer to remove the solvent.

2.2.6 Surface modification of pre-formed PLA microparticles using chitosan

PLA (MW 2000) microparticles were produced by non-solvent induced precipitation from a solution of PLA in acetone. Distilled water (10ml) was added dropwise to 5ml of a 2% w/v PLA solution which was stirred using a magnetic stirrer. Following solvent evaporation overnight, the PLA microparticles were modified with chitosan by incubation in (1%w/v) chitosan solution in 1% v/v lactic acid. Microparticles were also prepared using this technique and the polymer PLG 75:25.

2.2.7 Poly (vinyl alcohol) [PVA] - stabilised microparticles

Because PVA is a non-ionic surfactant, PVA stabilised microparticles were produced to act as a negative control. Microparticles were prepared using PLA (Mw 100,000) by the solvent evaporation process described in section 2.2.1. However, a 5% w/v solution of PVA in distilled water was used as a stabiliser.

2.2.8 PLG microparticles prepared without the addition of stabiliser

Control PLG 50:50 microparticles were prepared without the addition of stabiliser by the solvent evaporation process described in section 2.2.1. Following the initial homogenisation, PLG (5% w/v) in DCM (10ml) was added to distilled water (50ml) and homogenized at 9500rpm for a further 4 min. The resulting suspension was stirred overnight to evaporate the solvent.

2.2.9 Chitosan/DNA complexes

Complexes of DNA and low molecular weight chitosan (LMWC) were produced by incubation of LMWC (0.5% w/v) in lactic acid solution (1% v/v) with plasmid DNA (0.02% w/v) overnight with shaking (IKA Vibrax VXR system, Staufen, Germany).

2.3 Microparticle characterisation

2.3.1 Microparticle yield

The concentration of microparticles in each batch was calculated to assess reproducibility of the formulation process. An empty Eppendorf was weighed before 1ml of microparticle suspension in distilled water was aliquoted into the tube. The sample was frozen at −80°C for 30 min before lyophilisation in an Edwards Modulyo freeze dryer (Edwards, Tonowanda, NY, USA) overnight. The Eppendorf was weighed again and the microparticle concentration calculated by subtraction of the Eppendorf weight.

2.3.2 Surface charge measurements (zeta potential)

Zeta potential measurements were carried out in double distilled water (DDW) using a Zeta Plus zeta potential analyzer (Brook Haven Instruments Corporation, Holtsville, USA). An electric field was applied in the liquid and particles migrated to either the positive or negative pole. The direction in which the particles moved indicated their

charge and the velocity with which they moved was proportional to the magnitude of the charge. Each sample was analysed 10 times to provide a mean value of the zeta potential (mV).

2.3.3 Microparticle size

The size distribution of microparticles were measured by laser light scattering (Malvern Instruments, Malvern, UK). This method relies on the fact that the laser diffraction angle is inversely proportional to microparticle size. Microparticles were suspended in DDW and their size was measured by recirculating a sample in front of a laser beam in a stirred cell containing filtered DDW. Each sample was analysed three times to provide the particle size expressed as the volumetric mean diameter (μm).

2.3.4 Scanning electron microscopy

The size and morphology of the microparticles was analysed by scanning electron microscopy (SEM). A dilute sample of microparticles, suspended in distilled water, was gently applied to carbon tabs mounted on SEM specimen stubs and left to air dry overnight. The specimen stubs were coated with approximately 5nm of gold by ion beam evaporation (Emscope SC500) prior to examination in a Cambridge Instruments, UK Stereoscan 90B scanning electron microscope operated at 25 kV.

2.4 Quantitation of chitosan in chitosan-stabilised microparticles

2.4.1 Ninhydrin assay

The Ninhydrin assay for chitosan quantification was adapted from methods described by Curotto & Aros, and Prochazkova *et al.* (Curotto & Aros, 1993; Prochazkova *et al,* 1999). It is based on the premise that when chitin is deacetylated to chitosan free amino groups are produced. Ninhydrin reacts with primary amino groups to form a coloured

reaction product (Ruhemann's purple) which absorbs at 570nm. The strength of the absorbance signal will correlate with the concentration of chitosan.

The concentration of chitosan in chitosan-stabilised microparticles was estimated by measuring the concentration of the chitosan stabilising solution after microparticle formulation, and subtracting this from the initial stabiliser concentration. Washes from the formulation process were collected and combined. A 1ml aliquot of the wash solution was mixed with acetone (1ml), dichloromethane (1ml) and 1% v/v lactic acid (1ml) in a test tube. This step was included to break up possible PLG/chitosan complexes and solubilise the chitosan. The tube was shaken vigorously overnight (IKA Vibrax VXR system, Staufen, Germany) to allow evaporation of the acetone and the dichloromethane leaving a precipitate of PLG in a chitosan solution. Ninhydrin solution (1ml) was added to the chitosan solution at a 1:1 ratio. The tube was shaken and incubated in a boiling water bath for 20 min. Following incubation the tube was cooled and 200μl of the contents were transferred to a flat bottomed 96 well plate. The absorbance was read at 570nm on a plate reader (MRX, DYNEX Technologies, Ashford, UK). A calibration curve was produced by addition of 1ml of ninhydrin reagent to varying concentrations of chitosan solution in 1% v/v lactic acid. Calibration samples were treated in exactly the same way as the unknown samples. Chitosan concentration in the wash was determined from the calibration curve, and this value was multiplied by total wash volume. To calculate the total amount of chitosan incorporated in the microparticles, the wash value was subtracted from the initial chitosan concentration of the stabilising solution and expressed as a percentage of the weight of chitosan/weight of microparticles.

2.4.2 Chitosan release *in vitro*

Chitosan- stabilised microparticles (20mg) were incubated over 14 days in 1ml phosphate buffered saline (PBS) at 37 °C to assess whether chitosan was released from the particles. At intervals samples were centrifuged for 6 min at 3500rpm. The supernatant was removed and assayed for chitosan content by the Ninhydrin assay (section 2.4.1). The pellet was resuspended in fresh PBS and the release study continued.

2.5 DNA preparation

2.5.1 Sources of DNA

The expression vector used for the majority of these studies was pCMV-km-Luc (Chiron Corp., USA), a 6kb vector containing a kanamycin resistance gene and a luciferase gene under the transcriptional control of the CMV immediate early promoter. A second commercially available reporter plasmid (gWIZ) (Aldevron, USA) was used in some of the transfection studies. This plasmid was 6732bps in size and also expressed kanamycin resistance and luciferase under the control of the CMV promoter. The plasmid used for the *in vivo* experiments was HIV-1 pCMVkm p55 gag (Chiron Corp, USA).

2.5.2 Plasmid propagation, isolation and purification

Transforming *E.coli*

E. Coli competent JM109 cells (0.1ml) were transformed with the pCMV-km-Luc plasmid by addition of 1μg of plasmid to the bacteria followed by gentle mixing and incubation on ice for 30min. Cells were heat shocked at 42°C for 45s, followed by a further incubation on ice for 2min. Sterile LB broth (1ml) was added and the sample incubated for 1h at 37°C with vigorous shaking. The cells were spun at 2500rpm for 3min, most of the supernatant poured off and the cells resuspended in the remaininig liquid. The cells were pipetted and spread on an LB agar plate containing kanamycin (25μl/ml). The plates were incubated at 37°C overnight to allow colony growth.

Propagation and purification of plasmid DNA

A colony was selected from the agar plates containing kanamycin and grown up overnight in sterile LB broth (500ml) containing kanamycin (25μg/ml). Plasmid was purified using the Promega Wizard Plus maxiprep kit according to the manufacturers instructions. Briefly, cell suspension was centrifuged (10min, 14,000 x g) and

resuspended in 15ml cell resuspension solution (50mM Tris-Hcl pH7.5, 10mM EDTA, 100μg/ml RNase A). Cell lysis solution (0.2M NaOH, 1% SDS) was added (15ml) to the cell suspension and mixed gently. This was followed by the addition of 15ml of cell neutralisation solution (1.32M potassium acetate pH4.8) before centrifugation (15min, 14,000 x g). This mixture was filtered and isoproponal (half the total volume) was added and mixed. The solution was centrifuged (15min, 14,000 x g) and the pellet resuspended in 2ml TE buffer (10mM TRIS pH 8, 1mM EDTA). Resin (10ml) was added to the DNA solution, the mixture transferred to a maxicolumn and a vacuum applied. Under vacuum, column wash solution (80mM potassium acetate, 8.3mM, Tris-HCl pH7.5, EDTA 40mM) was added to the column (25ml) followed by 80% ethanol (5ml). The column was centrifuged (5min, 1,300 x g), the supernatant discarded, and the vacuum applied for a further 5min. Preheated water (70°C, 1.5ml) was added to the column and DNA eluted by centrifugation (5min, 1,300 x g). The DNA solution was filtered and centrifuged again (1min, 14,000 x g). DNA was stored at -20°C.

2.5.3 DNA quantification

DNA concentrations in unknown samples were determined using either UV spectrophotometry or the PicoGreen dsDNA quantitation assay.

Spectrophotometry

The concentration of DNA in unknown samples was measured by UV spectrophotometry using an absorbance at 260nm (OD_{260}). DNA samples were generally diluted 1:50 with DDW before an absorbance reading. An OD_{260} reading of 1.0 corresponds to approximately 50μg/ml of double-stranded DNA. The purity of the DNA was determined from the ratio of OD_{260} and OD_{280}, where a ratio between 1.8 and 2.0 was used to indicate pure DNA.

PicoGreen assay

The PicoGreen reagent is a fluorescent nucleic acid stain that can quantify double-stranded DNA in solution. The PicoGreen assay is more sensitive than spectrophotometry and can detect picogram amounts of DNA. Assays were performed according to the manufacturers' instructions except that the standard curve was produced by carrying out serial dilutions of the pCMV-km-Luc plasmid (500ng/µl) used for adsorption studies rather than using the lambda DNA standard provided. Briefly, 100µl of diluted PicoGreen reagent was added to 100µl of sample containing an unknown concentration of DNA and incubated at room temperature, in darkness for 2-5mins. Samples were transferred to a 96 well plate and the fluorescence measured (λ_{ex} 485, λ_{em}538) on a Spectra MAX GEMINI XS fluorescence plate reader (Molecular Devices, Workington, UK). DNA concentration was determined from the standard curve.

2.5.4 Assessment of integrity of purified DNA

Following purification of the pCMV-km-luc DNA, integrity of the plasmid was assessed by digestion with the restriction enzymes PvuII and EcoRI followed by agarose gel elctrophoresis. Intact purified plasmid digested with PvuII would result in 4 bands of DNA, 2870, 1477, 1310 and 360 base pairs in size (bp). EcoRI which cuts either side of the luciferase gene would give 2 bands, 1707 and 4310bp in size. Restriction enzyme (1.5µl) and restriction enzyme buffer (1.5µl) were added to an aliquot of plasmid solution containing 1µg of plasmid and made up to a volume of 10µl with sterile distilled water. The sample was vortexed and incubated at 37°C overnight. The reaction was stopped by incubation at 65°C for 10 min. Loading dye (1% xylene cyanole, 1% bromophenol blue, 40% glycerol) was added (3µl), and samples were run on a 1% agarose gel against uncut plasmid and a 1kb DNA step ladder. The agarose gel contained ethidium bromide (0.1µg/ml) and ultraviolet light was used to visualise the DNA (Syngene, Cambridge, UK). Data was captured using Genesnap software.

2.5.5 Preparation of rhodamine-labelled DNA

5-carboxy-x-rhodamine is a fluorescent DNA label (λ_{ex} 576, λ_{em}597). Plasmid DNA was labelled with rhodamine using the *Label* IT Nucleic Acid Labelling Kit (Mirus) according to the manufacturer's instructions. Briefly, DDW (35μl), 10X Mirus labelling Buffer A (5μl), 1mg/ml nucleic acid solution (5μl) and lastly *Label* IT Reagent (5μl) were added to an Eppendorf and incubated at 37°C for 1 hour. Unreacted labelling reagent was removed from labelled nucleic acid by ethanol precipitation (section 2.8.2). DNA was resuspended in 1X Mirus Labelling Buffer A and labelled nucleic acid was stored at –20°C.

2.6 Interaction of DNA with microparticles

2.6.1 DNA loading

Microparticles (20mg) were incubated with plasmid DNA (0.02% w/w) in distilled water overnight. Following incubation, microparticles were centrifuged (MSE Mistral 2000) for 6min (3500rpm) and the supernatant was collected. The concentration of DNA in the supernatant was measured by the PicoGreen assay and by UV spectrophotometry at OD_{260} (section 2.5.3). DNA loading of the microparticles was estimated by subtraction of this value from the DNA content of the incubation medium.

2.6.2 Confirmation of DNA loading by hydrolysis of microparticles

Microparticles loaded with DNA as described in section 2.6.1. were subjected to hydrolysis breakdown to confirm that DNA was associated with the microparticles. The microparticle suspension 1ml, (20mg/ml) in 1M NaOH and 5% SDS (w/v) was incubated overnight with vigorous shaking (IKA Vibrax VXR system). Following breakdown of the microparticle, solutions were diluted in 1M NaOH and 5% SDS and the DNA concentration was measured by UV spectrophotometry at OD_{260}.

2.6.3 DNA release *in vitro*

Microparticles (20mg) loaded with DNA as described in section 2.6.1. were incubated over 14 days in 1ml phosphate buffered saline (PBS) at 37 °C. At intervals samples were centrifuged for 6 min at 3500rpm. The supernatant was decanted off and assayed for DNA concentration by the PicoGreen assay or UV spectrophotometry at OD_{260} (section 2.5.3). The pellet was resuspended in fresh PBS and the release study was continued.

2.7 Integrity of DNA released from microparticles *in vitro*

2.7.1 Assessment of integrity by agarose gel electrophoresis

DNA-loaded microparticles were incubated in PBS at 37°C for 14 days as described in section 2.6.3. To determine whether DNA released from the microparticles retained a supercoiled conformation, samples were run on a 1% agarose gel and compared to intact DNA.

2.7.2 Bioactivity of DNA as assessed by transfection of 293T cells with FuGENE 6.

To establish whether DNA released from microparticles was still biologically active and could mediate protein expression in cells, DNA in release samples was complexed with the transfection reagent FuGENE 6 (F6). 293T cells were seeded in 24 well plates at a density of 1×10^5 cells/well and incubated at 37°C overnight. F6 was complexed with the DNA in the samples according to the manufacturer's instructions. Briefly, F6 was diluted in serum free (SF) medium. The recommended ratio of DNA: F6 was added to the SF medium (3:1 ratio) and incubated for 15-45min at room temp. The complex mixture (100μl) was added dropwise to cells and the plate swirled. The plate was returned to the incubator overnight. Following incubation cells were lysed and luciferase production was assessed by the luciferase assay (section 2.11.2)

2.8 Protection of adsorbed DNA by chitosan-stabilised microparticles

2.8.1 DNase I protection assay evaluated by UV analysis

This assay utilises the fact that intact DNA molecules possess hypochromicity and upon enzymatic digestion or heat-induced denaturation of the molecule, an increase in the absorbance at OD_{260} will be observed.

Plasmid DNA (10µg/ml) was incubated with chitosan-stabilised microparticles (1mg/ml) overnight with shaking. An aliquot of DNase I (20 units) was spiked into 1ml of the DNA/ microparticle mixture in a quartz cuvette and the absorbance at OD_{260} was read immediately on a spectrophotometer (MRX, DYNEX Technologies) before the cuvette was directly transferred to a 37°C water bath. Absorbance readings were taken at 5min intervals up to 45min. Assays on the naked plasmid and chitosan-stabilised microparticles were conducted in the same manner. Absorbance values for chitosan-stabilised microparticles were subtracted from the values for the DNA/microparticle mixture to account for any absorbance due to the microparticles alone at 260nm.

2.8.2 DNase I protection assay evaluated by agarose gel electrophoresis

Naked DNA (10µg) or plasmid/microparticle complex (10µg of plasmid incubated with 1mg/ml of microparticles) were incubated with DNase I (5 units). A sample of naked DNA and the complex suspension (100µl) were taken at 0, 20, 40 and 60 minutes post-incubation with DNase I and mixed with (100µl) of stop solution (200mM sodium chloride, 20mM EDTA, and 1% SDS). To dissociate the plasmid DNA from the microparticles, the reaction mixtures were incubated at 60°C overnight and DNA was extracted by phenol/chloroform extraction.

Phenol/chloroform extraction of DNA

An equal volume of phenol was added to 100μl isoamyl alcohol (IC) and vortexed for 30s. The phenol/IC solution PIC (200μl) was added to an equal volume of sample, vortexed and centrifuged to separate the aqueous phase which contained the DNA from the organic phase. The aqueous phase was carefully removed and collected in an eppendorf. Separation of the organic and aqueous layers by centrifugation was repeated three more times by addition of PIC (200μl), IC (400μl) and DDW (400μl) respectively. In each case the aqueous layer containing the DNA was removed to a separate eppendorf. DNA was precipitated from the collected samples by ethanol precipitation.

Ethanol precipitation of DNA

5M sodium chloride (10% of total volume) was added to each sample, followed by ice cold 100% ethanol (2 x total volume). The samples were incubated at -80°C for 30min. Precipitated DNA was recovered by centrifugation of the samples in a microfuge (Hawk 15/05 Sanyo MSE) for 10min (10,000rpm, 4°C). The ethanol was carefully removed and the pellet was washed once in 70% ethanol. The pellet was dried in a desiccator (Eppendorf Concentrator 5301, Helena Bioscences, Sunderland, UK) and resuspended in sterile TE buffer (pH 8). Following precipitation each sample was run on a 1% agarose gel.

2.9 Stabilisation of DNA-adsorbed microparticles by sugars or PVA prior to lyophilisation

2.9.1 Lyophilisation of stabilised formulations

Chitosan-stabilised microparticles were prepared as described in section 2.2.4 and DNA was adsorbed to the surface as described in section 2.6.1. To protect plasmid DNA during freeze drying, and to prevent aggregation of microparticles upon reconstitution, excipients were added to microparticle suspensions prior to lyophilisation. Microparticles

(20mg/ml) in distilled water were aliquoted out in 1ml volumes. Mannitol (4.5%w/v final concentration) and sucrose (1.5% w/v final concentration) were added to some of the aliquots whereas PVA (0.5 or 0.1% w/v final concentration) was added to others. Samples were frozen at −80°C for 30 min followed by lyophilisation in an Edwards Modulyo freeze dryer overnight.

2.9.2 Microparticle size following lyophilisation

Following lyophilisation microparticles were reconstituted in sterile distilled water (20mg/ml). Microparticle size was measured using a Malvern Mastersizer as described in section 2.3.4.

2.10 Cell culture

Cell culture was performed in a biological safety class II laminar flow cabinet. Cells were retained at 37°C with a constant CO_2 level of 5%. The majority of procedures were carried out in Dulbecco's MEM (25MM HEPES, w/o sodium pyruvate, with 4500MG/L Glucose, with Pyridoxine, Gibco, UK) supplemented with 1% penicillin (10,000 units/ml)/streptomycin (10,000 μg/ml) and 10% foetal calf serum (FCS). However, dendritic cell experiments were carried out in RPMI 1640 (1% L-glutamine) medium (Gibco,UK) supplemented with 1% penicillin(10,000 units/ml)/streptomycin (10,000 μg/ml) and 10% foetal calf serum (FCS). Media was stored at 4°C prior to use.

2.10.1 Cell lines

293T, a murine kidney epithelial cell line; C_2C_{12}, a human myoblast cell line and DC 2.4, a murine dendritic cell line were used in these studies. 293T and C_2C_{12} were established cell lines grown in the Medicines Research Unit at Aston University. DC 2.4 was an established cell line grown in the Department of Immunology at Trinity College Dublin.

2.10.2 Isolation and culture of bone marrow-derived dendritic cells (BMDCs)

Bone marrow isolation

Bone marrow was flushed from the femurs and tibias of Balb/c mice (6-8 weeks old). The bone marrow was washed and frozen (-80°C) in heat-inactivated FCS supplemented with 10% cell-culture grade dimethyl sulfoxide (DMSO) at a density of 2×10^7 cells/ml.

Generation of bone marrow-derived dendritic cells (BMDCs)

Frozen cell aliquots were thawed rapidly and washed to remove DMSO. Immature DCs were cultured in RPMI 1640 medium and 10% FCS supplemented with 10% supernatant from a GM-CSF expressing cell line (J558-GM-CSF). The cells were cultured for 3 days, pelleted by centrifugation (5 min, 1500rpm), and recultured with fresh medium and 10% GM-CSF cell supernatant on day 3. On day 7 of culture, mature BMDCs were collected, washed, and used for assays.

2.10.3 Cell Maintenance

293T and C_2C_{12} cells were grown as monolayers in 75 cm^3 flasks containing 20ml of culture medium (Dulbecco's MEM, 25MM HEPES, w/o sodium pyruvate, with 4500MG/L Glucose, with Pyridoxine). The medium was replenished every 3 days to avoid depletion of essential nutrients and increases in toxic substances. Confluent flasks were routinely sub-cultured at a ratio of 1:5 to prevent overgrowth and loss of surface contact. For adherent myoblast cells, medium was removed, cells were washed in PBS (10ml) and the cell-surface adherence was proteolytically cleaved by the addition of trypsin/EDTA (2.5ml). Flasks were incubated at 37°C for 5min before cells were resuspended in fresh medium (10ml) and aliquoted into fresh flasks. For partially adherent 293T cells, trypsin was not used. Cells were dislodged from the flask surface by tapping. The cell suspension was centrifuged (1500rpm, 5 min) and the pellet resuspended in fresh medium before aliquoting into fresh flasks.

DC 2.4 cells were grown as monolayers in 75 cm^3 flasks containing 20ml of RPMI 1640 culture medium supplemented with 10% FCS, 1% L-glutamine and 1% pen/strep. The medium was replenished every 3 days and confluent flasks were routinely sub-cultured at a ratio of 1:5. Cells were washed in PBS followed by the addition of trypsin/EDTA (2.5ml). Flasks were incubated at 37°C for 5min before cells were resuspended in fresh medium (10ml) and aliquoted into fresh flasks.

2.10.4 Storage and resuscitation of cell lines

Cells were stored by resuspending a cell pellet from a 75cm^3 culture flask in cryopreservation solution (0.1 ml DMSO, 0.9ml FCS). The cell solution was aliquoted into vials and stored at −80°C for up to 1 month. Cells were resuscitated by thawing at 37°C followed by dropwise addition of 1ml of warmed DMEM medium. The cells were transferred to a flask and fresh medium added. The flask was stored at 37°C/5% CO_2 for 24h before the medium was replaced to remove traces of DMSO.

2.10.5 Determination of cell numbers

Cell numbers were determined using a light microscope (Olympus CK2, Tokyo, Japan) and a haemocytometer. Cell pellets were resuspended in 10ml of DMEM medium and a small volume of cell suspension was pipetted onto the haemocytometer. Values are expressed as the average cell count from 3 fields of view.

2.11 Biological evaluation of DNA vaccine delivery vectors *in vitro*

2.11.1 Transfection of cell lines *in vitro*

Transfection experiments using DNA/microparticle complexes were performed using three different cell lines and primary BMDCs to assess the efficiency of transgene expression. 293T kidney epithelial cells were used as a standard cell line known to be transfected readily by soluble DNA. C_2C_{12} myoblasts were used as a model cell line as

the DNA vaccine was being designed for i.m. injection. In addition, efficient uptake of DNA by muscle cells has been reported *in vivo*. Finally, the DC 2.4 cell line and primary BMDCs were used to assess if cells of the immune response could be transfected *in vitro*. Cells were seeded in 24 well plates so that following incubation for 24h at 37°C, the cells would be approximately 50-70% confluent. 2ml of medium/well was used. For non-dividing primary BMDCs, cells were seeded at 1×10^5 cells/well. Following incubation, treatments were added in triplicate to the wells and the plate was swirled and returned to the incubator overnight. Naked DNA was used as a negative control and DNA/F6 complexes were used as a positive control. DNA/F6 complexes were prepared as described in section 2.7.2. Following incubation for 24h, medium was removed from one set of the triplicate wells and the cells were washed with PBS (1ml). Cell lysis reagent (100μl) was added to the wells and the plate rocked for 15 min at room temperature. The cell lysates were removed to eppendorfs and stored at −4°C. The plate was returned to the incubator. Cells in the remaining wells were lysed on either day two or three. Once all the lysates were collected luciferase activity was assessed by the luciferase assay (2.11.2). The cell numbers in each well were estimated using the BCA protein assay (2.11.3).

2.11.2 Luciferase assay

Luciferase activity in cells was measured using the luciferase assay system wherein the luciferase assay reagent oxidizes the luciferase gene product resulting in the production of light (measured in relative light units (RLU)). Lysate (20μl) was added to a 96 well, flat bottomed, opaque plate (Maxisorb, Nunc). Luciferase assay reagent (100μl) was added to each well and the plate read immediately on a Spectra MAX GEMINI XS spectrofluorimeter.

2.11.3 BCA protein assay

The BCA protein assay was used as an estimate of cell numbers/well to provide a measurement of luciferase activity per cell. Cell lysates (50 μl) were added to wells of a flat-bottomed, 96-well plate followed by 200 μl of BCA reagent (1:50 dilution of copper

(II) sulphate to bicinchoninic acid). The plate was incubated for 30min at 37°C before the absorbance at 570nm was measured. A calibration curve was produced by carrying out serial dilutions of a stock solution (1mg/ml) of ovalbumin (OVA) followed by incubation with BCA reagent. The concentration of protein in the lysates was determined from the calibration curve.

2.11.4 Interaction of chitosan-stabilised microparticles with myoblast cells

Myoblast cells were transfected with chitosan-stabilised microparticles as described in section 2.11.1. On days 1, 2 and 3 of the transfection procedure, cells were observed under a light microscope (Olympus CK2, Japan) to examine cell/microparticle interaction and assess the affects of microparticle presence on cell growth. Untreated cells were observed as a comparison.

2.11.5 Chitosan-stabilised microparticle transfection of cells *in vitro* with rhodamine-labelled DNA

Microparticles loaded with rhodamine-labelled-DNA were transfected into 293T cells following the procedure described in section 2.11.1. except that cells were seeded in 8 well chamber slides (Lab-Tek, Christchurch, New Zealand) in a total volume of 0.4ml medium. Additionally, following transfection cells were not lysed but were fixed in 4% w/v paraformaldehyde and the cell nuclei were stained with Hoechst 33342 dye (section 2.11.6).

2.11.6 Fluorescence microscopy of transfected cells

Cell fixing

Paraformaldehyde (2g) in 25ml DDW was heated with stirring to 60°C. At this temperature 1M NaOH was added dropwise until the liquid became clear. The resulting solution was allowed to cool. A 4% w/v solution of paraformaldehyde was prepared by

adding 25ml of the paraformaldehyde solution to 25ml of double concentration PBS. Transfected cells grown on slides were washed gently in PBS twice. Cells were fixed by incubation in paraformaldehyde (4%w/v) for 15 min.

Nuclear staining

Hoechst 33342 dye was used to stain the nuclei of transfected cells. Hoechst 33342 is a fluorescent dye (λ_{ex}350, λ_{em}461) that is capable of permeating the membrane of living cells. A stock solution of Hoechst (1mg/ml in 0.9% NaCl) was diluted to a final concentration of 0.001mg/ml in PBS. After fixing with paraformaldehyde (4%w/v) cells were washed in PBS and stained with Hoechst dye for 10-15 min. The cells were washed again gently in PBS. Coverslips were mounted on the slide using a fade resistant fluorescent mounting agent. Slides were stored at 4°C in the dark to reduce fading.

Visualisation

The slides were visualised on a fluorescent microscope (Zeiss Axioskop, Carl Zeiss Inc., Thornwood, NY, USA) and data was captured using Axiovision software.

2.12 Assessment of microparticle cytotoxicity *in vitro*

2.12.1 MTT assay of cell viability

This assay is based on the principle that the yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals which absorb at a wavelength of 570nm. Thus, the number of viable cells can be estimated by the intensity of the OD_{570} measurement. Cells were seeded at a density of 5×10^4 cells/well in 24 well plates and incubated at 37°C overnight. Following incubation, treatments were added in triplicate to the wells and returned to the incubator overnight. Some wells were left untreated to act as controls. 120μl of MTT reagent (2mg/ml) was added to one set of triplicate wells and incubated for a further 4hrs. Following incubation, medium was

removed and 750μl of DMSO was added to the wells to dissolve the formazan crystals. Aliquots of 200μl from each well were removed to a 96-well plate and the absorbance was measured at 570nm. This process was repeated on days 2 and 3. Cell viability (%) was calculated according to the following equation:

$$\text{Cell viability (\%)} = (OD_{570(sample)}/OD_{570(control)}) \times 100$$

Where the $OD_{570(sample)}$ represents the measurement from the treated wells and the $OD_{570(control)}$ represents the measurement from the untreated wells.

2.12.2 Assessment of cell numbers

In conjunction with the MTT assay for cell viability the affect of treatments on cell numbers was also assessed. Briefly, 293T cells were seeded as described in section 2.12.1 and treated in triplicate. Following 24h incubation 293T cells were scraped from the bottom of one well of the triplicate set and counted (section 2.10.5). This process was repeated on days 2 and 3. Since myoblasts are adherent, these cells were treated differently. After incubation, medium was removed from one of the triplicate wells and the cells were washed with PBS. Trypsin/EDTA (200μl) was added to the well and the plate was incubated for 5min at 37°C. The reaction was stopped with 1ml of medium and cells were counted as described above (section 2.10.5).

2.13 Evaluation of the immune response to particle adsorbed DNA in Balb/C mice

2.13.1 Immunisation

BALB/c mice in groups of 10 were immunised with either naked DNA, lyophilised, DNA-loaded microparticles stabilised with LMWC (1% or 3% w/v) or DNA-loaded microparticles in suspension stabilised with LMWC (1% or 3% w/v). Each formulation was administered at two doses (1 and 10μg DNA). Immunisations took place at weeks 0 and 4 to determine antibody levels in studies of humoral immunity. The microparticle

formulations were suspended in sterile water and a total of 100µl was administered per animal by two 50µl injections in the tibialis anterior (TA) muscles in the hind legs of each animal. The immunisation protocol for the CTL study involved a single injection (*n* = 5 mice per group) in the TA muscles followed by harvesting of splenocytes at the three week time point.

2.13.2 Sera and tissue collection

Mice were bled through the retroorbital plexus and the sera were separated for the immunoassays. Spleens were harvested and pooled from groups of mice before the CTL assay. Single-cell suspensions were prepared by meshing through steel or nylon meshes.

2.13.3 Immunoassay

Serum samples were assayed for IgG titres. HIV-1 p55 gag-specific serum IgG titres were quantified by an ELISA. Briefly, ELISA plates (96-well U bottom by Nunc Maxisorp) were coated with p55 protein overnight (5 µg/well). After blocking (5% goat serum, 25mM Tris, 10mM EGTA, 150mM KCl, 2mg/ml BSA, 0.3% Tween-20, pH 7.5) wells were coated with serially diluted serum samples in blocking buffer (1x PBS, 5% goat serum, 0.03% Tween). A serum standard was included in each assay for quantitation purposes. The samples were then incubated with 1: 40,000 dilution of a goat anti-mouse IgG-horseradish peroxidase. The samples were developed with tetramethylbenzidine for 15 min and stopped with 2M HCl. The absorbance (450nm) of each well was measured.

2.13.4 ELISPOT assay

From the pooled spleens of 5 mice, single-cell suspensions were added onto nitrocellulose or PVDF plates (Milipore) pre-coated with monoclonal rat anti-mouse anti-IFN-γ antibody. Plates were blocked (complete RPMI medium, pH 7.2, 10% FCS, 5mM Hepes, antibiotics), and incubated overnight with either gag-derived p7g peptide, anti-CD3 and anti-CD28 as a positive control for polyclonal T-cell activation or media only as

a negative control. Biotynylated anti-IFN-γ in P/T (PBS, 0.1% BSA,0.02% Tween) was added to the plates following washing, and incubated at room temperature for 2h. The plates were washed with P/T and incubated with DAB in Tris-HCl (pH7.5) buffer for 30min to visualise the spots. Plates were washed with de-ionized water and air-dried. Background spots from negative control (media only) wells were subtracted from wells activated by gag-p7g peptide. Positive control wells (polyclonally activated with anti-CD3 and anti-CD28) displayed 5-10-fold more spots than wells activated with gag-p7g peptide. The spots were counted with an automated ELISPOT reader developed by Chiron Corp, USA and using software from Alpah Inotech Corp (San Leandro, CA). Two to four wells per group/tissue were counted. Data is represented as the mean ± S.D. of two independent experiments with pools of five mice per group and expressed as the number of gag-p7g peptide specific IFN-γ-secreting cells per 10^7 Mononuclear cells (MNC).

2.14 Expression of data

Unless otherwise stated all error bars represent the standard deviation of the mean from three independent experiments.

3. Microparticle formulation and characterisation

3.1 Introduction

Numerous particulate formulations have been characterised for the delivery of encapsulated plasmid DNA to cells (Ando *et al,* 1999; Walter *et al,* 1999; Capan *et al,* 1999; Cohen *et al,* 2000; Perez *et al,* 2001). However, relatively few systems have been characterised and evaluated as carriers for the delivery of DNA adsorbed to the surface of microparticles (Eposito *et al,* 1999; Singh *et al,* 2000; Walter & Merkle, 2002). Due to the nature of this delivery system, detailed physicochemical characterisation of these formulations is perhaps even more critical in helping to understand how the vector may behave *in vitro* and *in vivo*. For example, since DNA is adsorbed to the surface, the charge of the microparticle could critically influence DNA loading and release characteristics. Surface charge will also affect interaction of the microparticle with the negatively charged surface of cells, and anionic serum proteins in the body. In addition, the size of the microparticle may influence which cells will be targeted by the delivery system. Microparticles are too large to be taken up via endocytosis or pinocytosis into non-phagocytic cells. However, a number of groups have demonstrated efficient phagocytosis by professional antigen presenting cells (APCs) of particles approximately 1μm in diameter, while larger particles are taken up less efficiently (Tabata & Ikada, 1988; Singh *et al,* 2000). Characterising the physicochemical stability of a novel formulation is also essential as long term storage or processes such as lyophilisation may alter critical components of the delivery system.

This chapter documents the experimental findings and data which were generated by a range of techniques employed to formulate distinct groups of polymer microparticles. The size and surface charge of the various formulations were measured for correlation with their performance as DNA vaccine delivery systems via the intramuscular (i.m.) route. As a positive control cationic PLG microparticles stabilised with CTAB were formulated as described by Singh *et* al (Singh *et al,* 2000). Negative controls included PLG microparticles stabilised with the non-ionic surfactant PVA, and PLG microparticles

formulated without the addition of stabiliser. Initial studies highlighted two potential formulations for DNA vaccine delivery; PLG microparticles stabilised with PVP-PDAEMA produced by the o/w solvent evaporation method described in section 2.2.2 and PLG microparticles stabilised with LMWC produced by the oil in water (o/w) solvent evaporation method described in section 2.2.4. These formulations both exhibited a net positive surface charge and were between 2-5μm in size.

3.2 Characterisation of microparticle preparations

3.2.1 Analysis of microparticle size and surface charge

The size and surface charge of microparticle formulations are shown in table 3.2.1. The smallest microparticles were produced using PLG and the anionic surfactant PVA (1.26μm) by the o/w solvent evaporation method described in section 2.2.7. By comparison PLG 50:50 microparticles formulated without stabiliser using the o/w solvent evaporation technique (section 2.2.8) were the largest (54.25μm), underlining the effect of lack of stabiliser which may result in particle aggregation. A reduction in size was observed for all PLG microparticles formulated with the addition of CTAB, chitosan or PVP-PDAEMA as stabilisers. However, despite the use of CTAB as a stabiliser in the o/w solvent evaporation technique (section 2.2.1), both PLA and PLG microparticles formulated using this surfactant were surprisingly large (44.14μm and 19.47μm respectively). Previous research found that PLG/CTAB microparticles were 1.45 ± 0.8μm in size (Briones *et al,* 2001). Microparticles formulated by a sodium precipitation method using chitosan alone (section 2.2.3) were also relatively large (21.10μm). PLG microparticles using chitosan as a stabiliser and the o/w solvent evaporation technique (section 2.2.4) were all between 3 and 6μm. However, in the cases where MMWC was used as a stabiliser as opposed to LMWC, microparticles tended to be greater than 5μm in size. A slight reduction in the size of chitosan-stabilised microparticles was observed if the core polymer PLG 75:25 was used as opposed to PLG 50:50. When microparticles were formulated by non-solvent induced precipitation, and were surface modified with

Formulation	Technique	Average size (μm)	Zeta potential (mV)
PLG/PVA	A	1.26 ± 0.00	−24.87 ± 1.42
PLG 50:50 no stabilizer	B	54.25 ± 1.90	−39.45 ± 1.99
PLA/CTAB	C	44.14 ± 12.2	+75.28 ± 1.78
PLG/CTAB	C	19.47 ± 1.13	+ 38.41 ± 2.11
Chitosan microparticles	D	21.10 ± 0.04	+18.06 ± 1.17
PLG 50:50/LMWC	E	4.51 ± 0.16	+38.38 ± 5.99
PLG 50:50/MMWC	E	5.62 ± 0.05	+20.03 ± 2.08
PLG 75:25/LMWC	E	3.90 ± 0.67	+29.66 ± 2.76
PLG 75:25/MMWC	E	5.15 ± 0.20	+15.76 ± 1.90
PLA surface modified with LMWC	F	10.43 ± 0.54	+42.79 ± 1.27
PLA surface modified with MMWC	F	5.61 ± 0.08	+31.18 ± 2.45
PLG surface modified with LMWC	F	19.70 ± 0.10	± 41.36 ± 1.21
PLG surface modified with MMWC	F	19.61 ± 0.27	± 36.84 ± 1.19
PLG/PVP-PDAEMA	G	3.56 ± 0.04	+30.66 ± 1.60

Table 3.2.1 Microparticle size and surface charge. The size distribution of microparticles was measured by laser light scattering. Microparticles were suspended in double distilled water (DDW). Particle sizes are expressed as the volumetric mean diameter (μm). Zeta potential measurements were carried out in DDW using a zeta potential analyzer. Each sample was analysed 10 times and the zeta potential is expressed as the mean ± s.e. Formulation technique A. O/W solvent evaporation method, section 2.2.7. B. O/W solvent evaporation method, section 2.2.8. C. O/W solvent evaporation method, section 2.2.1. D. Sodium precipitation method, section 2.2.3. E. O/W solvent evaporation method, section 2.2.4. F. Non-solvent induced precipitation method, section 2.2.6. G. O/W solvent evaporation method, section 2.2.2.

chitosan post-formulation (section 2.2.6), chitosan molecular weight did not affect the size of PLG microparticles (LMWC:19.70μm, MMWC: 19.61μm). By comparison, post-modified PLA microparticles were smaller when modified with MMWC (5.61 μm) than when modified with LMWC (10.43μm). The smallest cationic microparticles (3.56μm) were formulated using the o/w solvent evaporation technique (section 2.2.2) with PLG 75:25 as a core polymer and PVP-PDAEMA as a stabiliser.

PLG microparticles stabilised with PVA had a negative zeta potential (-24.87mV). This was not surprising as PVA is an anionic surfactant. Interestingly, when PLG microparticles were formulated without a stabiliser, the particles retained a negative charge (-39.45mV). It has been suggested that this could be due to the carboxylic end groups or charged cleavage products of the polymer (Walter & Merkle, 2002). Both PLA and PLG microparticles stabilised with the cationic surfactant CTAB exhibited a net positive surface charge (+75.28mV and +38.41mV respectively). PLA/CTAB microparticles were the most strongly cationic of the formulations described here. Microparticles formulated using chitosan alone were also cationic (+18.06mV) but were not as strongly charged as particles stabilised with CTAB. All PLG formulations stabilised using chitosan were positively charged, with zeta potentials ranging from +15.76mV (PLG 75:25/MMWC) to +38.38mV (PLG 50:50/LMWC). The stabiliser LMWC gave more strongly positively charged microparticles than MMWC suggesting that surface coverage of particles by LMWC was greater than coverage by MMWC. Surprisingly, the core polymer also seemed to have an affect on surface charge. Chitosan-stabilised particles made with PLG 50:50 as opposed to PLG 75:25 were more cationic. Surface modification of PLG and PLA microparticles with chitosan also resulted in the production of cationic microparticles. Particles surface modified with LMWC were more strongly cationic than those modified with MMWC once again indicating more effective surface coverage by LMWC. PLA and PLG microparticles surface modified with LMWC had zeta potentials of +42.79mV and +41.36mV respectively whereas PLA and PLG microparticles surface modified with MMWC had surface charge measurements of +31.18mV and +36.84mV respectively. PLG microparticles formulated using the polymer PVP-PDAEMA as a stabiliser had a net positive surface charge of +30.66mV.

On the basis of these initial studies chitosan-stabilised PLG microparticles and PLG/PVP-PDAEMA microparticles were characterised in more depth. These formulations were chosen because they were cationic and in both cases the size of the microparticles were within the range capable of targeting APCs (<10μm). Unless otherwise stated PLG 75:25 was used as the core polymer.

3.3 Further characterisation of PVP-PDAEMA stabilised microparticles

3.3.1 The effect of PVP-PDAEMA stabiliser concentration on microparticle recovery

Commercially, the efficacy of a vaccine must be balanced against cost of production and yield. Ideally, efficiency of recovery (%w/w) and biological efficacy will be high whilst production costs are low. The effect of PVP-PDAEMA stabiliser concentration in the water phase, during emulsification and solvent evaporation, on the % (w/w) recovery of PLG microparticles is shown in figure 3.3.1. As the concentration of stabiliser increased from 5% v/v to 20% v/v the recovery of microparticles decreased from approximately 95% w/w to approximately15% w/w. An intermediate level of microparticle recovery (approx. 45% w/w) was achieved when microparticles were stabilised with 10% v/v PVP-PDAEMA. Increasing the stabiliser concentration above 20% v/v had little affect on microparticle recovery which remained at approximately 15% w/w. The observed reduction in microparticle recovery may be attributed to the increased viscosity of the PVP-PDAEMA solution at higher stabiliser concentrations. Stabiliser function may be decreased at high concentrations if association between stabiliser molecules occurs and increased viscosity prevents formulation of an emulsion. Alternatively, at high stabiliser concentrations particles may be too small to sediment and as a result recovery by centrifugation could be hindered.

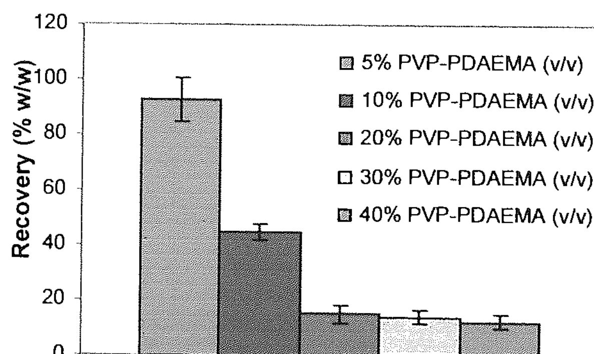


Figure 3.3.1 The effect of PVP-PDAEMA stabiliser concentration on PLG 75:25 microparticle recovery (%w/w). Sample (1ml) of microparticle suspension was frozen at −80°C for 30 min before lyophilisation overnight. The microparticle concentration was calculated by subtraction of the eppendorf weight from the weight of the sample following lyophilisation.

PVP-PDAEMA concentration (% v/v)	Average size (μm)	Zeta potential (mV)
5	3.56 ± 0.035	+ 30.66 ± 1.60
10	2.62 ± 0.021	+ 26.13 ± 1.08
20	3.28 ± 0.057	+ 22.53 ± 1.99
30	2.93 ± 0.031	+ 29.75 ± 2.82
40	3.40 ± 0.000	+ 36.78 ± 2.81

Table 3.3.2 The effect of PVP-PDAEMA concentration on PLG 75:25 microparticle size and surface charge. Microparticles were prepared by the o/w solvent evaporation method (section 2.2.2). The size distribution of microparticles was measured by laser light scattering. Microparticles were suspended in DDW. Particle sizes are expressed as the volumetric mean diameter (μm). Zeta potential measurements were carried out in DDW using a zeta potential analyser. Each sample was analysed 10 times and the zeta potential is expressed as the mean ± s.e.

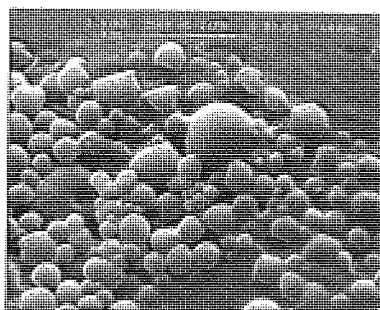


Figure 3.3.3 Scanning electron micrograph (SEM) showing the surface morphology of PVP-PDAEMA stabilised PLG 75:25 microparticles. Microparticles were stabilised with 20% v/v PVP-PDAEMA. A sample of microparticle suspension was left to dry overnight on an SEM specimen stub, coated in gold and examined on a scanning electron microscope (Cambridge Instruments, Stereoscan 90B) operated at 25kV.

3.3.2 The effect of PVP-PDAEMA concentration on microparticle size and surface charge

The effect of PVP-PDAEMA concentration on the size and surface charge of PLG microparticles is shown in table 3.3.2. As the polymer concentration increased from 5% v/v to 40% v/v little effect was seen on the size or surface charge of the microparticles. At all stabiliser concentrations microparticles were between approximately 2.5μm and 3.5μm in size. All the microparticles were cationic with zeta potential measurements ranging from + 22.53mV to +30.66mV except at the highest stabiliser concentration (40% v/v) where the zeta potential measurement rose to +36mV.

3.3.3 Morphology of PVP-PDAEMA stabilised microparticles

The surface morphology of PLG microparticles stabilised with 20% v/v PVP-PDAEMA is shown in figure 3.3.3. This SEM was representative of the results obtained for microparticles stabilised with 5, 10, 30 and 40% v/v PVP-PDAEMA (results not shown). The surface of the particles was smooth and the microparticles were predominantly spherical in shape. Although the results obtained using the particle sizer suggested that PLG/PVP-PDAEMA microparticles had a small size range (table 3.3.2), the SEM images revealed that although the majority of microparticles were approximately 3μm in size, there were also larger microparticles (approx 6μm), and a population of smaller microparticles that were 1μm or less in diameter. Larger particles may not have been detected by the sizer due to settling on the bottom of the cell, whereas the smaller particles (<1μm) may have been below the level of detection of the instrument.

3.4 Further characterisation of chitosan-stabilised microparticles

3.4.1 The effect of chitosan concentration on microparticle yield

The effect of LMWC concentration on the percentage recovery (w/w) of PLG microparticles is shown in figure 3.4.1. As the concentration of stabiliser was increased

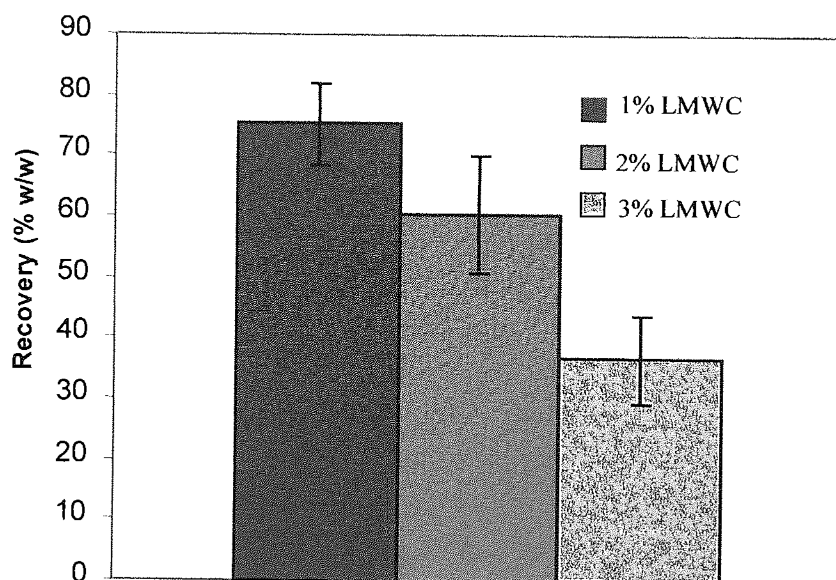


Figure 3.4.1 The effect of LMWC concentration on PLG 75:25 microparticle recovery (%w/w). Sample (1ml) was frozen at −80°C for 30 min before lyophilisation overnight. The microparticle concentration was calculated by subtraction of the eppendorf weight from the weight of the sample following lyophilisation.

from 1% w/v to 3% w/v the recovery of microparticles dropped from approximately 75% (w/w) to approximately 35% (w/w). Microparticles stabilised with 2% w/v LMWC resulted in intermediate recovery levels (60% w/w). These results suggest that microparticle recovery is inversely related to stabiliser concentration. As with PVP-PDAEMA stabilised microparticles the reduction in recovery may be attributed to the increased viscosity of the chitosan solution at higher stabiliser concentrations. Association of chitosan molecules at high concentrations may reduce the stabiliser function of the polymer preventing formation of an emulsion, or, microparticles may be too small to sediment by centrifugation at increased stabiliser concentrations.

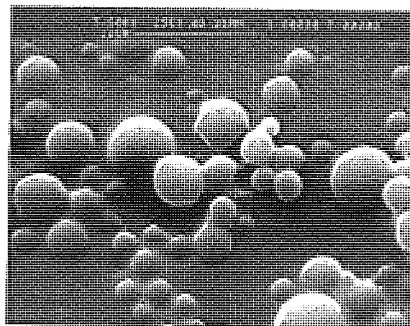
3.4.2 The effect of chitosan molecular weight and stabiliser concentration on zeta potential and microparticle size

The effect of chitosan molecular weight and stabiliser concentration on the size and zeta potential of PLG microparticles is shown in table 3.4.2. Microparticles stabilised with increasing concentrations of LMWC exhibited a slight but gradual reduction in microparticle size from 4.06μm at a 1% w/v stabiliser concentration to 2.89μm at a 3% w/v stabiliser concentration. This behaviour is in line with the often documented effect that increased stabiliser concentration results in a decrease in particle size using the o/w technique (Arshady *et al,* 1990; Scholes *et al,* 1993). This is considered to result from more sheer stress action on the droplets of polymer solution causing more effective size reduction. Conversely, microparticles stabilised with MMWC increased in size as stabiliser concentration increased, from 6.65μm when stabilised with a 1% w/v solution, to 7.31μm when stabilised with a 2% w/v solution. These findings concur with the results of Scholes *et al.* who observed increases in the size of PLG particles beyond a critical stabiliser concentration, due to non uniform flow conditions (Scholes *et al,* 1993). If the concentration of MMWC in solution was above 2% w/v, viscosity increased to such a degree that microparticles could not be produced as the solution could not be sheared. The surface charge of microparticles stabilised with LMWC increased as stabiliser concentration increased up to a concentration of 2.5% w/v. The zeta potential measurement increased from +29.66mV when particles were stabilised with a 1% w/v

Stabiliser concentration (% w/v)	Size (µm)		Zeta potential (mV)	
	LMWC	MMWC	LMWC	MMWC
1	4.06 ± 0.09	6.65 ± 0.19	+ 29.66 ± 2.76	+ 32.60 ± 3.83
2	3.89 ± 0.16	7.31 ± 0.42	+ 39.93 ± 1.46	+ 40.99 ± 3.56
2.5	3.34 ± 0.16	-	+ 47.79 ± 6.57	-
3	2.89 ± 0.28	-	+ 42.28 ± 1.68	-

Table 3.4.2 The effect of LMWC or MMWC concentration on PLG 75:25 microparticle size and surface charge. Microparticles were formulated using the o/w solvent evaporation method (section 2.2.4). The size distribution of microparticles was measured by laser light scattering. Microparticles were suspended in DDW. Particle sizes are expressed as the volumetric mean diameter (µm). Zeta potential measurements were carried out in DDW using a zeta potential analyser. Each sample was analysed 10 times and the zeta potential is expressed as the mean ± s.e.

A.



B.

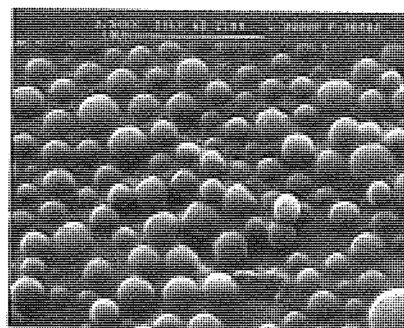


Figure 3.4.3 Scanning electron micrograph (SEM) showing the surface morphology of LMWC stabilised PLG 75:25 microparticles. (A) Microparticles were stabilised with 1% w/v LMWC (B) Microparticles were stabilised with 3% w/v LMWC. A sample of microparticle suspension was left to dry on an SEM specimen stub overnight, coated in gold and examined on a scanning electron microscope (Cambridge Instruments, Stereoscan 90B) operated at 25kV.

solution, to +47.79mV when the stabiliser concentration was 2.5% w/v. At a 3% w/v stabiliser concentration the surface charge dropped slightly to +42.28mV. The surface charge measurement of microparticles stabilised with MMWC also increased from +32.60mV to +40.99mV as stabiliser concentration increased from 1%w/v to 2% w/v. As the concentration of LMWC or MMWC (w/v) in the formulation increased, the zeta potential also increased suggesting greater coverage of the particle surface by stabiliser at higher concentrations.

3.4.3 The effect of chitosan concentration on microparticle morphology

The effect of LMWC concentration on the morphology of PLG microparticles is shown in figure 3.4.3. Microparticles stabilised with 1% w/v LMWC (Figure 3.4.3A) and microparticles stabilised with 3% w/v LMWC (Figure 3.4.3B) were smooth and spherical. As stabiliser concentration increased the uniformity of the particles increased and the size range decreased. This phenomenon is commonly observed in microparticles formulated using the o/w solvent evaporation method and results from more effective stabilisation of the particles at increased stabiliser concentrations (Arshady *et al,* 1990; Scholes *et al,* 1993). A discrepancy was seen between results obtained using the particle sizer (Table 3.4.2) and the SEM image of microparticles stabilised with 1% w/v LMWC (3.4.3A). The sizer results suggest that these microparticles were approximately 4μm in diameter, and although some of the microparticles on the SEM image were this size, smaller particles (approximately 1-2μm) were also evident. By comparison, the size of the microparticles stabilised with 2% w/v LMWC on the SEM image (3.4.3B) correlated well with results from the particle sizer (Table 3.4.2).

3.4.4 The effect of wash number on size and surface charge of microparticles

To establish whether PLG/LMWC microparticles were washed an adequate number of times to remove free chitosan and produce a stable surface for DNA adsorption, microparticles were washed five times by resuspension in DDW followed by

A.

Number of washes	Size		
	Stabiliser concentration (w/v)		
	1%	2%	3%
0	2.73 ± 0.04 μm	2.23 ± 0.06 μm	2.45 ± 0.24 μm
1	3.11 ± 0.01 μm	2.84 ± 0.05 μm	3.25 ± 0.06 μm
2	3.10 ± 0.07 μm	3.14 ± 0.10 μm	3.40 ± 0.02 μm
3	3.22 ± 0.03 μm	2.95 ± 0.09 μm	3.05 ± 0.10 μm
4	3.18 ± 0.02 μm	2.76 ± 0.01 μm	2.77 ± 0.31 μm
5	3.78 ± 0.01 μm	2.94 ± 0.04 μm	3.13 ± 0.05 μm

B.

Number of washes	Zeta potential		
	Stabiliser concentration (w/v)		
	1%	2%	3%
0	+ 63.60 ± 0.75 mV	+ 69.86 ± 3.20 mV	+ 81.07 ± 3.28 mV
1	+ 65.36 ± 3.12 mV	+ 70.33 ± 1.76 mV	+ 65.35 ± 1.48 mV
2	+ 36.92 ± 2.63 mV	+ 54.79 ± 3.20 mV	+ 48.11 ± 4.67 mV
3	+ 33.87 ± 0.74 mV	+ 45.36 ± 0.95 mV	+ 46.44 ± 1.91 mV
4	+ 36.14 ± 2.51 mV	+ 41.10 ± 0.60 mV	+ 46.75 ± 2.23 mV
5	+ 37.14 ± 1.03 mV	+ 43.92 ± 0.64 mV	+ 53.23 ± 1.99 mV

Table 3.4.4 The effect of wash number on the size and surface charge of PLG 75:25 microparticles stabilized with varying concentrations of LMWC. For formulation of microparticles see methods section 2.2.4. Following formulation, microparticles were centrifuged (6min, 3500rpm) and washed 5 times in DDW. (A) The size distribution of microparticles was measured by laser light scattering. Microparticles were suspended in DDW. Particle sizes are expressed as the volumetric mean diameter (μm). (B) Zeta potential measurements were carried out in double disitilled water (DDW) using a zeta potential analyzer. Each sample was analysed 10 times and the zeta potential is expressed as the mean ± s.e.

centrifugation (3500rpm, 6 min.), and size and zeta potential measurements were taken after each wash. The effect of wash number on the size of PLG microparticles stabilised with varying concentrations of chitosan is shown in figure 3.4.4A. The size of the microparticles stabilised with 1%, 2% and 3% w/v LMWC did not appear to be significantly affected by the number of washes. Irrespective of the number of washes, microparticles stabilised with 1% w/v LMWC fluctuated in size between 2.73 and 3.78μm. Likewise, microparticles stabilised with 2% w/v LMWC were between 2.23 and 3.14μm in diameter, and 3% w/v stabilised microparticles between 2.45 and 3.40μm.

The effect of wash number on the zeta potential of PLG microparticles stabilised with varying concentrations of LMWC is shown in figure 3.4.4B. In comparison to microparticle size, the wash number had an obvious effect on the surface charge of the particles. After the first wash, the zeta potential measurements of microparticles stabilised with all concentrations of LMWC were between +65 and +70mV. These high values suggested that a large quantity of loosely bound chitosan was still present at the microparticle surface. As the number of washes increased, a reduction in zeta potential was observed until particles had been washed 3 (microparticles stabilised with 1% w/v and 3% w/v LMWC) or 4 times (microparticles stabilised with 2% w/v LMWC). At this point the zeta potential measurements of microparticles stabilised with 1%, 2% and 3% w/v LMWC were +33.87mV, +41.10mV and +46.44mV respectively. Following subsequent washes, the zeta potential of each group of microparticles fluctuated around these values. These results suggest that microparticles should be washed a minimum of 3 times to produce a stable surface.

3.4.5 The effect of stabiliser concentration (w/v) on chitosan incorporation

The effect of increasing stabiliser concentration on LMWC incorporation in PLG microparticles is shown in figure 3.4.5. If the stabiliser concentration was increased from 1% w/v to 3% w/v, incorporation of chitosan increased from approximately 55% w/w to approximately 70% w/w. This increase was significantly different ($p< 0.01$). If these high

incorporation levels are accurate it suggests that the microparticles are effectively PLG/chitosan blends.

3.4.6 The effect of chitosan stabiliser concentration (w/v) on chitosan release from PLG microparticles

Following microparticle preparation, the presence of chitosan on the particle surface was indicated by the positive zeta potential. However, to confirm that the surface of the particle was stable for DNA adsorption, an *in vitro* study was conducted in PBS at 37°C to measure chitosan release from the microparticle. The effect of stabiliser concentration (%w/v) on cumulative chitosan release is shown in figure 3.4.6. Release of chitosan from the microparticles did occur but at very low levels. Cumulative release of just over 1% (w/w) of chitosan from microparticles stabilised with 1% w/v LMWC occurred over 14 days suggesting that the chitosan remained effectively bound to the particle. The cumulative release of chitosan from microparticles stabilised with 3% w/v LMWC reached approximately 2.2% by day 14 suggesting that a small fraction of loosely bound chitosan was associated with these particles.

3.4.7 Analysis of LMWC-stabilised PLG microparticle size following the addition of excipients and lyophilisation.

Excipients such as saccharides or surfactants are often added to formulations prior to lyophilisation in order to prevent aggregation of the microparticles upon reconstitution and to protect adsorbed DNA (Ando *et al,* 1999). However, these excipients can alter the physicochemical characteristics of a formulation. PLG/LMWC microparticles formulated using the o/w solvent evaporation technique (section 2.2.4) were incubated with plasmid DNA (0.02% w/w) overnight (section 2.6.1). The effect of the addition of excipients prior to lyophilisation, on the size of DNA loaded PLG/LMWC microparticles, following reconstitution in DDW are shown in table 3.4.7. Without the addition of excipients, lyophilised and reconstituted DNA loaded PLG/LMWC (1%w/v) microparticles were

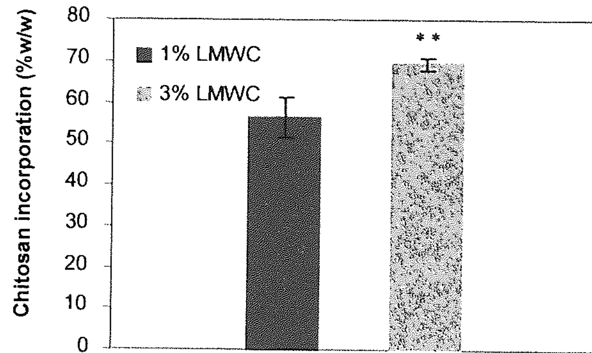


Figure 3.4.5 The effect of LMWC stabiliser concentration (% w/v) on chitosan incorporation (% w/w) in PLG 75:25 microparticles. Microparticles were formulated using the o/w solvent evaporation method (section 2.2.4).The concentration of chitosan in the microparticles was estimated by measuring the concentration of chitosan in the stabilising solution after microparticle formulation and subtracting this from the initial stabiliser concentration. Washes from the formulation process were collected and combined. A 1ml aliquot of the wash solution was mixed with acetone (1ml), dichloromethane (1ml) and 1% v/v lactic acid (1ml) in a test tube and the tube was shaken vigorously overnight. Ninhydrin solution (1ml) was added to the chitosan solution in lactic acid at a 1:1 ratio and incubated in a boiling water bath for 20 min. Following incubation the tube was cooled and the absorbance was read at 570nm.

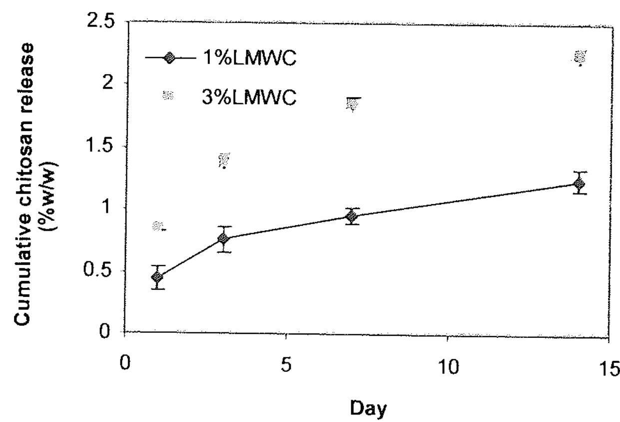


Figure 3.4.6.The effect of LMWC chitosan stabiliser concentration (% w/v) on cumulative chitosan release from microparticles over 14 days. Chitosan- stabilised microparticles (20mg) were incubated over 14 days in 1ml phosphate buffered saline (PBS) at 37 °C. At intervals samples were centrifuged (6 min, 3500rpm). The supernatant was removed and assayed for chitosan content by the Ninhydrin assay (method 2.4.1). The pellet was resuspended in fresh PBS and the release study continued.

Excipients (concentration in final volume)	Chitosan concentration (% w/v)	Size (μm)
None	1	19.01 ± 1.22
Mannitol (4.5% w/v) + sucrose (1.5% w/v)	1	16.44 ± 1.26
Mannitol (4.5% w/v) + sucrose (1.5% w/v)	3	16.40 ± 0.07
Mannitol (4.5% w/v), sucrose (1.5% w/v) + 9,000MW PVA (0.5% w/v)	1	16.06 ± 0.48
9,000MW PVA (0.5% w/v)	1	11.74 ± 0.23
13,000MW PVA (0.5% w/v)	1	13.43 ± 1.40
30,000MW PVA (0.5% w/v)	1	12.49 ± 0.54
9000MW PVA (0.1% w/v)	1	21.56 ± 0.34

Table 3.4.7. Size analysis of LMWC stabilised PLG 75:25 microparticles following lyophilisation. Microparticles were formulated using the o/w solvent evaporation method (section 2.2.4). Microparticles (20mg) were incubated with plasmid DNA (0.02%w/w) in distilled water overnight. Excipients were added to microparticle suspensions prior to lyophilisation. Microparticles (20mg/ml) in distilled water were aliquoted out in 1ml volumes. Mannitol (4.5% w/v final concentration) and sucrose (1.5% w/v in final concentration) were added to some aliquots whereas PVA (0.5 or 0.1% w/v in final concentration) was added to others. Samples were frozen at −80°C for 30 min followed by lyophilisation. Microparticles were reconstituted in sterile DDW (20mg/ml). The size distribution of microparticles was measured by laser light scattering. Particle sizes are expressed as the volumetric mean diameter (μm).

19.01μm in diameter. The addition of the sugars, sucrose and mannitol, prior to lyophilisation led to a reduction in the size of the microparticles to 16.44μm. Microparticles stabilised with 3% w/v LMWC were approximately the same size (16.40μm) under these conditions. When the sugars were added as excipients in conjunction with PVA 9,000 MW prior to lyophilisation the resulting size of the microparticles was approximately 16.06μm. The smallest microparticles (11.74μm) were produced when the non-ionic surfactant PVA 9,000 MW was used alone. Microparticles lyophilised following the addition of PVA 13,000 MW or PVA 30,000 MW were 13.43μm and 12.49μm in diameter respectively. These results suggest that PVA was more effective at reducing aggregation of microparticles following reconstitution than sucrose and mannitol. However, use of a surfactant in the formulation is undesirable. When the concentration of PVA 9,000 MW was reduced from 0.5% w/v to 0.1% w/v the size of the microparticles increased to 21.56μm.

3.5 Discussion

Key physicochemical factors which must be taken into consideration when evaluating microparticulate carriers for the delivery of DNA vaccines are the size and surface charge of the particles. It is well established that only microparticles < 10μm in diameter are capable of being efficiently phagocytosed by professional antigen presenting cells such as dendritic cells and macrophages (Tabata & Ikada, 1990) and there is much evidence to support the importance of these cells in the generation of potent immune responses following immunisation with genetic vaccines (Doe *et al,* 1996; Corr *et al,* 1999; Timmerman & Levy, 1999). In this study PLA, PLG and chitosan microparticles were produced which ranged in size from 1.26 – 54.25μm (table 3.2.1). Unsurprisingly, PLG microparticles produced without the addition of stabiliser were the largest, due to aggregation of disperse phase droplets during emulsification. Microparticles which fell within the size range capable of transfecting professional APCs included PVA stabilized PLG microparticles (1.26μm), LMWC and MMWC stabilised PLG microparticles (3.9-5.62μm), PLA microparticles surface modified with MMWC (5.61μm) and PLG microparticles stabilised with PVP-PDAEMA (3.56μm). The importance of microparticle

size in DNA vaccination was highlighted in a study by Singh *et al.* It was demonstrated that the size of DNA-loaded PLG/CTAB microparticles was directly related to the magnitude of the antibody response in i.m. injected mice. The greatest enhancement of antibody titres was observed in mice injected with the smallest microparticles (300nm), whereas mice injected with microparticles >30μm showed no enhancement of antibody levels (Singh *et al,* 2000). These results suggest that the smaller the delivery vehicle, the greater the enhancement of the immune response. In addition, smaller microparticles will also have a greater surface area for adsorption of DNA.

In order to efficiently adsorb plasmid DNA, a microparticulate delivery system must be positively charged at its surface. Binding will occur by electrostatic interaction of the negatively charged phosphate group on the DNA backbone with the polymer forming the particle or the cationic group of the stabiliser. In this study, all the microparticles produced had a net positive surface charge with the exception of PLG particles stabilised with the surfactant PVA, and PLG particles produced without the addition of stabiliser (table 3.2.1). PLA microparticles produced without the addition of stabiliser are also known to exhibit a negative zeta potential (Walter & Merkle, 2002). Consequently, cationic stabilisers used to formulate particles containing PLA or PLG as core polymers must be capable of compensating for this negative charge. In this study it was demonstrated that both the cationic surfactant CTAB and the polycation chitosan were capable of producing PLG and PLA microparticles with a net positive surface charge. The strength of the cationic charge on the particle surface may influence its effectiveness as a DNA vaccine delivery system. Strongly cationic microparticles may still retain a net positive surface charge following DNA adsorption. This may lead to enhanced electrostatic interaction of the particle with the negatively charged cell surface and could result in enhanced uptake of DNA by the cell (Mislick *et al,* 1996). Conversely, a net positive surface charge may also result in interaction of the microparticle with negatively charged serum proteins such as albumin (isoelectric point 4.9). For many cationic DNA delivery systems such as liposomes (Mahato *et al,* 1995) and PLL (Lollo *et al,* 1997), interaction with serum proteins leads to a reduction in transfection efficiency (Thiele *et al,* 2003).

From the initial physicochemical studies, microparticles within the desired size range and with a positive surface charge included LMWC and MMWC stabilised PLG microparticles. All subsequent studies were conducted using PLG 75:25 as the core polymer as opposed to PLG 50:50. Although PLG 75:25 has a longer resorption rate than PLG 50:50 (4-5 months as opposed to 1-2 months (table 1.8.1)) due to its higher lactide to glycolide content, PLG 75:25 was chosen because smaller microparticles were produced when this polymer was stabilised with LMWC (3.9μm). Since DNA is surface bound, the issue of degradation controlled release of encapsulated DNA was less important. However, if it was deemed necessary to increase the resorption rates of the carrier, for example to increase the rate of elimination from the body further it would be quite feasible to substitute PLG 50:50 for PLG 75:25 in this formulation. PLA microparticles stabilised with MMWC although exhibiting the desired size and charge characteristics were discounted for further studies due to the use of PLA as the core polymer in this formulation. The low resorption rate of PLA (12-16 months) may be unnecessarily long for the purposes of DNA vaccination and could increase the elimination time from the body. PLG 75:25 microparticles stabilised with the copolymer PVP-PDAEMA also fitted the desired physicochemical criteria and were therefore investigated in additional studies.

The decisions to further investigate PLG microparticles stabilised with either chitosan or PVP-PDAEMA were not based solely on the physicochemical data obtained in these studies. Chitosan was an attractive choice because the polymer is biodegradable (Struszczyk *et al,* 1991), biocompatible (Chandy & Sharma, 1990; Hirano *et al,* 1990), exhibits low immunogenicity and low toxicity (Arai *et al,* 1968; Brine *et al,* 1992; Carrero-Gomez & Duncan, 1997; Richardson *et al,* 1997). Chitosan is also well characterised having been investigated in many areas of biomedical research including gene delivery (Roemer & Friedmann, 1992; Lee *et al,* 1998; Leong *et al,* 1998; Köping-Högaard *et al,* 1998), burn treatment, wound healing (Peh *et al,* 2000; Berger *et al,* 2004) and as a hemostatic agent (Kim *et al,* 2003). Additionally, in vaccine development it has been suggested that chitosans may be capable of non-specifically stimulating the immune

system, although this affect appears to be dependent on the degree of deacetylation of the chitosan and the type of formulation. In mice and guinea pigs 70% deacetylated chitin administered by intraperitoneal injection was shown to possess adjuvant activity for antibody formation and the induction of cell-mediated immunity. The production of the cytokines interleukin, interferon and colony stimulating factor were also stimulated following intraperitoneal injection with this compound (Nishimura *et al,* 1985). Furthermore, the physicochemical structure of chitosan may aid destabilisation of cellular membranes (Yang *et al,* 2002) leading to enhancement of DNA uptake and release of DNA from endosomal compartments into the cell cytoplasm. Chitosan is polycationic when its primary amines are protonated at a pH lower than or equal to its pKa and it is probably the molecules significant degree of deacetylation which drives chitosan-induced membrane destabilisation (Fang *et al,* 2002).

In comparison to chitosan the copolymer Poly (N- vinylpyrrolidone/2-dimethylaminoethyl methacrylate), dimethyl sulfate quaternary (PVP-PDAEMA) is poorly characterised. However, the PVP component of the copolymer has both industrial and medical applications including use as a pharmaceutical excipient and a topical antiseptic in surgery and for skin and mucous membrane infections (Flynn, 2003). Additionally, studies suggest that PVP has potential for use in DNA vaccine delivery systems. The polymer acts as a lysomotropic agent and Ciftci *et al* demonstrated that PVP/DNA complexes enhanced transfection with minimal toxicity in cultured fibroblasts (Ciftci & Levy, 2001). However, in contrast to these findings Wang *et al* demonstrated a dose and time dependent toxicity to PVP K-30 in cultured HeLa cells (Wang *et al,* 2003). An *in vivo* study by Anwer *et al* demonstrated that antigen specific IgG titers could be raised in dogs and pigs injected i.m. or subcutaneously with PVP complexed to plasmid DNA expressing human growth hormone (Anwer *et al,* 1999). Like chitosan the second component of the copolymer, PDAEMA may have physicochemical characteristics which could enhance endosomal disruption and release of DNA into the cytoplasm. Walter *et al* produced cationic microparticles using a related polymer (2-dimethylaminoethyl) methacrylate, methylmethacrylate (DAEM) and suggested the enhanced transfection levels observed were a result of the polymers' pH dependent solubility (walter *et al,*

2002). They postulated that the microparticles remained intact at extracellular pH because the DAEM was insoluble. However, in the phagosomal compartment which was more acidic DAEM became more soluble leading to dissociation and release of the cationic polymer. In the phagosome if DAEM accepted protons, the pH may increase leading to deactivation of degradative enzymes. Membrane disruption may also occur at lower pH due to the cationic and amphiphillic properties of the polymer.

In order to establish whether it was possible to manipulate the physicochemical characteristics of the chosen formulations by altering stabiliser concentration, the effect on microparticle recovery, size, zeta potential and morphology was investigated. For both formulations an increase in stabiliser concentration led to a decrease in microparticle recovery (PVP-PDAEMA fig 3.3.1, chitosan fig. 3.4.1). From an economic point of view these results suggest that microparticles produced with lower stabiliser concentrations would be more cost effective. However, this must ultimately be balanced against which of the formulations is most biologically effective. Interestingly, stabiliser concentration did not significantly affect the size or zeta potential of microparticles stabilised with PVP-PDAEMA (table 3.3.2) and the morphology of the microparticles remained smooth and spherical (fig. 3.3.3). It may therefore be possible to reduce the concentration of copolymer in the formulation further whilst retaining the physiochemical properties of the microparticles, although there will be a minimum concentration of stabiliser necessary to achieve satisfactory stabilisation of polymer droplets in the continuous phase. In comparison, the size, zeta potential and morphology of PLG microparticles stabilised with chitosan were affected by stabiliser concentration. As stabiliser concentration was increased in LMWC-stabilised particles their size decreased probably as a result of increased sheer stress on the droplets of polymer solution and increased droplet stabilisation (table 3.4.2). This effect has commonly been observed for microparticles produced by the o/w solvent evaporation method. For example, Kawata *et al* (ref) described the production of Eudragit microcapsules stabilised with aluminium tristearate and demonstrated that as the concentration of stabiliser increased from 0.72% to 9.21% (w/v) the microcapsule size decreased from more than 1000μm to about 100μm (Kawata *et al,* 1986) . In comparison, in the present study the size of particles stabilised with

MMWC increased as stabiliser concentration increased (table 3.4.2). This phenomenon has previously been reported by Scholes *et al* who found that if stabiliser concentration increased above a critical level then PLG microparticles increased in size, due to non-uniform flow conditions (Scholes *et al,* 1993). In the present study, for both LMWC and MMWC stabilised microparticles an increase in zeta potential occurred as stabiliser concentration increased (table 3.4.2). The morphology of microparticles stabilised with increasing concentrations of LMWC was smooth and spherical but became more uniform at higher stabiliser concentrations (fig 3.4.3). The ability to manipulate the size and charge characteristics of chitosan stabilised microparticles by altering the chitosan concentration is an advantage as it allows more flexibility in the design of potential delivery vehicles for DNA vaccination. Since chitosan is toxicologically safe and manipulation of microparticles is possible by altering stabiliser concentration, *in vitro* stability studies of this formulation were undertaken.

The stability of the microparticles was assessed in two ways. Firstly, was the microparticle surface stable in solution and did the surface remain stable over time? These considerations are important for short term storage of the particles and for DNA adsorption. Secondly, when DNA-adsorbed microparticles were lyophilised for long term storage did the physicochemical characteristics of the particles remain the same upon reconstitution? In this study it was demonstrated that chitosan-stabilised microparticles should be washed a minimum of three times to produce a stable charged surface. The stability of the surface was assessed by changes in microparticle size (3.4.4A) and surface charge (3.4.4B) following each wash. The size of the microparticles stabilised with 1%, 2% and 3% w/v LMWC remained approximately constant throughout the wash process. However, the surface charge of the microparticles became less positive as the wash number increased up to three washes. These results suggested that initially, loosely bound chitosan was present at the surface of the particle but that this was removed by washing.

The stability of the microparticles in PBS at 37°C was also quantified by measuring the amount of chitosan initially incorporated in the particle and the amount of chitosan released from the particle. Incorporation of chitosan in microparticles stabilised with 3%

w/v LMWC compared to particles stabilised with 1% w/v LMWC was significantly higher ($p<0.05$, fig 3.4.5). However, in both cases the calculated level of chitosan incorporation was surprisingly high (58-68 % w/w). It is possible that chitosan/PLG polymer complexes were formed in the wash solution resulting in the observed turbidity, and that these complexes were not completely broken down by the lactic acid (1%w/v), DCM and acetone mixture. If this were the case there may have been 'masked' chitosan in the solution still complexed to PLG which could not be detected by the ninhydrin assay. As a result the true incorporation of chitosan in the microparticles may be much lower than the results described here.

Overall cumulative release of chitosan from the microparticles over 14 days was low (fig. 3.4.6), although cumulative release from microparticles stabilised with 3% w/v LMWC was slightly higher than from microparticles stabilised with 1% w/v LMWC. The low levels of chitosan released over 14 days suggests that in PBS at pH 7.4 the microparticles were stable. It was expected that chitosan release from the microparticles at this pH would be low as chitosan is only soluble at pH< 6.5. Although chitosan is insoluble at extracellular physiological pH, if microparticles were taken up into the acidic phagosomes of professional APCs chitosan would become more soluble. As a result greater release of the polycation, and consequently adsorbed DNA, could occur from the particle.

Commercially, the ability to store a DNA vaccine in a stable lyophilized form without loss of efficacy is critical to increase the shelf life of the product and reduce storage costs. In this study the effect of lyophilisation on the size of DNA-loaded, LMWC stabilised microparticles was investigated. It became apparent that even with the addition of a variety of commonly used excipients prior to lyophilisation to prevent aggregation, when microparticles were reconstituted following freeze drying the diameter of the microparticles increased above10μm (table 3.4.7). This behaviour may impair the ability of the microparticles to target phagocytic cells by size exclusion. The smallest microparticles were produced using the surfactant PVA MW 9,000 (11.74 μm). However,

the use of a surfactant in the formulation was undesirable and when the concentration of surfactant was reduced the size of the microparticles almost doubled (21.56μm).

In summary, from the physicochemical data PLG microparticles stabilised with chitosan or with PVP-PDAEMA showed the greatest potential for investigation as DNA vaccine delivery vectors. Both formulations were within the size range found to be effective for targeting professional APCs (<10 μm) and were cationic. Chitosan stabilised microparticles represented a particularly attractive formulation due to the ability to manipulate physicochemical characteristics by altering stabiliser concentration. Chitosan is also toxicologically safe and well characterised. It was possible to produce LMWC stabilised PLG microparticles with a stable surface although lyophilisation of DNA-loaded microparticles led to an increase in size which may reduce the efficacy with which the microparticles can target APCs.

4. DNA interaction with microparticle carriers

4.1 Introduction

The studies described in the previous chapter established chitosan-stabilised PLG microparticles (PLG/chitosan) and PVP-PDAEMA-stabilised PLG microparticles (PLG/PVP-PDAEMA) as the most promising formulations for the delivery of DNA vaccines. However, these choices were based predominantly on the accumulated physicochemical data. In order to make a definitive choice as to which formulation to take on to test transfection efficiency in cells *in vitro* and potentially *in vivo*, it was necessary to establish how each of the formulations interacted with plasmid DNA *in vitro*.

In adsorbing DNA to the surface of the microparticles it was anticipated that many of the problems associated with DNA encapsulation would be avoided. These problems included DNA loading at low levels (0.1-0.4% w/w) with low efficiency (20-50%), degradation of DNA during encapsulation and release leading to a reduction in the supercoiled conformation (Jones *et al,* 1997; Maruyama *et al,* 1997; Ando *et al,* 1999; Walter *et al,* 1999; Capan *et al,* 1999; Tinsley-Brown *et al,* 2000) and slow release of DNA limiting the amount available to transfect cells (Walter *et al,* 1999). In comparison, DNA adsorbed to the surface of PLG/CTAB microparticles was reported to adsorb with high efficiency (92%) and the DNA released from the microparticles retained its integrity (Singh *et al,* 2000). One potential advantage of DNA microencapsulation is that the DNA is better protected from degradative cellular enzymes (Capan *et al,* 1999). However, Singh *et al.* reported that DNA adsorbed to PLG/CTAB microparticles showed enhanced resistance to DNase 1 degradation *in vitro* in comparison to naked DNA (Singh *et al,* 2000).

In this chapter, a comparison of the *in vitro* DNA loading and release characteristics of selected formulations was examined. Particular emphasis was given to investigating the interaction of DNA with PLG/LMWC microparticles including establishing the role of

stabiliser (LMWC) concentration on DNA loading and release profiles as well as the potential of these microparticles to protect plasmid DNA from degradative enzymes.

4.2 DNA loading and *in vitro* release characteristics

4.2.1 DNA loading

Microparticles were incubated overnight in a plasmid DNA solution (0.02 %w/w) (section 2.6.1). DNA loading was estimated by subtraction of DNA in the supernatant from total DNA in the incubation medium. Figure 4.2.1 shows the loading level (% w/w) and the loading efficiency (%) for seven selected formulations. Chitosan microparticles adsorbed DNA with the greatest efficiency (99%), followed by PLG microparticles surface modified with MMWC (78%). PLG/LMWC, PLG/MMWC and PLG/PVP-PDAEMA microparticles all exhibited DNA loading efficiencies between 37 and 45%. Surprisingly, the negatively charged PLG/PVA microparticles were also capable of adsorbing plasmid DNA with an efficiency of 43%. In comparison to reported values of approximately 92% loading efficiency (Singh *et al*, 2000) PLG/CTAB microparticles were the least efficient at DNA adsorption (28% efficiency).

4.2.2 DNA release

Microparticles were incubated at 37°C in PBS over 14 days to measure the release of DNA. The cumulative release of DNA for the seven selected formulations is shown in figure 4.2.2. The *in vitro* release rate of the luciferase reporter plasmid from four of the formulations was initially rapid. PLG/CTAB, PLG/PVA, PLG/LMWC and PLG/PVP-PDAEMA microparticles released approximately 55%, 43%, 30% and 22% of total adsorbed DNA respectively by day 1. Subsequently, the rate of release was slower particularly for PLG/LMWC and PLG/PVP-PDAEMA microparticles. By day 14 these formulations had released approximately 48% and 55% of their total adsorbed DNA respectively. Higher cumulative release of DNA by day 14 was observed for PLG/CTAB

Formulation	Mean size, µm	Zeta potential, mV	Loading level, % w/w	Mean loading efficiency, %
PLG/PVA	1.26 ± 0.00	− 24.7 ± 1.42	0.43 ± 0.03	43.0
PLG/CTAB	19.47 ± 1.13	+38.41 ± 2.11	0.28 ± 0.16	28.0
Chitosan microparticles	21.10 ± 0.04	+18.06 ± 1.17	0.99 ± 0.01	99.0
PLG /MMWC	5.15 ± 0.20	+15.76 ± 1.90	0.43 ± 0.02	43.0
PLG/LMWC	3.90 ± 0.67	+29.66 ± 2.76	0.38 ± 0.04	37.8
PLG/PVP-PDAEMA (5%v/v)	3.56 ± 0.04	+30.66 ± 1.60	0.45 ± 0.03	45.0
PLG microparticles surface modified with MMWC	19.61 ± 0.27	+36.84 ± 1.19	0.78 ± 0.03	78.0

Table 4.2.1 Size, zeta potential, DNA loading level (% w/w) and mean DNA loading efficiency of selected microparticle formulations. Microparticle size was measured using a particle sizer (section 2.3.3) and zeta potential was measured on a zeta potential analyser (section 2.3.2) To estimate DNA loading microparticles (20mg) were incubated with plasmid DNA (0.02%w/w) in 1ml DDW. Supernatant was collected following centrifugation (3500rpm, 6min). The concentration of DNA in the supernatant was measured by spectrophotometry or the PicoGreen assay (method 2.5.3) and loading was estimated by subtraction of this value from the DNA content of the incubation medium.

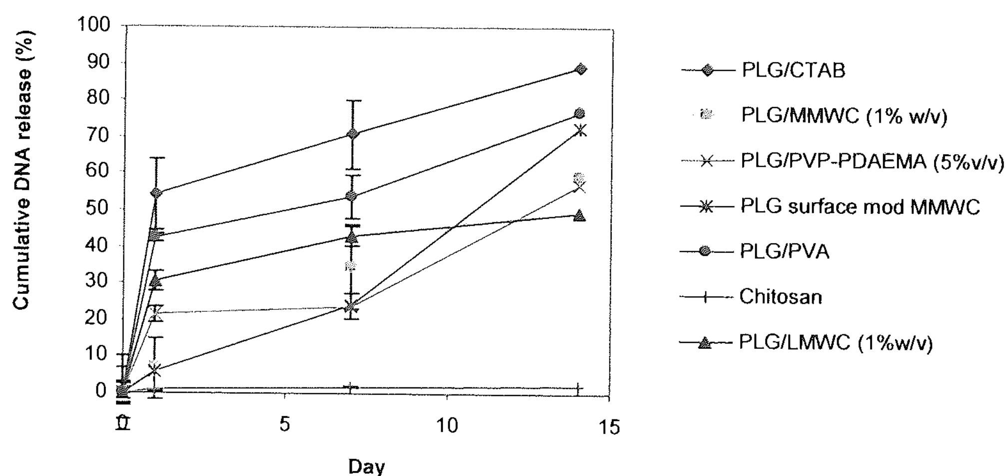


Figure 4.2.2 Cumulative release of DNA over 14 days from selected microparticle formulations with adsorbed plasmid DNA. Microparticles (20mg) loaded with DNA (0.02% w/w) were incubated over 14 days in 1ml phosphate buffered saline (PBS) at 37 °C. At intervals samples were centrifuged (6 min, 3500rpm) and the supernatant assayed for DNA concentration by spectrophotometry or the PicoGreen assay (method 2.5.3).The pellet was resuspended in fresh PBS.

particles (approx. 88%) and PLG/PVA microparticles (approx.72%). By comparison, cumulative release of DNA from PLG/MMWC and PLG microparticles surface modified with MMWC by day 1 was low (approx 5%) and release of DNA from the microparticles over 14 days was more gradual. However, by day 14 PLG/MMWC microparticles had released approximately 55% of adsorbed DNA and microparticles surface modified with MMWC had released approximately 70%. Release of DNA from chitosan microparticles was negligible throughout the duration of the experiment, indicating strong binding affinity between the DNA and the chitosan.

4.3 Interaction of chitosan-stabilised microparticles with DNA

PLG microparticles stabilised with LMWC were chosen to investigate further based on the physicochemical data obtained in chapter 3 and the DNA loading and release studies in this chapter (section 4.2). In summary, PLG/LMWC microparticles were chosen because they were highly cationic (+38.41mV), they were less than 5μm in diameter so had the potential to target professional APCs, DNA loading (approx. 0.4% w/w) and loading efficiency (approx. 40%) were reasonable, and although initial release of bound DNA was quite high (approximately 40% by 24h) a significant quantity of DNA was retained on the microparticle over the 14 day release study (approx. 50%). Table 4.3 gives a summary of PLG/LMWC microparticle size, surface charge, DNA loading efficiency (%), and cumulative DNA release (%).

4.3.1 The effect of stabiliser concentration on DNA loading characteristics

In order to determine whether the DNA loading characteristics of PLG/LMWC microparticles could be controlled to reduce the carrier concentration if necessary, the concentration of LMWC stabiliser in the formulation was varied between 1% and 3% w/v. The effect of LMWC concentration on DNA loading efficiency is shown in figure 4.3.1. As the chitosan stabiliser concentration increased from 1% w/v to 2% w/v DNA loading efficiency also increased from approximately 30% to just over 40%. If the

Formulation	Mean size (µm)	Zeta potential (mV)	Mean DNA loading efficiency (%)	Cumulative DNA release (%) D1	Cumulative DNA release (%) D14
PLG/LMWC	3.9 ± 0.67	29.66 ± 2.76	37.8	30.5 ± 2.6	49.4 ± 2.8

Table 4.3 PLG/LMWC microparticle size, surface charge, DNA loading efficiency and cumulative DNA release (%). Microparticle size was measured using a particle sizer (section 2.3.3) and zeta potential was measured on a zeta potential analyser (section 2.3.2). Microparticles (20mg) were incubated with DNA (0.02%w/w) in DDW (1ml). DNA loading was estimated by assaying the DNA concentration of the supernatant following centrifugation (3500rpm, 6min) (section 2.5.3). Cumulative DNA release was estimated by incubation of DNA-loaded microparticles in PBS at 37 °C for 14 days. At intervals samples were centrifuged (6 min, 3500rpm) and the supernatant assayed for DNA concentration by spectrophotometry or the PicoGreen assay (method 2.5.3)

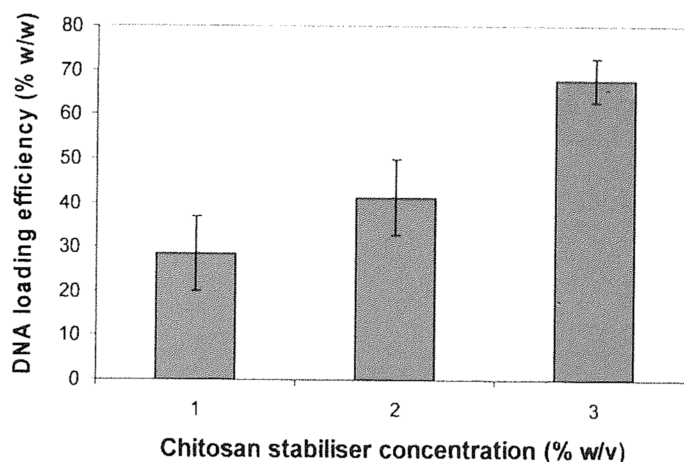


Figure 4.3.1 Effect of chitosan stabiliser concentration on DNA loading efficiency. PLG microparticles were formulated with varying concentrations of LMWC (method 2.2.4). Microparticles (20mg) were incubated with plasmid DNA (0.02% w/w) in DDW (1ml). Supernatant was collected following centrifugation (3500rpm, 6min). The concentration of DNA in the supernatant was measured (method 2.5.3) and loading was estimated by subtraction of this value from the DNA content of the incubation medium.

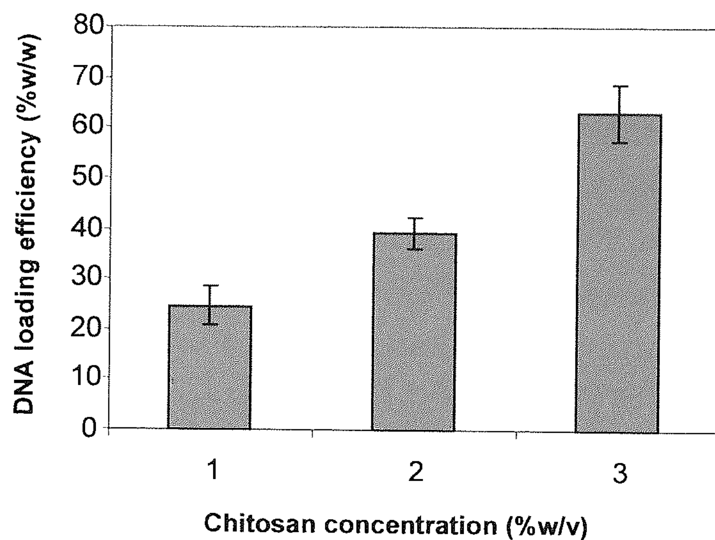


Figure 4.3.2 Confirmation of DNA loading efficiency by microparticle hydrolysis. Microparticle suspension 1ml (20mg/ml) was incubated overnight in 1M NaOH and 5% SDS (w/v) with shaking. Following break down of the microparticles, solutions were diluted in 1M NaOH and 5% SDS and the DNA concentration was measured by UV spectrophotometry at 260nm.

chitosan stabiliser concentration was increased further to 3% w/v then the loading efficiency increased dramatically to approximately 70%.

4.3.2 Confirmation of DNA loading levels by microparticle hydrolysis

In order to confirm that the DNA-loading efficiencies determined by supernatant analysis after DNA adsorption were accurate, microparticles were hydrolysed (1M NaOH and 5% SDS) and DNA loading was measured by UV sectrophotometry at an absorbance of 260nm. The effect of LMWC concentration on the percentage of loaded DNA is shown in figure 4.3.2. As expected, the loading of DNA increased as the stabiliser concentration increased in line with the above findings (section 4.3.1). At a 1% w/v stabiliser concentration, DNA loading was approximately 25%. At a 2 % w/v stabiliser concentration approximately 40% of DNA was loaded, and at a 3% w/v stabiliser concentration loading increased to approximately 65%. These values correlate well with the loading efficiencies determined by DNA analysis of the supernatant following DNA adsorption (fig 4.3.1).

4.3.3 The effect of low molecular weight chitosan (LMWC) concentration (w/v) on DNA adsorption.

Microparticles were incubated in solutions of salmon testes DNA in DDW of varying concentrations, from 0.01% - 0.15% w/v. The effect of LMWC stabiliser concentration (1% and 3% w/v) on DNA loading is also evaluated in figure 4.3.3. Figure 4.3.3A shows the concentration of DNA (μg/mg) adsorbed to the microparticles following incubation in various concentrations of DNA solution (% w/v). For both formulations an increase in the DNA concentration of the incubation medium led to an increase in the amount of adsorbed DNA. At all DNA solution concentrations investigated, more DNA was adsorbed to 3% LMWC stabilised microparticles than to 1% LMWC stabilised microparticles although in most cases this difference was not significant. However, when microparticles were incubated in a 0.075% w/v DNA solution, 14μg/mg of DNA was adsorbed to 3% w/v LMWC stabilised particles whereas only 4.9μg/mg was adsorbed to

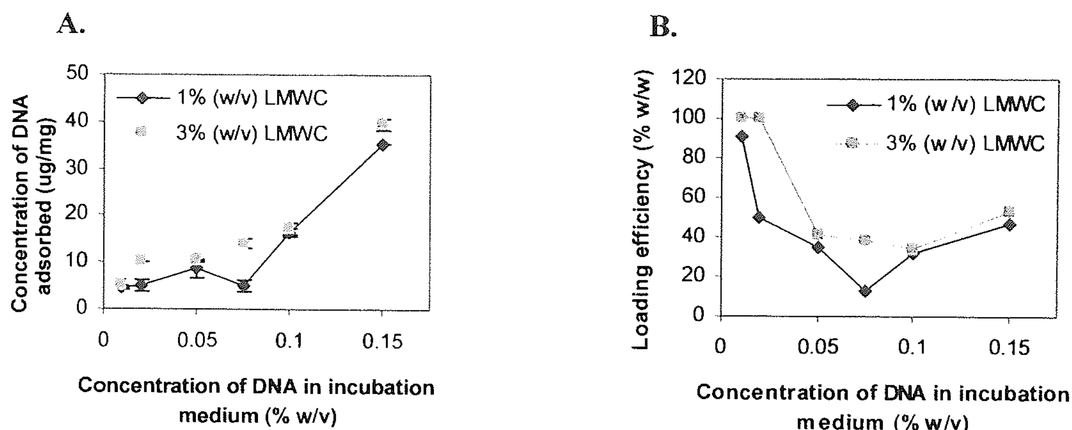


Figure 4.3.3 Effect of microparticle stabiliser concentration (w/v), and concentration of DNA in the incubation medium (w/v) on DNA adsorption. PLG microparticles were formulated with either 1% w/v LMWC or 3% w/v LMWC (method 2.2.4). Microparticles (20mg) were incubated in various concentrations of DNA solution (0.01 % w/v – 0.15% w/v) in 1ml DDW. Supernatant was collected following centrifugation (3500rpm, 6min). The concentration of DNA in the supernatant was measured by spectrophotometry or the Picogreen assay (method 2.5.3) and loading was estimated by subtraction of this value from the DNA content of the incubation medium. (A) Concentration of DNA (μg/mg) loaded on the microparticles. (B) Efficiency with which DNA was loaded on the microparticles (% w/w).

Stabiliser concentration (% w/v)	DNA loading Level (% w/w)	Size (μm)		Zeta potential (mV)	
		Blank	DNA-loaded	Blank	DNA-loaded
1	0.29 ± 0.08	3.85 ± 0.28	15.89 ± 1.10	+ 26.36 ± 1.13	− 42.73 ± 1.97
3	0.68 ± 0.05	3.02 ± 0.28	6.64 ± 6.32	+ 48.61 ± 1.85	+ 42.38 ± 3.03

Table 4.3.4 Effect of DNA loading on microparticle size and surface charge. Microparticles (20mg) stabilised with 1% LMWC or 3% LMWC were incubated with plasmid DNA (0.02 %w/w) overnight in 1ml DDW. Following incubation the supernatant was collected by centrifugation (3500rpm, 6min) and DNA loading was estimated by assaying the DNA concentration of the supernatant (method 2.5.3).The size distribution of microparticles was measured by laser light scattering. Microparticles were suspended in DDW. Particle sizes are expressed as the volumetric mean diameter (μm). Zeta potential measurements were carried out in DDW using a zeta potential analyser. Each sample was analysed 10 times and the zeta potential is expressed as the mean ± s.e.

1% w/v LMWC stabilised particles. At the highest concentration of DNA in the incubation medium (1.5% w/v) approximately 40µg/mg of DNA was adsorbed to 3% w/v LMWC microparticles and approximately 35µg/mg was adsorbed to 1% w/v LMWC microparticles. Results indicate a high binding affinity of DNA for the microparticles and in this study the maximum concentration of DNA that could be adsorbed to the particles was not reached.

Figure 4.3.3B shows the efficiency with which DNA was loaded on the microparticles (%w/w). At all concentrations of DNA in the incubation medium 3% w/v stabilised microparticles exhibited higher DNA loading efficiencies than 1% w/v microparticles. This difference was most significant when microparticles were incubated with a 0.02% w/v DNA solution. Microparticles stabilised with 3% w/v LMWC exhibited nearly 100% loading efficiency, whereas the loading efficiency of 1% w/v stabilised microparticles was approximately 50%. As the concentration of the incubation medium increased (≥ 0.05% w/v), the difference in loading efficiencies between the two formulations became less pronounced except when the concentration of the incubation medium was 0.075% w/v at which point the DNA loading efficiency of 3% w/v stabilised microparticles was approximately 40% and the loading efficiency of microparticles stabilised with 1% w/v LMWC was approximately 12%. Initially, an increase in DNA concentration in the incubation medium (%w/v) led to a rapid decrease in loading efficiency for both formulations. However, at the higher concentrations of DNA in the adsorption medium (≥ 0.075 % w/v for 1% w/v stabilised microparticles and ≥ 0.1% w/v for 3% w/v stabilised microparticles) DNA loading efficiency began to increase. This may indicate that the DNA loading efficiency is reaching a plateau level or, alternatively, at higher concentrations complexes may form between DNA molecules resulting in more efficient plasmid adsorption.

4.3.4 The effect of DNA loading on microparticle size and zeta potential

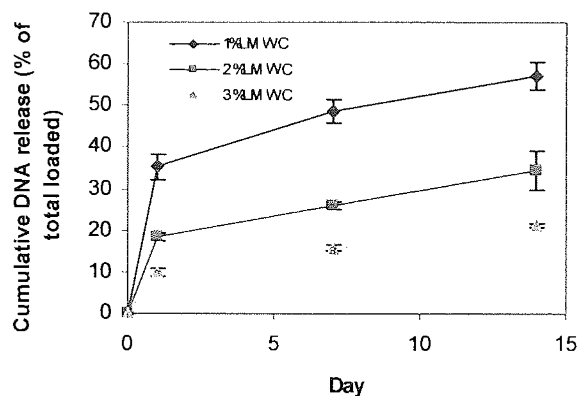
The effect of DNA adsorption on the size and zeta potential of PLG/LMWC microparticles is shown in table 4.3.4. When DNA was adsorbed to microparticles

stabilised with 1% w/v LMWC the size of the microparticles increased from 3.85μm to 15.89μm indicating aggregation. By comparison, although an increase in size from 3.02 μm to 6.64μm occurred when DNA was adsorbed to microparticles stabilised with 3% w/v LMWC the increase was not as pronounced. The zeta potential of 1% w/v LMWC stabilised microparticles following DNA adsorption decreased from +26.36mV to −42.73 mV. This suggests that DNA binding led to masking of the positive surface charge of the microparticles. In comparison only a slight reduction in zeta potential from +48.61mV to +42.38mV occurred when DNA was adsorbed to the surface of 3% w/v LMWC stabilised microparticles indicating that the stabiliser charge dominates the overall surface charge of the carrier/DNA complex.

4.3.5 The effect of stabiliser concentration on DNA release profiles.

Microparticles were incubated at 37°C in PBS over 14 days to measure the release of DNA. The effect of LMWC on the percentage cumulative release of DNA over 14 days is shown in figure 4.3.5A. As the concentration of stabiliser increased, the cumulative release of DNA from the microparticles decreased. Microparticles stabilised with 1% w/v LMWC initially showed rapid release of adsorbed DNA with approximately 35% released by day 1. Subsequently, the rate was slower but by day 14 approximately 55% of the adsorbed DNA had been released. Microparticles stabilised with 2% w/v LMWC released approximately 20% of adsorbed DNA by day 1 and about 35% by day 14. Microparticles stabilised with 3% w/v LMWC released the lowest percentage of adsorbed DNA. At day 1 approximately 10% of the total adsorbed DNA had been released and by day 14 this level had only risen to approximately 15%. Figure 4.3.5B shows the cumulative concentration of DNA (μg/mg) released from the microparticles over 14 days. For all formulations the greatest quantity of DNA was released by day 1 followed by more gradual release of DNA over the 14 days. At day 1 the concentration of DNA released from1%, 2% and 3% w/v LMWC stabilised microparticles was 1.16, 0.98 and 0.65 μg/mg respectively. The higher the concentration of stabiliser used in the formulation, the less DNA was released over 14 days. By day 14 microparticles stabilised with 1% w/v LMWC had released 1.87μg DNA/mg, microparticles stabilised with 2

A.



B.

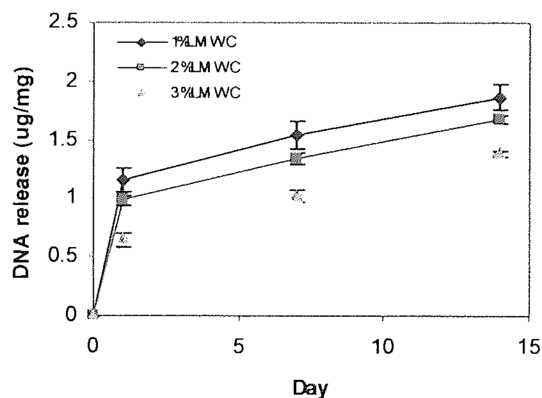


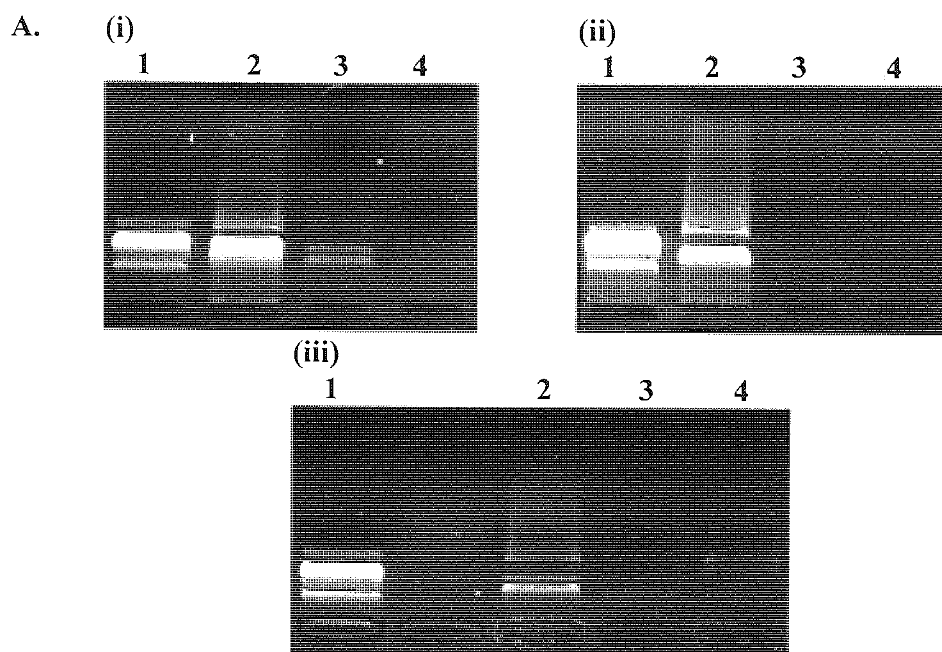
Figure 4.3.5 Effect of stabiliser concentration on DNA release profiles. DNA-loaded microparticles (20mg) stabilised with 1%, 2% or 3% w/v LMWC were incubated over 14 days in 1ml phosphate buffered saline (PBS) at 37 °C. At intervals samples were centrifuged (6 min, 3500rpm) and the supernatant assayed for DNA concentration (method 2.5.3).The pellet was resuspended in fresh PBS. (A) Cumulative DNA release (% of total adsorbed). (B) Cumulative concentration of DNA released (μg DNA/mg of microparticles).

% w/v LMWC had released 1.67μg of DNA/mg and microparticles stabilised with 3% w/v LMWC had released 1.39μg of DNA /mg.

4.3.6 Confirmation that DNA released from chitosan-stabilised microparticles was biologically active.

Luciferase plasmid DNA released *in vitro* from the surface of PLG/LMWC microparticles at various time points was evaluated for integrity on a 1% agarose gel. Figure 4.3.6A shows DNA released from (i) 1% w/v LMWC stabilised microparticles (ii) 2% w/v LMWC stabilised microparticles and (iii) 3% w/v LMWC stabilised microparticles. In each case unformulated DNA was run in lane 1 and release samples from days 1, 7 and 14 were run in lanes 2, 3 and 4 respectively. For all three formulations DNA in samples released at each time point was comparable to unformulated DNA (lane 1). However, some degradation of the DNA did occur during the 14 day release study. In lanes containing release samples (lanes 2-4), smearing was observed. This suggests that degraded DNA was present because it did not run as a distinct band. Despite DNA degradation occurring in samples collected as early as day 1, for each formulation luciferase plasmid DNA in a supercoiled conformation (fastest running band) was still observed in day 14 samples (lane 4).

To confirm that the DNA released from PLG/LMWC microparticles was intact and able to be expressed in cells, *in vitro* gene expression studies were performed with luciferase plasmid released from PLG/LMWC microparticles at days 1, 7 and 14. DNA was complexed with the transfection reagent FuGENE 6 (F6) and transfected 293T kidney cells were assayed for luciferase activity by measuring relative light units (RLU). Because the quantity of DNA released from the particles was different at each time point RLU values were corrected for the concentration of DNA in the sample. As a control, cells were transfected with the pGEM plasmid (10μg/ml) which did not contain the luciferase gene. Figure 4.3.6B shows the bioactivity of DNA released from 1% w/v stabilised microparticles and 3% w/v stabilised microparticles at days 1, 7 and 14. It was



B

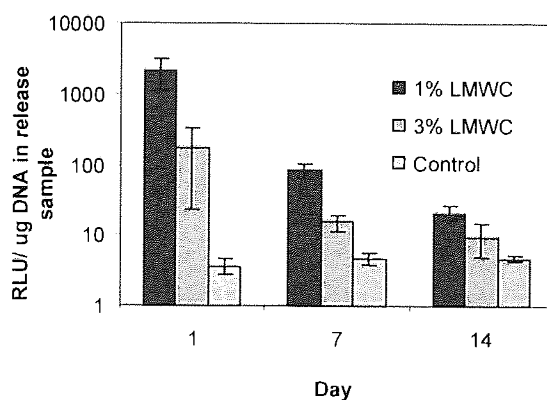


Figure 4.3.6 Integrity and bioactivity of luciferase plasmid DNA released from PLG/LMWC microparticles. Microparticles (20mg) loaded with luciferase plasmid DNA were incubated over 14 days in 1ml phosphate buffered saline (PBS) at 37 °C. At intervals samples were centrifuged (6min, 3500rpm) and the supernatant removed. The concentration of DNA in the release samples was assayed by UV spectrophotometry or the Picogreen assay (method 2.5.3).(A) To determine whether DNA released from the microparticles retained its integrity, release samples were run on a 1% agarose gel and compared to intact DNA. (B) To determine whether released DNA was biologically active, DNA in release sample (500μl) was complexed with the transfection reagent FuGENE 6 and added to 293T cells. Cells were incubated overnight. Following incubation, cells were lysed and luciferase production was assessed by the luciferase assay (method 2.11.2). RLU values were corrected for the concentration of DNA in the samples. 293T cells were also transfected with 500μl of control plasmid pGEM solution (10μg/ml) which did not contain the luciferase gene.

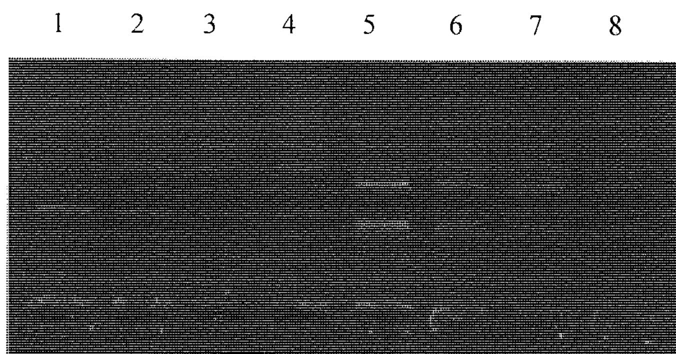
apparent that luciferase plasmid DNA released from 1% w/v and 3% w/v stabilised microparticles over 14 days was biologically active and that protein could be expressed in 293T cells as indicated by the enhanced RLU values when compared to control values. Interestingly, it appeared that DNA released over 14 days from microparticles stabilised with 1% LMWC (w/v) was more biologically active than DNA released from 3% LMWC (w/v) stabilised microparticles. This could indicate that higher concentrations of chitosan in the formulation damage the bioactivity of the DNA or, that at higher stabiliser concentrations more DNA is released in complex with chitosan and this may sterically hinder the interaction of the DNA with the transfection reagent F6.

4.3.7 Protection of adsorbed DNA from degradation by DNaseI *in vitro*

Plasmid DNA is easily degraded by endonucleases such as DNase I, which is one of the obstacles for the delivery of plasmid DNA *in vitro* or *in vivo*. Therefore, the stability of DNA in the presence of DNase I is one of the essential parameters for successful gene delivery. Figure 4.3.7A shows the results of a DNase I protection assay carried out to confirm that PLG/LMWC microparticles protected plasmid DNA from nucleases. When naked plasmid DNA was incubated with DNase I (5 units) gradual degradation of the plasmid occurred over 60 min (4.3.7A, lanes 1-4). By comparison, DNA adsorbed to the surface of PLG/LMWC microparticles was partially protected from enzyme degradation over the 60min incubation period (4.3.7A, lanes 5-8). This protection was particularly evident at the later time points of 40 min (lane 7) and 60 min (lane 8). If DNA in these lanes was compared to naked DNA at the equivalent time points (lanes 3 and 4 respectively) much clearer bands were observed in the lanes containing DNA from PLG/LMWC microparticles.

Figure 4.3.7B shows the results of a second DNase I protection assay based on the fact that as plasmid DNA is degraded its absorbance at 260nm increases. Microparticle/DNA

A.



Time (min) 0 20 40 60 0 20 40 60

B.

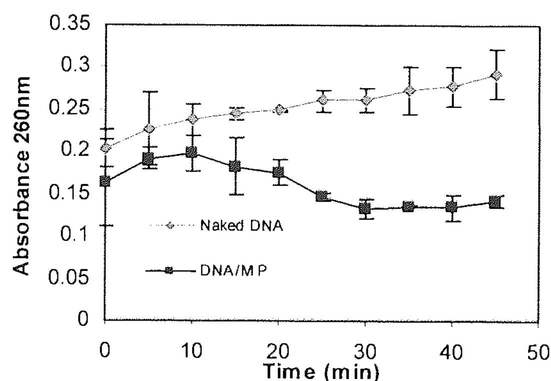


Figure 4.3.7 Protection of adsorbed DNA from DNase I degradation *in vitro*. (A). Naked DNA or DNA/microparticle complexes were incubated with DNase I (5 units) at room temperature. Samples were taken at 0, 20, 40 and 60 minutes post-incubation and mixed with stop solution (200mM sodium chloride, 20mM EDTA, and 1% SDS) followed by incubation overnight at 60°C to dissociate plasmid DNA from the microparticles. DNA was extracted by phenol/chloroform extraction and samples run on a 1% agarose gel. Lanes 1-4; naked DNA. Lanes 5-8; DNA from microparticle complex. (B). DNase I (20 units) was spiked into 1ml of the DNA/ microparticle mixture and the absorbance at 260nm was monitored at 37°C over 45min. Assays on the naked plasmid and blank chitosan-stabilised microparticles were conducted in the same manner and absorbance values for the blank particles were subtracted from the values for the DNA/microparticle mixture to account for any absorbance due to the microparticles alone at 260nm.

complexes or naked DNA were spiked with DNase I (20 units) and incubated at 37°C for 45 min. Absorbance values for blank PLG/LMWC microparticles were subtracted from the values for the DNA/microparticle mixture to account for any absorbance due to the microparticles alone at 260nm. Over the first 10 minutes an initial increase in absorbance was observed for both naked DNA and DNA complexed with PLG/LMWC microparticles. Subsequently the absorbance of the naked plasmid DNA continued to increase throughout the sampling time suggesting degradation of the DNA by the DNaseI. By comparison, the absorbance of the samples containing complexed DNA began to drop after 10mins and levelled off at 30min suggesting that the microparticles afforded the DNA some protection from DNase I. The initial increase in absorbance of complexed DNA observed in the first 10mins may have occurred as a result of free DNA in the sample being degraded.

4.3.8 Analysis of the integrity of DNA from LMWC/PLG microparticles following the addition of excipients and lyophilisation.

Commercially it is essential that DNA vaccine formulations can be stored long term without loss of stability. This is best achieved if the preparation can be lyophilised, but unless excipients are added prior to lyophilisation damage may occur to the DNA (Ando *et al,* 1999). Figure 4.3.8 shows the effects of the addition of different excipients on the integrity of DNA released from PLG/LMWC microparticles following lyophilisation. In chapter 3 (section 3.4.7), it was demonstrated that PVA 9000 MW (0.5% w/v final concentration) was the best excipient for preventing microparticle aggregation upon reconstitution in DDW. However, the DNA from lyophilised PLG/LMWC microparticles following the addition of PVA 9,000 MW appeared to undergo some degradation as indicated by smearing of the bands on elecrophoresis (lane 2). Degradation of DNA was prevented if mannitol (4.5% w/v final concentration) and sucrose (1.5% w/v final concentration) were added in conjunction with PVA 9000 MW (lane 3) and DNA remained in a predominantly supercoiled conformation (fastest running band). The addition of the sugars as excipients without PVA was also found to prevent DNA degradation during lyophilisation of PLG/LMWC microparticles with adsorbed DNA

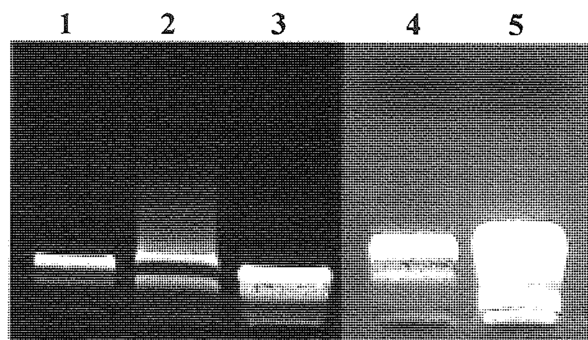


Figure 4.3.8 Analysis of DNA integrity released from PLG/LMWC microparticles following the addition of excipients prior to lyophilisation. Excipients were added to DNA-adsorbed microparticle suspensions prior to lyophilisation. Microparticles (20mg/ml) were aliquoted out in 1ml volumes. Mannitol (4.5% w/v final concentration) and sucrose (1.5% w/v final concentration) were added to some of the aliquots whereas PVA 9,000 MW (0.5% w/v final concentration) was added to others. Samples were frozen at −80°C followed by lyophilisation overnight. Microparticles were reconstituted in DDW, centrifuged (6min, 3500rpm) and a sample of the supernatant was run on a 1% agarose gel. Lanes 1 and 4; Unformulated luciferase plasmid DNA. Lane 2; DNA from microparticles with the excipient PVA added. Lane 3; DNA from microparticles with the excipients mannitol, sucrose and PVA added. Lane 5; DNA from microparticles with the excipients sucrose and mannitol added.

Formulation	Excipients (concentration in final volume)	Size (μm) following lyophilisation	Reduction in DNA integrity following lyophilisation
PLG/1%LMWC	PVA 9000 MW (0.5% w/v)	11.74 ± 0.23	YES
PLG/1%LMWC	PVA 9000 MW (0.5%w/v), mannitol (4.5% w/v) + sucrose (1.5% w/v)	16.06 ± 0.48	NO
PLG/1%LMWC	Mannitol (4.5% w/v) + sucrose (1.5% w/v)	16.44 ± 1.26	NO

Table 4.3.8 Analysis of PLG/LMWC microparticle size and the integrity of released DNA following the addition of excipients prior to lyophilisation. Excipients were added to microparticle suspensions prior to lyophilisation. Samples were frozen at −80°C for 30 min followed by lyophilisation. Microparticles were reconstituted in sterile DDW (20mg/ml). The size distribution of microparticles was measured by laser light scattering. Particle sizes are expressed as the volumetric mean diameter (μm). DNA integrity was assessed by running a sample of supernatant on a 1% agarose gel following centrifugation of reconstituted microparticles.

(lane 5). Although the band was overexposed due to the high concentration of DNA, no smearing was observed in this lane. Table 4.3.8 gives a summary of PLG/LMWC size and the integrity of released DNA following the addition of excipients prior to lyophilisation.

4.4 Discussion

PLG microparticles stabilised with PVP-PDAEMA (PLG/PVP-PDAEMA) and PLG microparticles stabilised with LMWC (PLG/LMWC) in particular exhibited promising physicochemical characteristics (particle size and surface charge) for delivery of adsorbed DNA vaccines. However, particle interaction with plasmid DNA and loading and *in vitro* release characteristics of five of the original formulations were investigated. PLG microparticles stabilised with PVA (PLG/PVA) were selected as a negative control because of their negative surface charge and PLG/CTAB particles were selected as a positive control because efficient DNA loading (approximately 92%) and release from these cationic particles had previously been reported (approximately 75% of adsorbed DNA released by day 14 at 37°C in PBS) (Singh *et al,* 2000). Chitosan microparticles were selected to investigate the interaction between a 'solid' chitosan substrate and DNA. PLG/MMWC microparticles were investigated in addition to PLG/LMWC microparticles to establish if the molecular weight of the chitosan stabiliser had an effect on DNA loading and release characteristics. Finally, although other surface modified particles were smaller, PLG microparticles surface modified with MMWC were chosen out of this group because the resorption rate of PLG is faster than PLA. Table 4.2.1 gives a summary of the characteristics of the chosen formulations.

When the various microparticle formulations were incubated with DNA, a broad range of loading efficiencies was observed (table 4.2.1). However, the majority of the formulations adsorbed DNA with about 40% efficiency. Surprisingly, PLG/PVA particles were also capable of adsorbing DNA with reasonable efficiency (43%). Lower loading efficiencies were expected because it was presumed that the negatively charged surface of the particle would repel the negatively charged phosphate groups on the DNA.

Interestingly, previous reports have shown that DNA can adsorb to negatively charged blank PLGA (27.4% efficiency) and PLA (36.9% efficiency) microparticles (Walter *et al,* 2002). This suggests that DNA may be capable of adsorbing to the core polymer independently of electrostatic interactions. Similar behaviour has commonly been recorded for albumin adsorption to microparticles where high levels of adsorption occur despite unfavourable electrostatic conditions (Norde & Lyklema, 1991; Al-Shakhshir *et al,* 1995; Rinella *et al,* 1996) suggesting the involvement of hydrophobic or van der Waals interactions. In spite of reasonable adsorption efficiencies however, lower transfection efficiencies were reported by Walter *et al.* in cells treated with anionic as opposed to cationic particles (Walter *et al,* 2002). The lowest DNA loading efficiencies in the present study were observed for PLG/CTAB microparticles (28%). These values were far lower than values reported by Singh *et al* (92% efficiency) (Singh *et al,* 2000). In addition, the mean size of the PLG/CTAB microparticles described by Singh's group (1.54 ± 1.13μm) were much smaller than the PLG/CTAB particles in the present work (19.47 ± 1.13μm) suggesting that the formulations were distinct. Chitosan microparticles adsorbed DNA with the greatest efficiency (approx 99%). It is generally accepted that chitosan binds DNA electrostatically (Mao *et al,* 1996). Efficient DNA loading on chitosan particles was therefore expected as the glocosamine backbone of chitosan has a high density of amino groups which, if protonated, would bind the negatively charged phosphate groups on the DNA. PLG particles surface modified by adsorption of MMWC were the second most efficient formulation for DNA adsorption (78% efficiency) after chitosan (99%). The high DNA loading efficiency of this formulation can be attributed to electrostatic interaction between DNA and chitosan bound to the PLG core particle.

Release of DNA from a number of the formulations (PLG/CTAB, PLG/PVA, PLG/PVP-PDAEMA, PLG/LMWC) was characterized by an initial burst over 24h followed by gradual release of more tightly bound DNA as time progressed (fig 4.2.2). If the *in vitro* release profiles of these formulations were mimicked *in vivo*, released DNA would be rapidly available to interact with target cells and stimulate the immune response, although the amount would depend on the nature of the carrier particle. However, the DNA release profile of a formulation *in vivo* could seriously be altered by factors such as serum

components in the bloodstream, or low pH and degradative enzymes if the particles are taken up by endocytosis. The release profiles of PLG particles surface modified with MMWC and PLG/MMWC microparticles followed a slightly different pattern. Initial release of plasmid by day 1 was low (5.9% and 6.8% respectively), although gradual release of DNA occurred throughout the two week time course. The molecular weight of a polymer is known to influence polymer-DNA complexation (Boussif *et al,* 1995; Kabanov *et al,* 1995; Richardson *et al,* 1999) and the results presented here suggest that the higher molecular chitosan may initially bind DNA more tightly than LMWC. The strong binding of DNA to chitosan microparticles was confirmed by the 14 day release study (fig 4.2.2). Nearly all the adsorbed DNA remained bound to the particles over 14 days supporting the theory that tight DNA/chitosan complexes are formed through hydrogen bonding and ionic interactions (Hayatsu *et al,* 1997, Richardson *et al,* 1999).

Taking into consideration the physicochemical data and DNA loading and release profiles, PLG/LMWC microparticles were chosen as the focus for further research. Chitosan microparticles were rejected because they were too large, and although DNA loading efficiency was very high (>99%), DNA release from the particles was negligible. Although the DNA release profile for optimal immune stimulation is unknown it is speculated here that significant DNA release may be important. Because DNA must enter the cell nucleus and be translated for protein expression to occur, if the carrier does not release DNA the nucleic acid may be prevented from entering the nucleus or if nuclear entry does occur and DNA is still complexed to carrier translation may be impeded. PLG/PVP-PDAEMA microparticles exhibited similar physicochemical and DNA loading and release characteristics to PLG/LMWC microparticles but were not investigated further because of the limited data available on the copolymer PVP-PDAEMA. PLG microparticles surface modified with MMWC exhibited high DNA loading efficiency but were not chosen because initial DNA release was low and this may diminish the magnitude of the early response to immunisation. High initial *in vitro* release of adsorbed DNA (35%) from PLG/CTAB microparticles by 24h was reported by Singh *et al* and these particles were found to significantly enhance immune responses in mice suggesting that initial DNA release may be an important factor in immune stimulation (Singh *et al,*

2000). In addition in the present study PLG microparticles surface modified with MMWC were >5μm so would not be efficient at targeting APCs. PLG/MMWC microparticles were rejected in favour of PLG/LMWC microparticles because MMWC stabilised particles were larger (>5μm) and initial DNA release was low. The use of LMWC also provided more control over particle size by variation of the concentration of stabiliser solution. Additionally, PLG/LMWC microparticles were favoured because there is evidence that DNA complexed with lower molecular weight chitosans shows enhanced transfection efficiency relative to DNA complexed with higher molecular weight chitosans. Sato *et al* demonstrated that the transfection efficiency in A549, B16 melanoma and HeLa cells treated with chitosans > 100kDa was lower than in cells treated with 15kDa or 52kDa chitosans (Sato *et al,* 2001; Ishii *et al,* 2001).

Further investigation into the DNA loading efficiency of PLG/LMWC microparticles indicated that the mean loading efficiency of particles stabilised with 1% LMWC was approximately 30% (fig 4.3.1). This value was relatively low in comparison with the mean loading efficiencies previously reported by other groups for DNA adsorption to the surface of PLG/CTAB (92%), PLG/PEI (72- 99%) and PLG/ 50%DEAEM (52%) microparticles (Singh *et al,* 2000; Walter & Merkle, 2002). However, direct comparison with other systems is very difficult as adsorption conditions vary. The type of DNA used and the concentration of DNA in the incubation medium (fig 4.3.3) can have a profound effect on DNA loading efficiency. The low DNA loading efficiency of PLG/1% LMWC microparticles was more comparable to the DNA encapsulation efficiencies (20-50%) achieved when PLG microparticles were produced using the double emulsion, solvent evaporation process (Jones *et al,* 1997; Capan *et al,* 1999; Tinsley-Brown *et al,* 2000). In the present study only by increasing the concentration of chitosan stabiliser in the formulation was it possible to increase the DNA loading efficiency above levels usually achieved by DNA encapsulation (fig 4.3.1). At the highest chitosan stabiliser concentration (3%w/v) the DNA loading of PLG/LMWC microparticles was relatively efficient (70%) when adsorbed from low DNA concentration solutions. These results support earlier findings that increasing stabiliser concentration leads to an increase in chitosan incorporation in the microparticle (fig 3.4.5). More LMWC would be expected

to be present at the particle surface leading to an increase in DNA loading efficiency. The DNA loading efficiencies of PLG/LMWC particles were confirmed by hydrolysis of the microparticles (fig. 4.3.2).

DNA adsorption on PLG/LMWC microparticles was further investigated by altering the concentration of DNA in the incubation medium (fig 4.3.3). As expected, as the particles were incubated with increasing concentrations of DNA, the quantity adsorbed to the particle surface increased, although the maximum concentration of DNA that could be adsorbed to the particles was not reached (fig 4.3.3A). In addition, as the DNA concentration of the incubation medium increased, loading efficiency decreased (fig 4.3.3B). Efficient DNA loading is an important economic consideration. Reducing the concentration of DNA required in the incubation medium and reducing wastage of DNA by efficient adsorption would ultimately lead to a reduction in production costs. From the performance aspect, if the microparticles can adsorb high loads of DNA this will potentially allow more DNA to be delivered per unit weight of microparticle carrier, thus reducing the quantity of non-active required to elicit the desired immune response in the recipient. Increasing the stabiliser concentration in PLG/LMWC microparticle formulations resulted in higher DNA loading efficiencies at all DNA concentration levels in the adsorption medium. Interestingly, the loading efficiencies of particles incubated with single stranded salmon testes DNA (0.02% w/v) (fig 4.3.3) were quite different to the loading efficiencies observed in particles incubated with luciferase plasmid DNA (0.02% w/v) (fig. 4.3.1). Incubation with salmon testes DNA resulted in higher DNA loading efficiencies for both 1% w/v LMWC and 3% w/v LMWC stabilised particles (50% and 100% salmon testes DNA v 30% and 70% luciferase plasmid DNA respectively) suggesting that single stranded DNA adsorbs to the particles more efficiently than plasmid DNA. These findings provide further evidence that the DNA loading efficiencies of microparticles are influenced by the type of plasmid or the type of DNA used. Briones *et al* reported that p55gag plasmid adsorbed to PLG/CTAB particles with 96% efficiency whereas the adsorption efficiency of gp140 plasmid was only 74% (Briones *et al,* 2001).

Adsorption of luciferase plasmid DNA to the surface of microparticles led to an increase in their size (table 4.3.4). This increase was most notable in microparticles stabilised with 1% LMWC where the mean diameter of the particles increased from approximately 4μm to over 15μm. In addition, these particles became negatively charged suggesting that adsorption of DNA led to masking of the particles cationic surface charge. The resulting increase in size upon DNA adsorption indicates that particle aggregation is occurring which may inhibit uptake of PLG/1%LMWC particles by professional APCs. This effect may be compounded by the negative surface charge of the particles which could repel negatively charged cell surface components, thus inhibiting interaction between the two. When DNA was adsorbed to microparticles stabilised with 3% w/v LMWC the particles had a mean diameter of less than 10μm and they retained a net positive surface charge. As previously mentioned, increased chitosan incorporation at the 3% w/v stabiliser concentration (fig 3.4.5) probably results in more chitosan localised at the particle surface. If the number of positively charged amine groups on the chitosan coating out number the negatively charged phosphate groups on the adsorbed DNA this could explain why very little reduction in zeta potential was observed when DNA was adsorbed to PLG/3% LMWC microparticles. These particles therefore have greater potential for interaction with and uptake by professional APCs. However, due to their net positive surface charge these particles may also be more likely to interact with negatively charged serum proteins in body fluids.

One factor which may contribute to the potency of a DNA vaccine is prolonged release of active DNA from the microparticle resulting in longer-duration transfection of cells. However, a number of DNA formulations previously shown to be active *in vivo* have exhibited rapid release of a significant fraction of DNA between 1-24h under *in vitro* conditions (Singh *et al,* 2000; Walter *et al,* 2002). In addition, immediate release of a core coated tetanus toxoid from PLGA microspheres *in vitro* was shown to elicit a long lasting immunogenic response (Tobio *et al,* 2000). The findings of Singh *et al* suggest that fast initial release of DNA may be an important factor in immune stimulation and could lead to a prolonged response, although it is possible that immunity occurs as a result of retained DNA. If initial release is important, then the release profile of microencapsulated

DNA, characterised by small quantities of DNA released over long periods, may not be ideal for DNA vaccination (Walter *et al,* 1999). In comparison, in the present study, the release of DNA from PLG/LMWC microparticles was characterised by an initial burst within the first 24h followed by more gradual release over the proceeding days. By varying incorporation of chitosan in the particles, formulations with different release characteristics could be produced (fig 4.3.5). Increasing chitosan concentration resulted in a decrease in the cumulative release of adsorbed DNA (fig 4.3.5A) and a decrease in the cumulative quantity of DNA released (fig 4.3.5B) from the particles. For vaccination the optimum DNA release profile is still unknown but could be investigated further using PLG/chitosan microparticle systems. Responses to burst release (PLG/LMWC) versus gradual release (PLG/MMWC) formulations could be directly compared (fig 4.2.2) to establish the optimum DNA release profile. Alternatively, by washing PLG/LMWC microparticles the burst effect could be removed and immune responses compared to unwashed PLG/LMWC formulations.

In addition to slow release of DNA, another problem associated with microencapslation is that the DNA is damaged during the encapsulation process and during release (Ando *et al,*1999; Walter *et al,* 1999; Tinsley-Brown *et al,* 2000). Primarily, released DNA is damaged as a result of degradation of the PLG/PLA polymers. This leads to the accumulation of oligomers that cause the environment within the microparticle to become acidic (Tobio & Alonso, 1998; Fu *et al,* 2000). In this acidic environment plasmid DNA is unstable (Berstrom *et al,* 1998). Adsorbing DNA to the surface of PLG/LMWC particles results in released DNA being protected from acidic degradation products of the core polymer. Previously, Singh *et al* demonstrated the release of intact DNA from the surface of PLG/CTAB microparticles over 14 days (Singh *et al,* 2000). In the present study although some degradation of plasmid released from PLG/LMWC microparticles was observed (fig4.3.6A) there was still supercoiled DNA present at day 14. The importance of supercoiled DNA for the efficient transfection of cells has been reported in many studies (Weintraub *et al,* 1986; Yamaizumi *et al,* 1983; Xu & Szoka, 1996; Middaugh *et al,* 1998). Confirmation that luciferase plasmid DNA released from PLG/LMWC particles retained its functional integrity was obtained by gene expression

assays in transfected 293T cells (fig 4.3.6B). Interestingly, DNA released from microparticles stabilised with 1% (w/v) LMWC was more biologically active than DNA released from particles stabilised with 3% (w/v) LMWC. It is possible that the higher concentration of chitosan in the formulation damages released DNA thus reducing gene expression. However, the integrity of DNA released from 1% and 3% stabilised microparticles as assessed by agarose gel electrophoresis (fig 4.3.8) was comparable. Alternatively, as a result of the higher incorporation of chitosan in PLG/3%LMWC microparticles (fig 3.4.5) a higher quantity of released DNA may be complexed with chitosan preventing efficient binding of DNA with the F6 transfection reagent, thus reducing the transfection efficiency.

One potential advantage of DNA microencapsulation over surface adsorbed delivery is that the DNA may be better protected within the cell from nuclease degradation (Jong *et al,* 1997; Mathiowitz *et al,* 1997; Smith *et al,* 1997). However, it appears that particulate carriers may also offer protection to surface adsorbed DNA. Singh *et al* reported enhanced resistance to DNase I of DNA adsorbed to the surface of PLG/CTAB particles (Singh *et al,* 2000). Similarly, in the present study, PLG/LMWC microparticles were shown to protect luciferase plasmid DNA from DNase I degradation *in vitro* (fig. 4.3.7). This protection may occur as a result of the formation of protective complexes of the DNA with chitosan on the surface of the particles. Richardson *et al* reported that complexation of DNA with highly purified chitosan fractions (MW <5000, 5000-10 000 and > 10000 Da) at a charge ratio of 1:1 resulted in almost complete inhibition of degradation by DNAase II (Richardson *et al,* 1999). It was suggested that this protection may be a result of a change in the tertiary DNA structure upon complexation which caused steric hindrance to the degradative enzyme. Chitosan-DNA nanoparticles have also been shown to protect DNA against DNase I degradation *in vitro* (Mao *et al,* 2001).

Damage to DNA occurring as a result of freezing and lyophilisation of microparticles for long term storage is a common problem (Ando *et al,* 1999). However, based on the knowledge that sacchrides were known cryoprotectants for proteins during lyophilisation (Carpenter *et al,* 1994; Suzuki *et al,* 1997) Ando *et al* developed a technique that

prevented buffer salt crystal formation and nicking of DNA during these processes. This was achieved by the addition of saccharides as excipients to formulations prior to lyophilisation. The results described in the present study (fig 4.3.7) support Andos findings. The addition of PVA as an excipient, although previously having been shown to prevent aggregation of PLG/LMWC particles upon reconstitution (table 3.4.7), did not protect DNA during lyophilisation. By comparison, the addition of the saccharides mannitol and sucrose resulted in DNA retaining a primarily supercoiled conformation following lyophilisation. Although cryoprotection of microparticles using sugars resulted in greater aggregation of microparticles following lyophilisation (>16μm) than if PVA 9000 MW was used (approx 11μm), protection of the DNA was deemed to be a priority (table 4.3.8). As a result lyophilised microparticles used in future studies were made with the addition of sucrose and mannitol prior to lyophilisation.

In summary, based on the initial physicochemical data and DNA loading and release studies, the interactions of PLG/LMWC microparticles with DNA were studied in detail in this chapter. By altering the concentration of stabiliser in the formulation it was possible to alter the efficiency of DNA loading on the particles and the DNA release profiles. The physicochemical characteristics of PLG/LMWC microparticles following DNA loading were also dependent on stabiliser concentration. PLG/LMWC particles protected adsorbed DNA from endonuclease degradation *in vitro* and DNA released from the particles was biologically active. Protection of DNA during freezing and lyophilisation of the particles was achieved by the addition of saccharide excipients.

5 Biological evaluation of PLG/LMWC microparticles as a vaccine delivery system

5.1 Introduction

In the preceding chapters the physicochemical properties and DNA loading and release characteristics of various microparticulate carriers were evaluated. In particular research focused on the potential of PLG microparticles stabilised with low molecular weight chitosan (PLG/LMWC) for the delivery of DNA vaccines. In this chapter the interaction of these particles with cells *in vitro* has been examined.

The cellular interaction and transfection mechanisms of non-viral microparticulate DNA carriers is an area which is poorly understood. Questions still remain as to whether particles cross the cell membrane and if so how, or whether particles interact locally with the cell surface leading to enhanced DNA uptake across the membrane (Luo and Saltzman, 2000a). Inside the cell DNA or particles may be taken up into endosomes that then fuse with lysosomes containing degradative enzymes. If this occurs then how does DNA escape from these cellular compartments into the cytoplasm, and how do large DNA molecules cross the nuclear membrane? Some groups have attempted to elucidate these pathways (Luo & Saltzman, 2000b; Ishii *et al,* 2001; Walter & Merkle, 2002) but evidence is inconclusive and it is probable that mechanisms of transfection differ between carriers. In the first part of this chapter the interaction with, and transfection efficiency of PLG/LMWC microparticles in non-phagocytic 293T mouse kidney epithelial and C_2C_{12} human myoblast cells was studied.

In the second part of this chapter the cellular toxicity of PLG/LMWC microparticles was investigated. Although *in vitro* cationic carriers for DNA are often effective at enhancing transfection levels in cells, high transfection efficiency is frequently associated with cytotoxicity. Examples of cytotoxic vectors include poly (L-lysine) PLL (Han *et al,* 2000), high molecular weight polyethyleneimine (PEI) (Godbey *et al,* 1999) and cationic

lipids (Li & Huang, 1997; Brown *et al,* 2001). Cytotoxicity can preclude the use of such agents *in vivo.* By comparison, chitosan/ DNA complexes have frequently been reported as exhibiting low cytotoxicity *in vitro* (Richardson *et al,* 1999; Lee *et al,* 2001; Gao *et al,* 2003).

5.2 Evidence of microparticle interaction with the cell surface

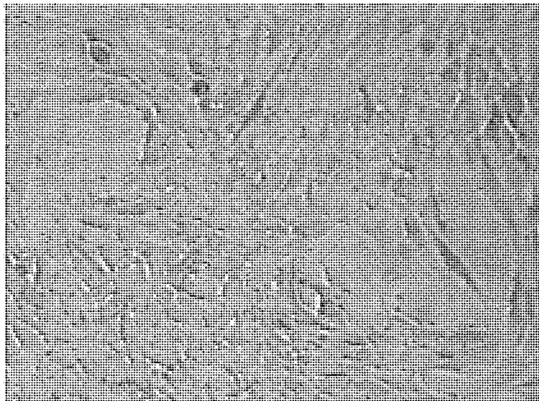
Figure 5.2B shows the interaction of C_2C_{12} myoblast cells treated with PLG/3%LMWC microparticles under the light microscope. The cells were incubated with the microparticles for 24h before observation. Figure 5.2A shows untreated myoblast cells after the same time period. For both 293T cells (results not shown) and myoblast cells the same phenomena was observed. The microparticles were attracted to the cell surface, probably due to electrostatic interactions, whereas in areas where there was no cell growth there were no microparticles. Another interesting visual observation was that cells treated with microparticles appeared to grow at a slower rate than treated cells. This led to speculation that the microparticles may be having a deleterious effect on cell growth.

5.3 *In vitro* luciferase gene expression in 293T epithelial cells and C_2C_{12} myoblast cells.

5.3.1 Optimisation of DNA concentration and plating procedure for luciferase gene expression in 293T cells

In order to asses whether PLG/LMWC microparticles could transfect 293T cells and to establish optimal transfection conditions, experiments on DNA dose and plating procedures were undertaken. Figure 5.3.1A shows luciferase expression in cells incubated with 2μg of free DNA or DNA adsorbed to PLG/LMWC particles, 24h after seeding. On all three days luciferase expression was considerably higher in cells treated with DNA-loaded PLG/LMWC microparticles than in cells treated with naked DNA. At maximum expression on day 2, levels were 70 fold higher than in cells treated with naked DNA ($p<0.01$). On day 1 the difference in expression was also statistically significant

A.



B.

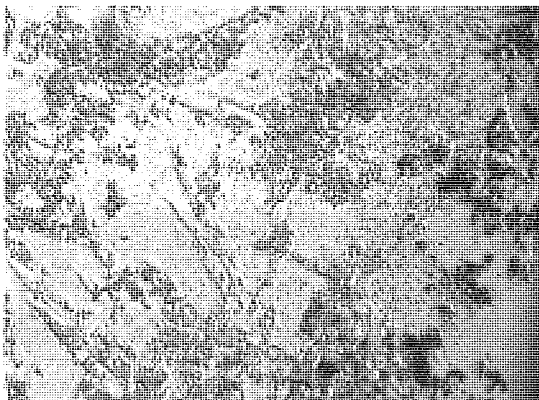
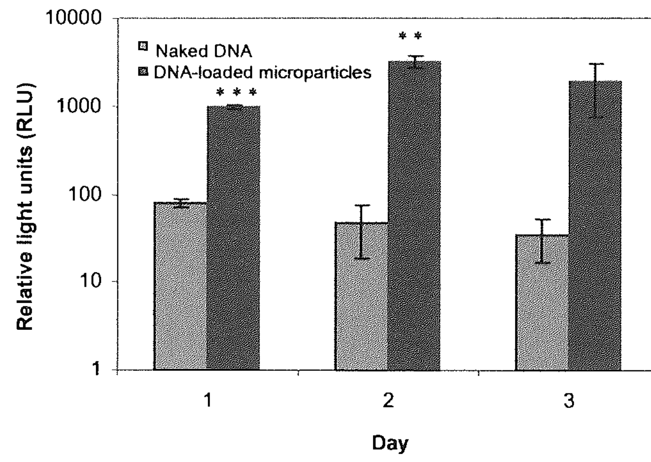


Figure 5.2 Evidence of microparticle interaction with the cell surface of C_2C_{12} myoblasts. C_2C_{12} myoblast cells were incubated at 37°C with DNA-loaded PLG3%/LMWC microparticles for 24h. Microparticles were viewed using a light microscope (× 200 magnification). (A) Control C_2C_{12} myoblast cells incubated without the addition of microparticles. (B). C_2C_{12} myoblast cells incubated with DNA-loaded PLG/3%LMWC microparticles.

A.



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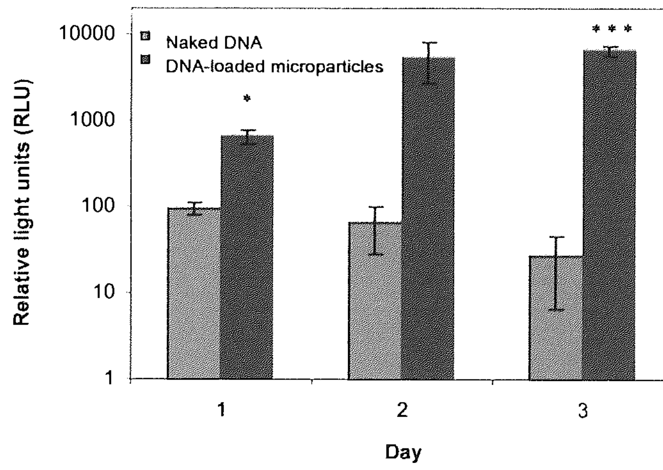

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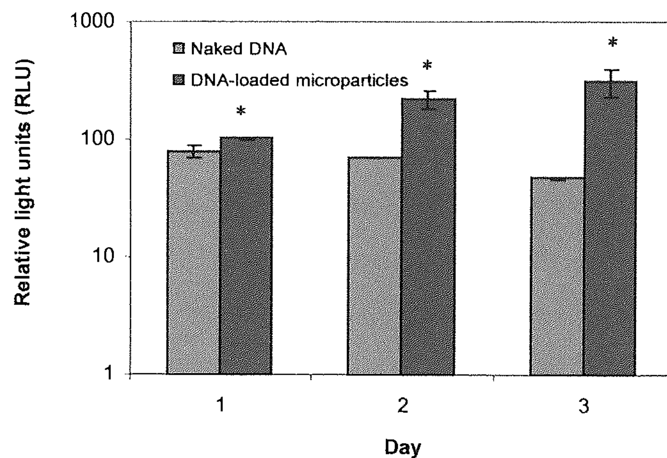


D.

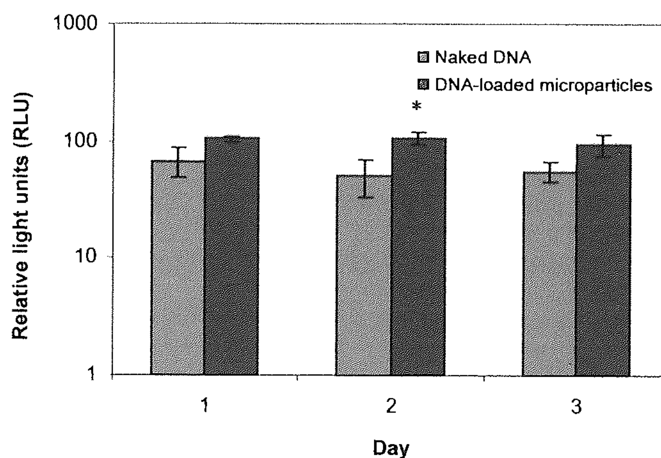


Figure 5.3.1 The effect of DNA concentration and plating procedure on luciferase expression (RLU) in 293T cells. Cells were incubated with luciferase plasmid DNA-loaded PLG/LMWC microparticles or naked luciferase plasmid DNA for 3 days. Each day medium was removed from one set of wells and the cells were washed with PBS (1ml). Cells were incubated with lysis reagent (100µl) for 15 minutes at room temperature. The cell lysates were assayed for luciferase expression (method 2.11.2). (A) Cells were seeded, and incubated for 24h before the addition of microparticles or naked DNA (2µg DNA/well). (B) Cells were seeded, and incubated for 24h before the addition of microparticles or naked DNA (10µg DNA/well). (C) Cells were mixed with microparticles or naked DNA prior to seeding (2µg DNA/well). (D) Cells were mixed with microparticles or naked DNA prior to seeding (10µg DNA/well). *Denotes p-values <0.05 **Denotes p-values <0.01 ***Denotes p-values <0.005 compared to naked DNA results.

($p<0.005$). Figure 5.3.1B shows luciferase expression in cells incubated with 10 μg of DNA, 24h after seeding. Once again on all three days, luciferase expression in cells treated with DNA-loaded PLG/LMWC microparticles was considerably higher than in cells treated with naked DNA. At maximum expression on day 3, levels were 250 fold higher than in cells treated with naked DNA ($p<0.005$). On day 1 the difference in expression was also significantly different ($p<0.05$).

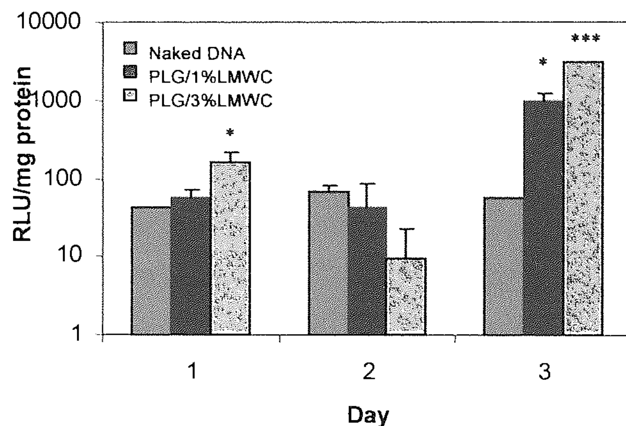
Figures 5.3.1C and 5.3.1D show cells incubated with 2μg and 10μg of DNA respectively. However, naked DNA or DNA-loaded microparticles were mixed with 293T cells prior to seeding. At the lower dose, cells treated with DNA-loaded PLG/LMWC microparticles exhibited significantly higher expression of luciferase at each time point compared to cells treated with naked DNA ($p<0.05$), although maximum expression levels (day 3) were only 6.5 fold higher. By comparison, at the 10μg dose although expression was slightly higher in cells treated with DNA-loaded microparticles, the maximum difference in expression on day 2 was only 2 fold ($p<0.05$). In both cases (5.3.1C and 5.3.1D) the RLU values in cells mixed with DNA or microparticles prior to plating were considerably lower than the RLU values in cells that were treated with naked DNA or microparticles 24h after cell seeding (5.3.1A and 5.3.1B). This suggests that mixing of microparticles with cells prior to seeding could interfere with cell attachment and spread, potentially reducing the surface area available for DNA uptake and reducing luciferase expression. The optimal dose for transfection was questionable with little difference in luciferase expression observed between cells treated with either dose. When cells were treated following seeding (5.3.1A and 5.3.1B) the 10μg DNA dose resulted in the highest levels of expression. However, when cells were mixed with DNA or DNA-loaded microparticles prior to seeding (5.3.1C and 5.3.1D) the highest RLU values were seen in cells treated with the 2μg DNA dose.

5.3.2 The effect of LMWC stabiliser concentration on luciferase expression in 293T and C_2C_{12} myoblast cells

The effect of low molecular weight chitosan (LMWC) stabiliser concentration on luciferase expression (RLU/mg protein) in 293T cells is shown in figure 5.3.2A. On day 1 the difference in luciferase expression between cells treated with naked DNA or DNA-loaded PLG/1%LMWC microparticles was not significant. In contrast, expression in cells treated with PLG/3%LMWC microparticles was approximately 4 fold higher ($p<0.05$). On day 2 luciferase expression in cells treated with either of the microparticle formulations was not significantly higher than in cells treated with naked DNA. In fact a distinct drop in levels of expression was observed in cells treated with PLG/3% LMWC. On day 3 luciferase expression in cells treated with PLG/1%LMWC or PLG/3%LMWC microparticles was 17 fold ($p<0.05$) or 55 fold ($p<0.005$) higher respectively than expression levels in cells treated with naked DNA. On day 3 cells treated with PLG/3%LMWC reached expression levels as high as 3000 RLU/mg protein in comparison to levels of 1000 RLU/mg protein in cells treated with PLG/1%LMWC, and 60 RLU/mg protein in cells treated with naked DNA.

The effect of LMWC stabiliser concentration on luciferase expression in myoblast cells is shown in figure 5.3.2B. These results indicate that myoblasts behave differently to 293T cells. On day 1 no luciferase expression was observed, irrespective of how the cells had been treated. On day 2 cells treated with naked DNA still expressed no luciferase. In contrast luciferase expression in cells treated with PLG/1%LMWC microparticles was 530 fold higher ($p<0.01$), and expression in cells treated with PLG/3%LMWC microparticles was 1320 fold higher ($p<0.005$). However, even at these levels (approximately 530 RLU/mg protein for PLG/1%LMWC microparticles and 1320 RLU/mg protein for PLG/3%LMWC microparticles) expression was lower than the maximum levels in 293T cells. On day 3, luciferase expression had returned to zero in cells treated with either of the microparticle formulations, but very low levels of expression were seen in cells treated with naked DNA

A. **293T murine epithelial cells**



B. **C_2C_{12} human myoblast cells**

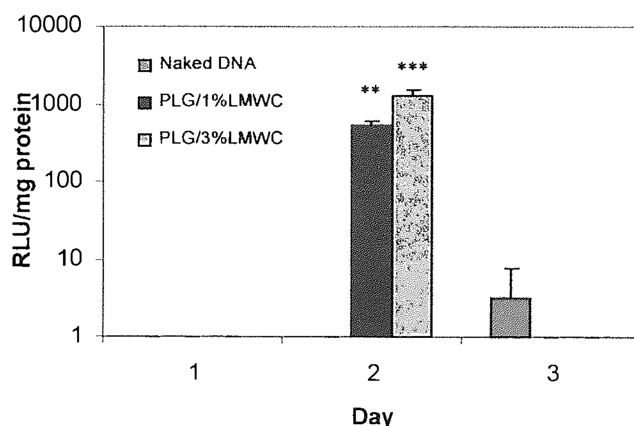


Figure 5.3.2 The effect of LMWC concentration on luciferase gene expression *in vitro*. Cells were seeded and incubated for 24h at 37°C. Cells were treated with naked luciferase plasmid DNA, DNA-loaded PLG/1%LMWC microparticles or DNA-loaded PLG/3%LMWC microparticles (10μg DNA/well) and incubated at 37°C. Each day medium was removed from one set of wells and the cells were washed with PBS (1ml). Cells were incubated with lysis reagent (100μl) for 15 minutes at room temperature. The cell lysates were assayed for luciferase expression (method 2.11.2), and luciferase expression was quantified in terms of RLU/mg of protein (method 2.11.3). (A) 293T mouse kidney epithelial cells. (B) C_2C_{12} human myoblast cells. *Denotes p-values <0.05 **Denotes p-values <0.01 ***Denotes p-values <0.005 compared to naked DNA results.

5.3.3 The effect of extending incubation time on luciferase gene expression in 293T cells

To determine whether the expression of luciferase in 293T cells was transient, the time of exposure of particles to the cells was extended from 3 to 6 days. The results are shown in figure 5.3.3. Cells treated with DNA-loaded PLG/3% LMWC microparticles expressed significantly higher levels of luciferase than cells treated with naked DNA on all days ($p<0.01$ on days 1, 3 and 6, $p< 0.05$ on day 2). Even on day 6 expression levels were 4 fold higher than in cells treated with naked DNA. As a positive control cells were transfected with DNA complexed to the lipid based transfection reagent F6. On days 1-3, luciferase expression in cells treated with F6/DNA was approximately 60-70 fold higher than in cells treated with PLG/LMWC3% microparticles, and on day 6 expression was 17 fold higher. On days 1, 2, and 3 expression in cells treated with F6 was significantly higher statistically ($p<0.005$) than in cells treated with naked DNA.

5.3.4 Confirmation that all components of the PLG/LMWC microparticle are required for efficient luciferase gene expression in 293T cells

The effect of microparticle components on luciferase gene expression in 293T cells is shown in Figure 5.3.4. On days 2 and 3 the highest expression of luciferase was observed in cells treated with DNA-loaded PLG/LMWC microparticles, and on all 3 days expression was significantly higher than expression in cells treated with naked DNA ($p<0.005$ days 1 and 2, $p< 0.01$ day 3). When blank PLG/LMWC microparticles were added to cells immediately followed by naked DNA (microparticles + DNA), expression of luciferase was not significantly higher than in cells treated with naked DNA alone. In contrast, an enhancement in luciferase expression was observed in cells treated with chitosan/DNA complexes produced by incubation of chitosan in lactic acid solution (0.5% w/v) with plasmid DNA (0.02% w/v) (section 2.2.9). On day 1 luciferase expression levels were 9 fold higher ($p<0.05$) than in cells treated with naked DNA, and on day 2 levels were 6 fold higher ($p<0.01$).

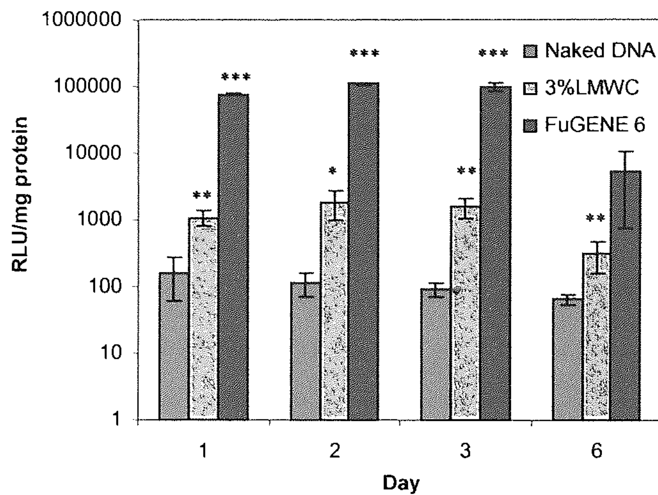


Figure 5.3.3 The effect of extended incubation time on transfection levels in 293T cells. Cells were treated with naked luciferase plasmid DNA, DNA-loaded PLG/3%LMWC microparticles or FuGENE 6/DNA complexes (10μg DNA/well) and assayed for luciferase expression (method 2.11.2) on days 1, 2, 3 and 6. The cell numbers in each well were estimated using the BCA protein assay (method 2.11.3). *Denotes p-values <0.05 **Denotes p-values <0.01 ***Denotes p-values <0.005 compared to naked DNA results.

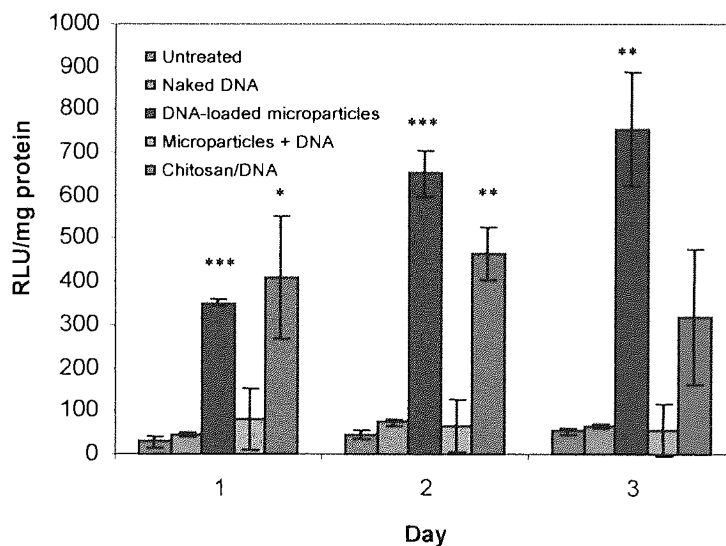


Figure 5.3.4 The effect of microparticle components on transfection efficiency in 293T cells. Cells were incubated with naked luciferase plasmid DNA, DNA-loaded PLG/3%LMWC microparticles, blank PLG/3%LMWC microparticles and plasmid DNA or chitosan/DNA complexes (10μg DNA/well). Control wells were left untreated. Cells were assayed for luciferase expression on days 1, 2 and 3 (method 2.11.2). The cell numbers in each well were estimated using the BCA protein assay (method 2.11.3). *Denotes p-values <0.05 **Denotes p-values <0.01 ***Denotes p-values <0.005 compared to naked DNA results.

5.3.5 The effect of PLG/LMWC microparticle lyophilisation on luciferase gene expression in 293T and C_2C_{12} myoblast cells

The effect of microparticle lyophilisation on luciferase gene expression in 293T cells transfected with DNA-loaded PLG/3%LMWC particles is shown in figure 5.3.5A. Luciferase expression in cells treated with non-lyophilised DNA-loaded PLG/3%LMWC microparticles was 23 fold higher on day 2 ($p < 0.005$) and 10 fold higher on day 3 ($p < 0.01$) than in cells treated with naked DNA. In contrast, luciferase expression in cells treated with lyophilised DNA-loaded PLG/3%LMWC microparticles was comparable to levels observed in cells treated with naked DNA on all 3 days.

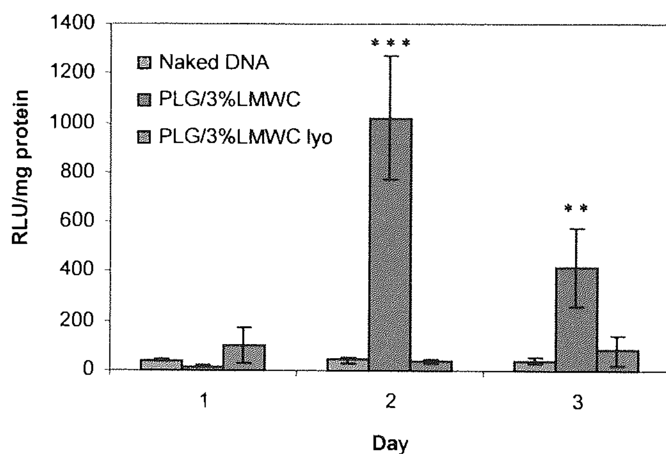
Luciferase gene expression in cells treated with lyophilised microparticles was also studied in myoblasts and the results are shown in figure 5.3.5B. Once again cells treated with the non-lyophilised formulation showed enhanced luciferase expression over cells treated with naked DNA after day 1. On day 2 expression levels were 3 fold higher ($p<0.01$) and on day 3 they were 1.5 fold higher ($p<0.01$). Although on day 1 luciferase expression in cells treated with lyophilised microparticles was higher than in cells treated with naked DNA or non-lyophilised PLG/3%LMWC microparticles, the difference was not significant. On days 2 and 3 expression levels were comparable to naked DNA. These results suggest that lyophilisation of PLG/3%LMWC microparticles impairs their ability to efficiently transfect cells.

5.4 Fluorescence microscopy

5.4.1 Confirmation that DNA adsorbs to the surface of PLG/LMWC microparticles

Confirmation by fluorescence microscopy that plasmid DNA was adsorbed to the surface of PLG/LMWC microparticles is shown in figure 5.4.1. The rhodamine labelled DNA could clearly be identified as a fluorescent band around the particles on days 1 (5.4.1A), 2 (5.4.1B) and 3 (5.4.1C) post incubation with 293T cells. Although DNA is known to be

A.



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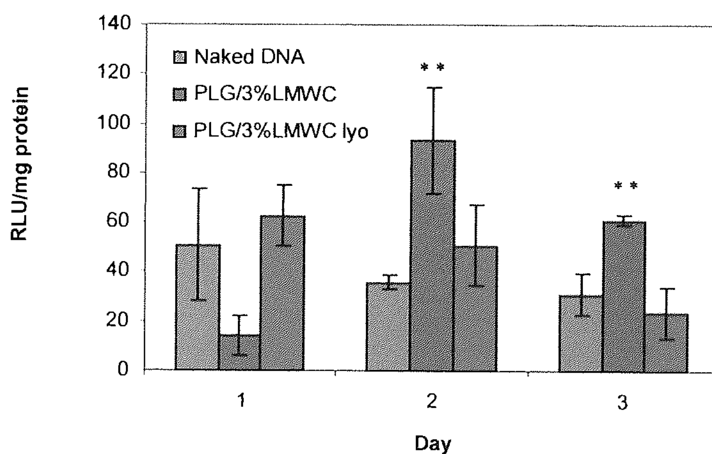
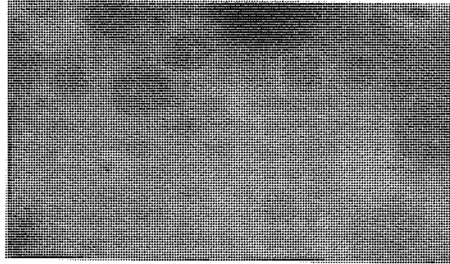


Figure 5.3.5 The effect of lyophilisation on the transfection efficiency of PLG/LMWC microparticles. Cells were treated with naked luciferase plasmid DNA, DNA-loaded PLG/3%LMWC microparticles or lyophilised and reconstituted DNA-loaded PLG/3%LMWC microparticles (10μg DNA/well). Cells were assayed for luciferase expression (method 2.11.2) on days 1, 2, 3. The cell numbers in each well were estimated using the BCA protein assay (method 2.11.3). (A) 293T mouse kidney epithelial cells. (B) C_2C_{12} human myoblast cells **Denotes p-values <0.01 ***Denotes p-values <0.005 compared to naked DNA results.

A.



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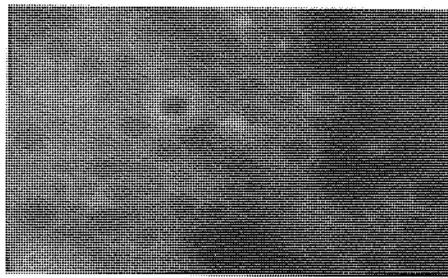

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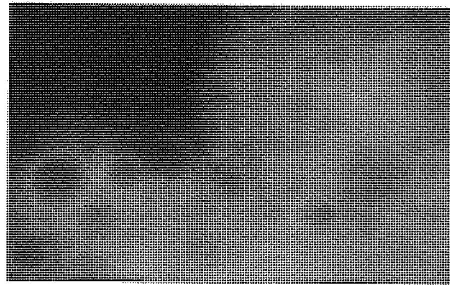


Figure 5.4.1 Confirmation that fluorescently labelled DNA adsorbs to the surface of PLG/LMWC microparticles. PLG/3%LMWC microparticles were incubated with rhodamine-labelled p55gag DNA for 12h at room temperature. For fluorescence labelling of DNA see method 2.5.5. DNA-loaded microparticles were transfected into 293T cells in 8-well chamber slides. On days 1, 2 and 3 post transfection slides were washed in PBS to remove unbound particles, cells were fixed with 4% w/v paraformaldehyde and slides were visualised under a fluorescence microscope (×1000 magnification). (A) 24h post - incubation. (B) 48h post - incubation. (C) 72h post - incubation.

released from the surface of the microparticles (section 4.3.5) no obvious shedding of DNA or chitosan/DNA complexes was observed over the three days.

5.4.2 Evidence that PLG/LMWC microparticles interact with cells and that adsorbed DNA may enter the cytoplasm of transfected 293T cells

In order to try and elucidate the mechanism of cell transfection of PLG/LMWC microparticles, 293T cells were transfected with particles loaded with rhodamine labelled DNA. The nuclei of the cells were counter stained with Hoechst 33342 to try and establish the cellular localisation of the DNA following transfection. Figure 5.4.2 shows fluorescent images of 293T cells on days 1 (5.4.2A), 2 (5.4.2B) and 3 (5.4.2C) post-transfection with PLG/LMWC microparticles. Unfortunately, from these images it was impossible to say whether the DNA was actually inside the cells or whether it was bound to particles lying on top of them. It is possible that the brighter patches of red, for example seen in images 5.4.2A (ii), 5.4.2B (i) and 5.4.2C (i) were due to DNA-loaded microparticles lying on top of cells, and that the fainter red images were produced by free DNA within the cell cytoplasm. Clearly however, there was an association between the labelled DNA and the cells as demonstrated by the co-localisation of the rhodamine with the Hoechst 33342 fluorescence. By day 3 (figure 5.4.2C (ii)) there was a suggestion that the labelled DNA may have associated with the nuclei of some cells (indicated by arrows).

5.5 Assesment of cytotoxicity of PLG/LMWC microparticles

5.5.1 Assesment of cell viability in 293T cells treated with PLG/LMWC microparticles or microparticle components

The effect of components of PLG/LMWC microparticles or complete microparticle preparations on the viability of 293T cells as measured by the MTT assay for cell activity (section 2.12.1) is shown in figure 5.5.1A. To compare toxicity cells were also treated with PLA lamellae and with the cationic lipid based transfection reagent FuGENE 6 (F6).

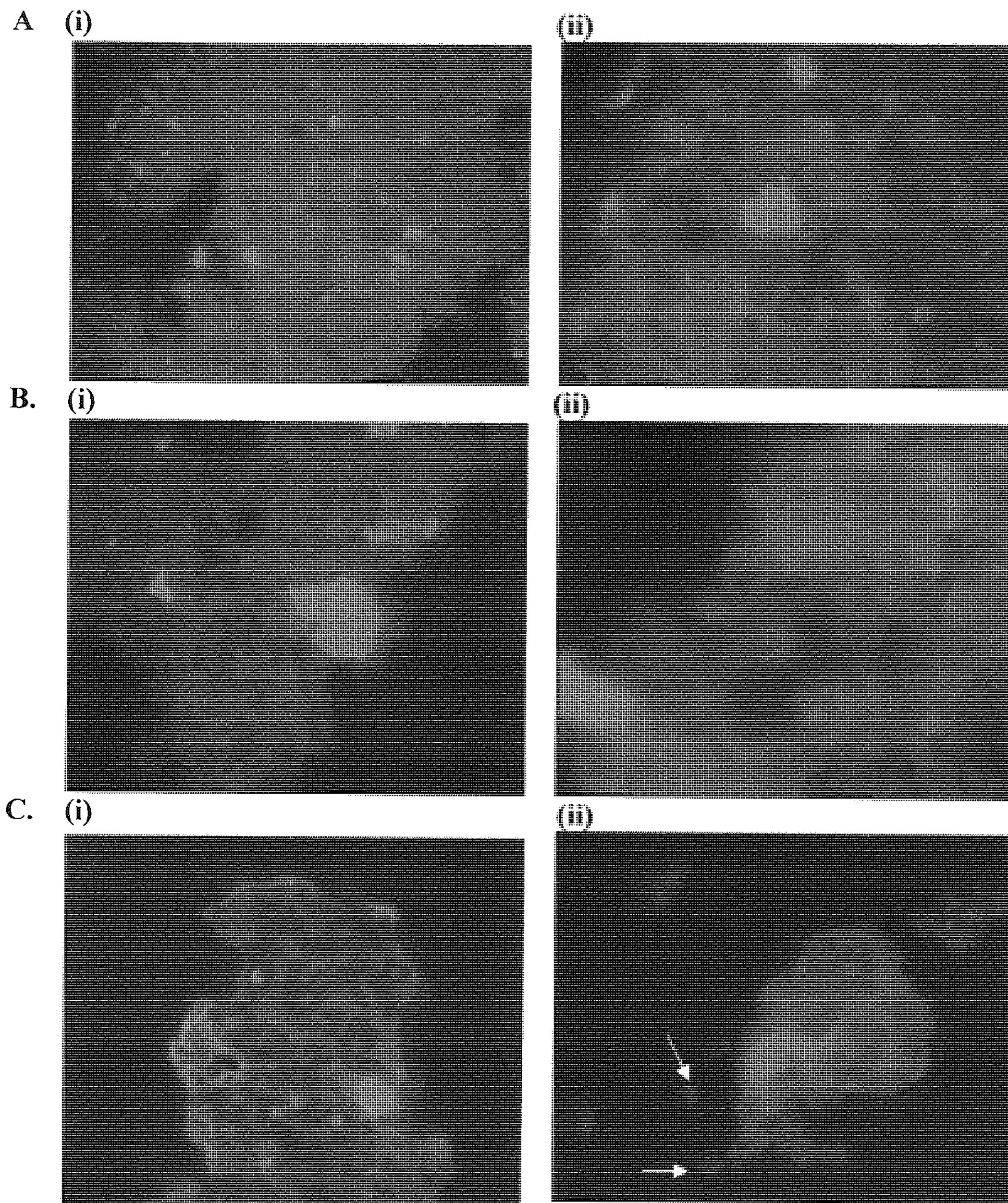
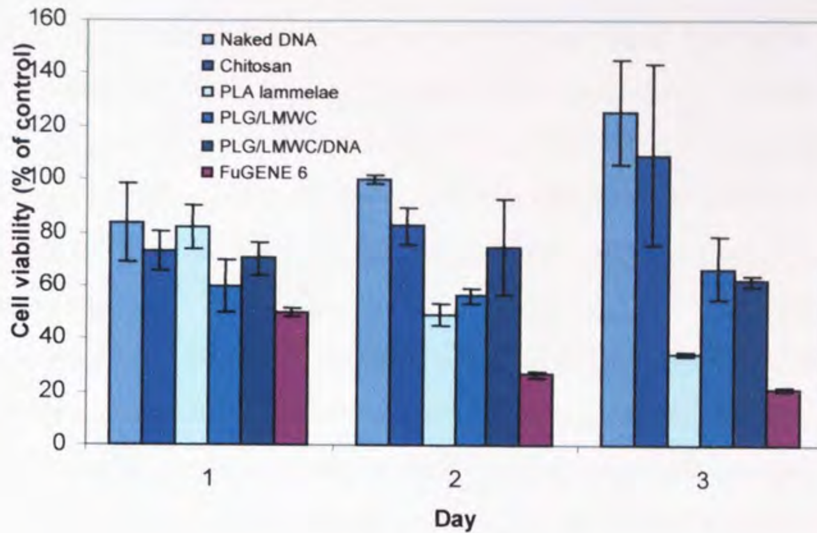


Figure 5.4.2 Evidence that DNA-loaded PLG/LMWC microparticles associate with 293T cells. PLG/LMWC microparticles were incubated with rhodamine-labeled p55gag DNA for 12h at room temperature. For fluorescent labelling of DNA see method 2.5.5. DNA-loaded microparticles were transfected into 293T cells in 8 well chamber slides. On days 1, 2 and 3 post transfection slides were washed in PBS to remove unbound particles, cells were fixed with 4% w/v paraformaldehyde and the cell nuclei were stained with Hoechst 33342 dye (method 2.11.5). Slides were visualised under a fluorescent microscope. (A) 24h post - transfection. (B) 48h post - transfection (C) 72h post - transfection. In each case (i) × 400 magnification. (ii) × 1000 magnification.

A.



B.

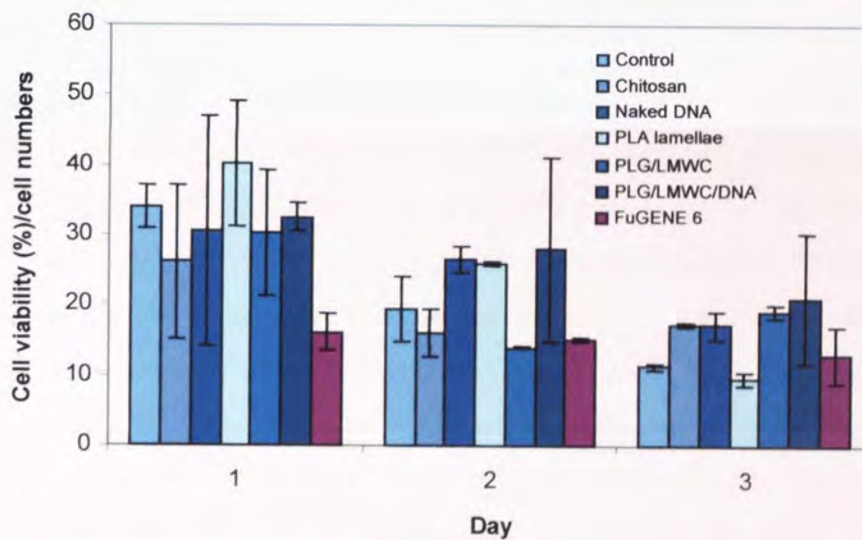


Figure 5.5.1 The effect of PLG/LMWC microparticles or microparticle components on cell viability in 293T cells. Cells were incubated with naked luciferase plasmid DNA, chitosan in lactic acid solution (1% w/v), PLA lamellae, blank PLG/1%LMWC microparticles, DNA-loaded PLG/1%LMWC microparticles or FuGENE 6/DNA complexes (10μg DNA/well). Control cells were left untreated (A) On days 1, 2 and 3 cell viability was assessed by the MTT assay. MTT reagent (120μl) was added to the wells and incubated for 4hrs. Medium was removed and DMSO (750μl) added to the wells. Absorbance was measured at 570nm. (B) Cell counts were also performed each day. Medium was removed and cells were scraped from the bottom of the wells. Cells were resuspended in medium (1ml) and counted on a haemocytometer.

Control cells were left untreated. On day 1 all treatments resulted in a slight decrease in cell viability. The decrease was greatest in cells treated with blank PLG/LMWC (60% viability) or F6/DNA (50% viability). For all other treatments viability remained between 70 and 80%. In cells treated with naked DNA viability increased from approximately 80% on day 1 to about 125% by day 3. Viability in cells treated with soluble chitosan (1% w/v) also increased over the 3 days from 75% to 110%. This was surprising as it suggests that chitosan stimulates cell growth and proliferation. The viability of cells treated with blank PLG/LMWC microparticles remained at approximately 60% on days 1 and 2 increasing slightly to 70% on day 3. Cells treated with DNA-loaded PLG/LMWC remained between 65 and 70% throughout. In comparison, the viability of cells treated with PLA lamellae went down from 80% on day 1 to approximately 30% by day 3 suggesting the irregular nature of these particles may cause cellular damage. The lowest viability levels were seen in cells treated with F6/DNA with a drop from 50% on day 1 to 15% by day 3.

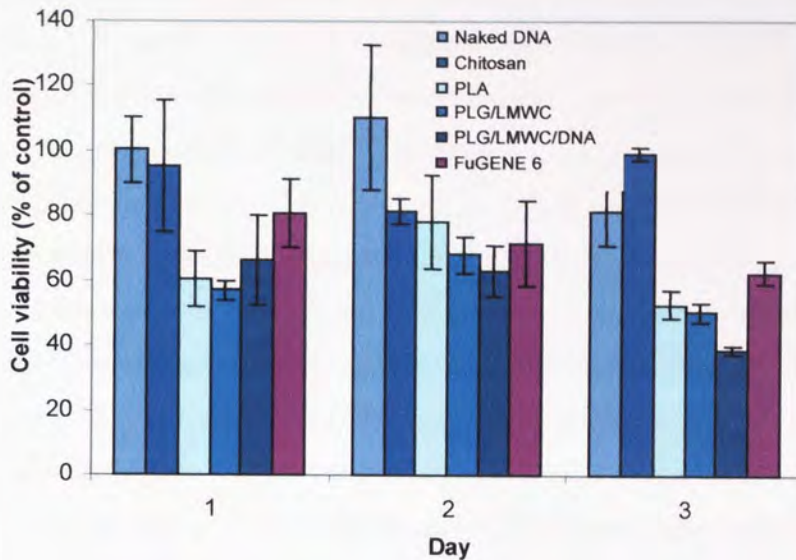
In order to establish whether the observed toxicity was a result of cell death, or inhibition of cell growth, cell counts were conducted on 293T cells treated identically. Figure 5.5.1B shows the cell viability (% of control) of 293T cells following correction for cell numbers. In general, when cell numbers were taken into consideration the viability of treated cells was either not significantly lower, or was higher, than levels in untreated control cells on each of the days. This suggests that in treated 293T cells a reduction in viability tends to correlate with a reduction in cell numbers. However, in some cases significantly reduced viability was observed compared to control cells. On day 1 the viability of cells treated with F6 was approximately 15 % compared to 33 % in control cells, and on day 2 both cells treated with PLG/LMWC or F6 had reduced viability (both approx 15 %) compared to control cells (approx. 20%). Generally, over the 3 days for both treated and untreated cells, a gradual reduction in % viability/cell number was observed. Since untreated control cells were affected as well as treated cells this suggests that the cell culture conditions may have had a detrimental impact on cellular activity as measured by the MTT assay.

5.5.2 Assesment of cell viability of C_2C_{12} myoblast cells treated with microparticles or microparticle components

The effect of components of PLG/LMWC microparticles or complete microparticle preparations on the viability of C_2C_{12} myoblast cells is shown in figure 5.5.2A. On days 1 to 3 cells treated with DNA remained between 85% and 100% viable. Likewise, the viability of cells treated with soluble chitosan (1% w/v) did not drop below 80% over the 3 days. The viability of cells treated with PLA lamellae was 60% on day 1, rising to approximately 80% on day 2 and dropping back to 55% on day 3. Following a similar pattern the viability of cells treated with blank PLG/LMWC particles rose from approximately 55% (day 1) to 65% (day 2) and then dropped to 50% (day 3). Cells treated with DNA-loaded PLG/LMWC remained approximately 65% viable on days 1 and 2 dropping to 40% on day 3. In comparison to 293T cells, F6 did not appear to affect the viability of myoblasts to the same extent. Viability of cells treated with F6 went down from 80% on day 1 to 60% by day 3. This suggests that transfection reagents may be variably toxic depending on cell type.

Figure 5.5.2B shows the viability of myoblast cells (% of control) following correction for cell numbers. As observed for 293T cells, when cell numbers were taken into consideration the differences in viability observed between untreated and treated cells were much reduced. On days 1 and 2 there was no significant difference between treated and untreated cells with the exception of cells treated with F6. On day 2 these cells showed slightly reduced viability (1.3%) compared to control cells (2.2%). The viability of all treated and untreated cells by day 3 was approximately the same, with a very slight reduction in viability observed in cells treated with particulate formulations or F6. As with 293T cells a general reduction in cell viability over the time course was observed independent of whether cells were treated or untreated (control) suggesting once again that cell culture conditions may not be optimal for cell activity as measured by the MTT assay.

A.



B.

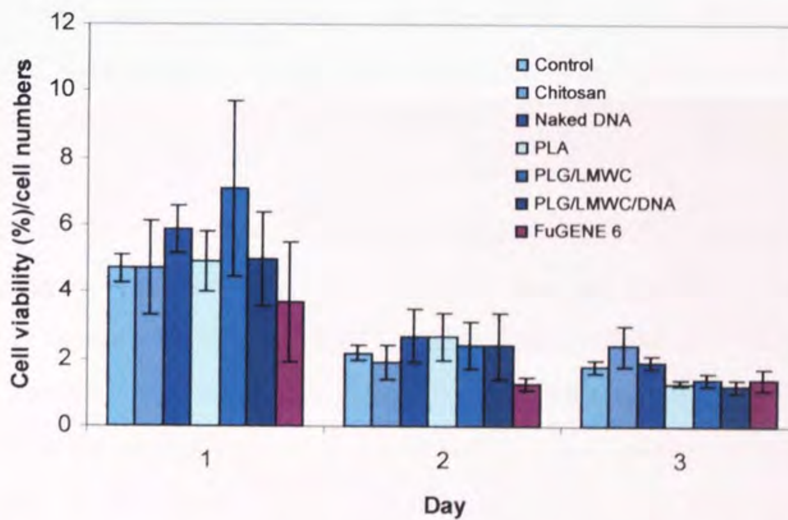


Figure 5.5.2 The effect of PLG/LMWC microparticles or microparticle components on cell viability and cell numbers in C_2C_{12} myoblast cells. Cells were incubated with naked luciferase plasmid DNA, chitosan/DNA complexes, blank PLG/1%LMWC microparticles, DNA-loaded PLG/1%LMWC microparticles or FuGENE 6/DNA complexes (10μg DNA/well). (A) On days 1, 2 and 3 cell viability was assessed by the MTT assay. MTT reagent (120μl) was added to the wells and incubated for 4hrs. Medium was removed and DMSO (750μl) added to the wells. Absorbance was measured at 570nm. (B) Cell counts were also performed each day. Cells were washed with PBS and incubated with trypsin/EDTA (200μl, 5min.). Cells were resuspended in medium (1ml) and counted on a haemocytometer.

5.5.3 The effect of plating procedure on microparticle cytotoxicity

In addition to potentially sub-optimal cell culture conditions, one theory for the reduced viability and cell numbers of 293T and myoblast cells treated with PLG/LMWC microparticles is that the microparticles form a 'blanket' over the cells which impedes their growth and proliferation. Figure 5.5.3 shows an assessment of cell numbers and cell viability to determine whether cells seeded on top of microparticles grow better than cells seeded before the addition of microparticles. Figure 5.5.3A shows the cell viability in 293T cells. In this case, cell viability on each of the days was not significantly affected by whether the cells were seeded before adding the microparticles or whether cells were seeded on top of the microparticles. Likewise, the cell numbers obtained from both plating procedures (figure 5.5.3B) were not significantly different on any of the days. When cells were seeded prior to incubation with the microparticles cell viability peaked on day 2 and cell numbers peaked on day 3. When these results were compared to transfection data it was observed that transfection levels in 293T cells also peaked on day 2 or 3 post-incubation (figs 5.3.2A, 5.3.3, 5.3.4, 5.3.5A) suggesting a potential link between cell viability and transfection efficiency.

Figures 5.5.3C and 5.5.3D show the viability and cell numbers respectively of myoblasts plated using the two procedures. For this cell line it appears that the plating procedure may have had an affect on cell viability (5.5.3C). If the particles were plated first a gradual increase in cell viability from 40 to 90% was observed. In comparison, if microparticles were added after cells were seeded then the viability fell from 80 to 60% over the 3 days. A similar pattern was seen for cell numbers. If microparticles were plated first then the cell numbers rose from 60% of control numbers to 130% over the 3 days. If the cells were seeded before the microparticles, cell numbers decreased from approximately 85% of the control on day 1 to 60% on day 2 then recovered to 85% on day 3. These results suggest that the 'blanket' effect may result in some suppression of growth in myoblast cells. In contrast to 293T cells, when myoblasts were seeded prior to incubation with the microparticles, both cell viability and cell numbers were at their

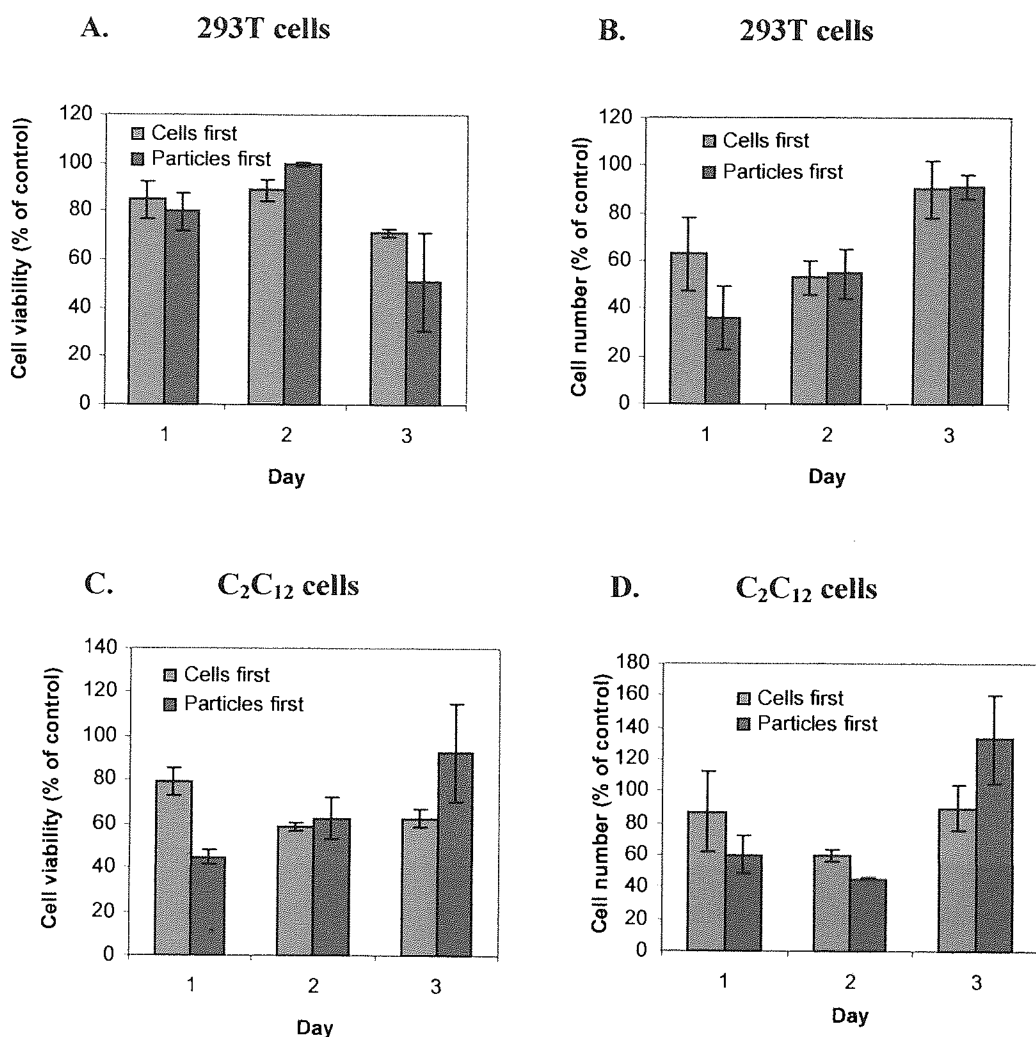


Figure 5.5.3 The effect of plating procedure on cell viability and cell numbers in 293T and C_2C_{12} myoblast cells treated with PLG/LMWC microparticles. Cells were incubated with PLG/1%LMWC microparticles. Cells were either seeded prior to incubation with the microparticles, or microparticles were first allowed to settle on the bottom of the well before cells were seeded on top of them. On days 1, 2 and 3 cell viability was assessed by the MTT assay. MTT reagent (120µl) was added to the wells and incubated for 4hrs. Medium was removed and DMSO (750µl) added to the wells. Absorbance was measured at 570nm. Cell counts were also performed each day. (A) 293T cell viability. (B) 293T cell numbers. (C) C_2C_{12} cell viability. (D) C_2C_{12} cell numbers.

lowest on day 2. Conversely, transfection levels in myoblast cells peaked on day 2 (figs 5.3.2B, 5.3.5B) suggesting an inverse relationship between cell viability and transfection efficiency in this cell line.

5.6 Discussion

Efficient endocytosis of cationic gene delivery vehicles by cells is thought to occur subsequent to electrostatic interactions between the two. Attraction of the cationic polymer to the negatively charged cell surface leads to cell binding and spontaneous endocytosis mediated by anionic cell surface proteoglycans (Mislick & Baldeschwieler, 1996). There is evidence that DNA-loaded PLG/3%LMWC microparticles are attracted to the cell surface of myoblast (fig 5.2) and 293T cells (results not shown). Although these particles are too large (>100nm) to be endocytosed by non-phagocytic cells, electrosatatic interaction of the chitosan with the cell surface probably occurs. In addition it has been suggested that non-ionic interactions between the carbohydrate backbone of chitosan and the cell membrane may occur (Vankatesh & Smith, 1998). Furthermore, there is evidence that chitosan has a perturbing effect on cell membranes (Fang *et al,* 2001; Yang *et al,* 2002). A combination of these effects may result in enhanced DNA uptake mediated by PLG/LMWC microparticles and surface localized chitosan molecules that can interact with and disrupt the cell membrane.

PLG/LMWC microparticles were capable of transfecting non-phagocytic 293T cells more efficiently than naked DNA (fig 5.3.1). Because particles > 100nm are too large to be endocytosed or pinocytosed by non-phagocytic cells this result was surprising. However, previous reports have also demonstrated efficient transfection of non-phagocytic cells by larger PLG/DAEM, PLA/PEI and tripalmitin solid lipid particulate carriers with adsorbed DNA (Walter *et al,* 2002; Erni *et al,* 2002). One theory for this efficient transfection is that *in vitro* microparticles will settle down on top of adherent cells leading to a local increase in DNA concentration at the cell surface (Luo & Saltzman, 2000b). Subsequently, contact-induced DNA transfer into cells may occur which does not require uptake of the microparticles themselves. The possible membrane perturbation caused by chitosan may further enhance this effect. This theory is further supported by the present

findings, as much higher levels of microparticle mediated transfection were observed in cells that were allowed to adhere to wells before incubation with PLG/LMWC microparticles (figs 5.3.1 A and B) compared to transfection levels in cells that were mixed with microparticles prior to seeding (figs 5.3.1 C and D). Cells mixed with microparticles prior to seeding would not be exposed to the same local increases in DNA concentration because particles would not necessarily be concentrated at one surface of the cells.

The enhanced levels of luciferase gene expression observed in cells treated with DNA-loaded PLG/LMWC microparticles did not appear to be dependent on the DNA dose (fig 5.3.1). In subsequent experiments a 10μg DNA dose/well was routinely used because the maximum RLU value was achieved at this concentration (fig 5.3.1B). The ability of PLG/LMWC microparticles loaded with the lower DNA dose to elicit the same magnitude of response as particles loaded with the higher dose in 293T cells was very encouraging. One of the ultimate aims in developing effective DNA vaccine delivery systems for human vaccination is to reduce the DNA dose whilst maintaining the potency of the vaccine.

Increasing the concentration of chitosan stabiliser used for microparticle formulation led to an increase in transfection efficiency in both 293T cells (fig 5.3.2A) and myoblast cells (fig 5.3.2B). At higher stabiliser concentrations, if increased chitosan incorporation (fig 3.4.5) results in more LMWC localized at the particle surface this may result in greater membrane destabilization and enhanced DNA uptake compared to particles with a lower concentration of chitosan at their surface. The zeta potential of the microparticle formulations may also influence their efficacy. Following DNA adsorption the surface of PLG/1%LMWC microparticles became negatively charged (table 4.3.4). By comparison, DNA-loaded PLG/3%LMWC microparticles retained a net positive surface charge which may improve the ability of this formulation to interact with the cell surface leading to enhanced transfection.

An alternative explanation for the enhanced transfection of non-phagocytic cells mediated by larger particles was proposed by Walter *et al.* This group suggested that rather than whole microparticles being endocytosed, small (<100nm) DNA/stabiliser complexes were released from the surface of extracellular PLA/PEI microparticles and these complexes were endocytosed (Walter *et al,* 2002). The same year Erni *et al* described efficient transfection of non-phagocytic 293 cells by DNA-loaded cationic solid lipid microparticles (SLM) and attributed these results to the detachment and cellular uptake of nanocomplexes of cationic lipid and DNA released from the surface of the SLM (Erni *et al,* 2002). Potentially the efficient transfection of non-phagocytic cells by PLG/LMWC microparticles could result from the release of chitosan/DNA complexes from the particle surface followed by cellular endocytosis of the complexes. Chitosan/DNA complexes and chitosan nanoparticle-DNA delivery vehicles are known to be efficient at transfecting a variety of cell types *in vitro* (Vankatesh & Smith,1997; Erbacher *et al,* 1998; Sato *et al,* 2001; Mao *et al,* 2001,). In chapter 3 it was demonstrated that chitosan was released from the surface of microparticles, albeit it in small quantities (fig 3.4.6). In addition, more chitosan was released from PLG/3%LMWC particles than from PLG/1%LMWC particles which correlates with the higher transfection observed in the former system. However, by contrast less DNA was released from particles stabilised with the higher chitosan concentration (fig 4.3.5) although if DNA was released in complex with chitosan it is possible that it was not detected by the DNA quantitation assay described here.

In addition to efficient transfection of 293T cells, PLG/LMWC microparticles were also capable of transfecting myoblast cells efficiently (fig 5.3.2B). PLG/LMWC microparticles are designed to be delivered via the intramuscular route, therefore the ability of the microparticles to transfect myoblast cells *in vitro* may be critical in predicting the potency of the vaccine *in vivo*. Targeting of the vaccine to professional APCs was a critical factor in designing the DNA delivery vector. Strong evidence suggests that direct transfection and priming of these cells results in a potent immune response to encoded antigens. However, there is also evidence supporting the importance of myocyte cell transfection in the immune response elicited by i.m. injected DNA

vaccines. I.m. injection of plasmid DNA encoding reporter genes has resulted in protein expression in myocytes (Wolff *et al,* 1990), and similar administration of DNA encoding antigens led to the induction of humoral and cell mediated immunity (Ulmer *et al,* 1993; Donnelly *et al,* 1994; Donnelly *et al,* 1995). Ulmer *et al* proposed that the transfer of antigen from myocytes to professional APCs could occur, and that this may preclude the requirement for direct transfection of professional APCs by DNA vaccines (Ulmer *et al,* 1997; Fu *et al,* 1997). The ability of PLG/LMWC microparticles to transfect myoblast cells *in vitro* suggests that the particles may also be capable of transfecting myocytes *in vivo.*

Another factor which may affect the potency of a vaccine *in vivo* is the length of time that the protein is expressed following transfection. However, it must be taken into consideration that gene expression patterns are closely related to the type and stability of the gene product, for example whether the protein is secreted or membrane bound. 293T cells treated with PLG/LMWC microparticles exhibited maximum luciferase expression 2 to 3 days post-transfection but expression levels were still elevated in comparison to cells treated with naked DNA 6 days post-transfection (fig 5.3.3). Similar *in vitro* luciferase gene expression patterns have been described by groups using chitosan/DNA delivery systems. Erbacher *et al* demonstrated late gene expression (72-96h) in HeLa cells treated with chitosan/DNA complexes compared with very transient gene expression using a PEI based system (Erbacher *et al,* 1998). Mao *et al* analyzed luciferase expression in HEK293 cells treated with chitosan-DNA nanoparticles and found that maximal gene expression occurred around 3-5 days. However, luciferase was still detectable in the cells 30 days after transfection after seven passages (Mao *et al,* 2001). This may provide support for the hypothesis that transfection of PLG/LMW microparticles is mediated by released chitosan/DNA complexes. In comparison to 293T cells, myoblast cells exhibited very transient luciferase expression when treated with PLG/LMWC microparticles (fig 5.3.2B). Cell type has also been reported to affect the transfection efficiency of chitosan/DNA complexes (Erbacher *et al,* 1998; Corsi *et al,* 2003). The varying cellular membrane composition between cell types will affect binding affinity and internalization in the case of nanoparticle carriers and is probably responsible for the observed

differences. It has also been reported that cells deficient in proteoglycan synthesis are more difficult to transfect using cationic lipid/DNA complexes suggesting that successful internalization of a gene carrier is dependent on this molecule (Mislick & Baldeschwieler, 1996).

The most efficient transfection of 293T cells was observed when cells were treated with the catonic lipid reagent FuGENE 6 (F6) complexed with DNA (fig. 5.3.3). However, although luciferase expression levels were approximately 60-70 fold higher than in cells treated with PLG/LMWC microparticles, one of the practical problems for *in vivo* gene delivery mediated by cationic liposomes is that gene expression is inhibited by serum (Goldman *et al,* 1997). In this experiment complexation of F6 and DNA occurred in serum free medium prior to incubation with the cells which were incubated in medium containing 10% FCS. By comparison PLG/LMWC microparticles with adsorbed DNA were added directly to the cells and were still capable of transfecting them relatively efficiently. In systems where chitosan/DNA complexes have been used, high transfection efficiency in the presence of serum has frequently been reported (Mao *et al,* 2001; Sato *et al,* 2001). This finding also suggests that transfection may occur as a result of release of chitosan/DNA complexes from the surface of PLG/LMWC microparticles.

There are a number of potential advantages in using PLG/LMWC microparticles for DNA vaccine delivery over chitosan/DNA complexes or chitosan-DNA naonoparticle vectors. Although chitosan delivery systems are very effective *in vitro*, cell transfection *in vivo* has been less successful. Furthermore, although chitosan based nasal vaccines have been investigated (Illum *et al,* 2001) and Roy *et al* described immunological protection to peanut allergy in a murine model using chitosan-pDNA nanoparticles (Roy *et al,* 1999), *in vivo* vaccine applications have been limited. The size of chitosan/DNA complexes (<1μm) means they are not capable of targeting professional APCs by size exclusion. Stimulation of potent humoral and cellular immune responses in a variety of animal models by PLG/CTAB microparticles was in part attributed to the ability of these particles to target dendritic cells (Denis Mize *et al,* 2000). In the present study, 293T cells treated with PLG/LMWC microparticles exhibited enhanced transfection levels compared

to cells treated with chitosan/DNA complexes (fig 5.3.4). It has previously been noted that particles of size <100nm have a fast, Brownian motion which may prevent them from associating with cells (Chuck & Palsson, 1996). This effect may in part explain the lower transfection efficiency observed in cells treated with chitosan/DNA complexes in the present study. Because PLG/LMWC microparticles sediment on to the cell, transfection may be enhanced as a result of increased local DNA concentration at the cell surface. Alternatively, if microparticle transfection is mediated by the release of chitosan/DNA complexes, once again the particle may act as a concentrator at the cell surface enhancing spontaneous endocytosis of these complexes. Interestingly, cells incubated with a mixture of blank particles and luciferase plasmid DNA did not exhibit enhanced luciferase expression. This suggests that the interaction of the particle with the DNA and the cell surface is critical for efficient transfection.

Unfortunately, upon lyophilisation of PLG/LMWC microparticles the ability of the particles to transfect 293T or myoblast cells was lost (fig 5.3.5). In chapter 4, DNA from lyophilised particles was shown to remain intact (fig 4.3.8) but the size of microparticles following lyophilisation underwent an increase (table 3.4.7). However, DNA-loaded PLG1%/LMWC microparticles prior to lyophilisation were already too large (approximately 15.9 μm) to be taken up effectively by non-phagocytic cells, so an increase in size should not necessarily affect transfection efficiency. Interestingly, Roy *et al* demonstrated that lyophilised chitosan-DNA nanoparticles aggregated on resuspension in saline leading to reduced transfection efficiency (Roy *et al,* 1997). It is therefore probable that aggregation of PLG/LMWC microparticles results in the observed loss of transfection efficiency. Aggregation may prevent DNA or chitosan/DNA complexes being efficiently released from the surface of the microparticles, or these complexes may themselves aggregate and become too large to enter the cell by endocytosis. From a commercial point of view stability, especially the ability of PLG/LMWC microparticles to be lyophilised and maintain function, is critical and is an issue which would have to be redressed if the product were to be manufactured industrially.

Following cell surface interaction, many studies have attempted to elucidate the cellular trafficking of DNA/carrier complexes within the cell. In HeLa cells Erbacher *et al* demonstrated spontaneous endocytosis of chitosan/DNA complexes into small vesicles and large endosome-like compartments (Erbacher *et al,* 1998). If transfection mediated by PLG/LMWC microparticles is reliant on endocytosis of chitosan/DNA complexes, then how are these complexes released from the endosome? Reports suggest that the ability of poycation-DNA complexes such as poly (L-lysine), polyamidoamine and polyethylenimine to escape the endosome can be correlated with the buffering capacity of the polymer between pH 5 and 7 (Tang & Szoka, 1997). In theory, chitosan/DNA complexes have a high ability for proton accumulation. Endosome buffering by chitosan/DNA complexes may perturb the endosomal membrane and as the pH decreases, complexes may be released from the endosome. However, evidence is inconclusive and it has been reported that in comparison to PEI, chitosan has very limited buffering capacity (Mao *et al,* 2001). In addition, chitosan may serve as a type of hydrogel which swells as pH decreases, leading to membrane rupture (Ishii *et al,* 2001).

Following release from the endosome, entry of DNA into the nucleus is critical for transfection. Ishii *et al* confirmed that chitosan/DNA complexes were endocytosed and then accumulated in the nucleus using fluorescently labelled plasmid and chitosan (Ishii *et al*, 2001). Nuclear localisation of microinjected or endocytosed DNA/PEI complex has also been demonstrated (Pollard *et al,* 1998; Godbey *et al,* 1999b). In these studies, intact complex found within the cell nuclei demonstrated that dissociation of PEI and DNA prior to nuclear entry was not necessary. It was proposed that fusion with the nuclear membrane occurred as a result of the phospholipid coating of the complex, resulting in release of PEI/DNA into the nucleus (Godbey *et al,* 1999b).

In an attempt to elucidate the transfection mechanism of PLG/LMWC microparticles, plasmid DNA was fluorescently labelled before loading on the particles. This confirmed that DNA was adsorbed to the surface of PLG/LMWC microparticles (fig 5.4.1) and supported the DNA release data (fig 4.3.5) as large quantities of DNA remained bound to the microparticles after 3 days. There was also evidence of intracellular association of the

DNA with 293T cells following transfection in either particle bound or free form (fig 5.4.2), and evidence of DNA localisation within the cell nucleus on day 3. However, unlike Walter *et al* who detected small fluorescent spots inside the cells which appeared to be released DNA/PEI complexes (Walter & Merkle, 2002) chitosan/DNA complexes were not obviously detectable.

Many cationic DNA vectors used *in vitro* are not suitable for *in vivo* use because of their toxicity. The toxicity of chitosan, one of the major components of the PLG/LMWC microparticle formulation has previously been evaluated in many gene delivery systems *in vitro*. Some studies have suggested that higher molecular weight chitosan, or some chitosan derivatives (e.g chitosan hydrochloride) have cytotoxic and haemolytic properties (Carrero-Gomez *et al,* 1997), although purified, lower molecular mass fractions of chitosan were not found to be cytotoxic (Richardson *et al,* 1999). However, the majority of studies have demonstrated chitosan/DNA complexes or chitosan DNA-nanoparticles to exhibit low toxicity to a variety of cell lines (Sato *et al,* 2001; Mao *et al,* 2001; Lee *et al,* 2001; Thanou *et al,* 2002; Corsi *et al,* 2003).

In the present study, the cell viability of both 293T (fig 5.5.1) and myoblast cells (fig 5.5.2) treated with soluble LMWC (50-190kDa) at the maximum possible concentrations found in PLG/LMWC microparticles remained high. In fact, chitosan was actually found to enhance cell viability. Howling *et al* previously demonstrated stimulation of fibroblast proliferation in the presence of serum when cells were incubated with chitosan and suggested chitosan may interact with growth factors in the serum to enhance their effects. (Howling *et al,* 2001). Some toxicity was observed in cells treated with microparticulate systems irrespective of chitosan content or DNA loading. Similarly, Walter *et al* demonstrated higher toxicity of particle bound PEI compared to soluble PEI (Walter *et al,* 2002). Because cells incubated with chitosan or DNA alone did not result in a notable loss of viability, the observed toxicity was attributed to the particulate nature of the formulations rather than to an individual component. It is proposed here that PLG/LMWC microparticles act as a 'blanket' over the cells which may prevent normal cell growth by blocking cellular functions. This theory is supported by the light

microscope images of myoblast cells showing retardation of growth in cells incubated with microparticles compared to control cells (fig 5.2). Interestingly, when cell viability was expressed per cell little difference was observed between treated and untreated cells in both 293T (fig 5.5.1B) and myoblast (fig 5.5.2B) cell lines. This finding suggests that the observed toxicity due to the microparticles is a result of impeded cell growth rather than cell death. In addition, a decrease in viability over the 3 days was observed in untreated as well as treated 293T and myoblast cells. This suggests that sub-optimal culture conditions may also in part be responsible for the toxicity observed in PLG/LMWC microparticle cells particularly at later time points.

In both cell types, in particular 293T cells, the greatest toxicity was observed in cells treated with F6/DNA. This supports evidence that the use of cationic lipids for gene delivery results in cellular toxicity, and is one of the problems restricting use of this type of vector *in vivo*. As with transfection efficiency, cellular toxicity appears to be dependent on cell type with 293T cells being more susceptible to F6 toxicity than myoblasts. In myoblast cells the transfection efficiency of DNA-loaded PLG/LMWC microparticles appears to be inversely related to cytotoxicity. Whilst transfection levels peaked at day 2 post-incubation (figs 5.3.2B, 5.3.5B), at this time point viability and cell numbers were at their lowest levels (fig 5.5.3 C and D). These findings suggest that increased transfection efficiency is associated with increased toxicity. Corsi *et al* suggested a similar relationship between DNA-chitosan nanoparticle toxicity and transfection efficiency in HEK 293, MG63 and MSC cells *in vitro* (Corsi *et al,* 2003). In addition, it is well established that as PEI molecular weight increases PEI/DNA complexes becomes more efficient at transfecting cells but are also more cytotoxic (Godbey *et al,* 1999). Surprisingly, the opposite trend was observed in the 293T kidney epithelial cell line. Maximum transfection levels occured 2 or 3 days post incubation with DNA-loaded PLG/LMC microparticles (figs 5.3.2A, 5.3.3, 5.3.4, 5.3.5A) when cell viability and cell numbers respectively were at their highest levels (fig 5.5.3A and B). A reduction in microparticle toxicity was observed in myoblast cells that were plated on top of sedimented microparticles as opposed to addition of particles to plated cells (fig 5.3.3). A similar reduction in toxicity was not observed for 293T cells. This suggests that the

interaction of PLG/LMWC microparticles with either the apical or basal surface of 293T cells impedes their growth. In contrast, although initially growth was slow in myoblast cells, by day 3 recovery had occurred. The initial lag in recovery may be a result of the time required for cell adhesion to the particles.

In summary, DNA-loaded PLG/LMWC microparticles can efficiently transfect non-phagocytic 293T and myoblast cells *in vitro* over a 3 day timescale. The transfection efficiency of the microparticles can be enhanced by increasing LMWC concentration. Lyophilisation of the microparticles results in loss of transfection ability. The transfection mechanism of the microparticles in non-phagocytic cells requires further elucidation but may result from electrostatic interaction of the particle with the cell membrane leading to spontaneous endocytosis of DNA or chitosan/DNA complexes. The mechanism by which DNA escapes the endosome and enters the nucleus is still unclear. PLG/LMWC particles do cause some cytotoxicity but this appears to be a result of reduced cell activity rather than cell death. In addition, PLG/LMWC mediated cytotoxicity appears to be related to cell type and transfection efficiency.

6. Interaction of PLG/LMWC microparticles with cells of the immune response *in vitro* and *in vivo*

6.1 Introduction

In chapter five, DNA-loaded PLG/LMWC microparticles were shown to successfully transfect non-phagocytic cells *in vitro*. Although transfection of non-phagocytic cells, particularly myocytes, may be useful in helping to promote an immune response to i.m. injected DNA vaccines, transfection of professional antigen presenting cells (APCs) such as dendritic cells (DCs) by DNA vaccines could result in direct priming of cytototoxic T lymphocytes and helper T cells. The importance of direct transfection of dendritic cells following immunization with the gene gun was first reported by Porgador *et al* (1998). In the same year it was shown that DCs efficiently took up particles *in vivo* (Banchereau & Steinman, 1998). However, the transfection of DCs *in vitro* by non viral methods is notoriously difficult and only a few attempts have resulted in significant numbers of transfected APCs. (Diebold *et al*, 1999; Irvine *et al*, 2000; Denis-Mize *et al*, 2000).

In vivo DNA vaccines have frequently been shown to be effective at stimulating cellular and humoral immune responses in mice. For example, encapsulation of a beta-galactosidase (beta-gal) vaccine within PLG microparticles administered to mice via the parenteral route resulted in enhanced antibody titers and increased T-cell responses (McKeever *et al*, 2002). However, in humans it appears that DNA vaccines are frequently more effective at priming T-cell responses than humoral immune responses. Wang *et al* reported that vaccination of humans with plasmid DNA encoding a malaria protein resulted in enhanced cytotoxic T lymphocyte (CTL) responses in recipients but no production of antibodies against the protein (Wang *et al*, 1998). PLG/CTAB microparticles with DNA adsorbed to the surface have been shown to induce potent CTL responses and enhanced antibody responses to encoded HIV proteins in both small animal models (mice and guinea pigs) and non-human primates (Singh *et al*, 2000; O'Hagan *et al*, 2001). These findings suggest that cationic microparticles may be effective at stimulating both humoral and cellular immunity in humans.

In this final chapter, the interaction of PLG/LMWC microparticles with cells of the immune response *in vitro* and *in vivo* was studied. The ability of PLG/LMWC to transfect the DC cell line 2.4 and primary bone marrow derived DCs (BMDCs) was assessed, as was the induction of cellular and humoral immunity in a mouse model immunized with HIV-1 p55 gag DNA-loaded PLG/LMWC microparticles.

6.2 *In vitro* transfection of dendritic cells with PLG/LMWC microparticles

6.2.1 Luciferase expression in the murine dendritic cell line DC 2.4

Following successful transfection of non-phagocytic cell lines (section 5.3.2), the potential of PLG/LMWC microparticles to transfect the murine dendritic cell line 2.4 was investigated. Because the function of phagocytic cells is to engulf foreign material, expression of luciferase in DC cells incubated with microparticles was expected to occur more quickly than in non-phagocytic cells therefore the 3 day time point used in previous transfection experiments was replaced by an earlier 6h time point. DC 2.4 cells were incubated for up to 2 days with luciferase plasmid DNA formulations, and transfection efficiency was assessed by the luciferase assay (section 2.11.2). Figure 6.2.1 shows the effects of microparticle stabiliser concentration, microparticle lyophilisation and microparticle components on luciferase gene expression. As a positive control cells were also transfected with FuGENE 6/DNA. FuGENE 6 is a commercially available lipid based transfection reagent capable of efficiently transfecting a variety of cell lines *in vitro*. The experimental findings showed clearly that PLG/LMWC microparticles were unable to efficiently transfect DC 2.4 cells and expression levels were comparable to levels in untreated cells and cells treated with naked DNA. Unlike in non-phagocytic cells, increasing stabiliser concentration had no effect on gene expression, and none of the microparticle components were capable of transfecting DC 2.4 cells. Only cells

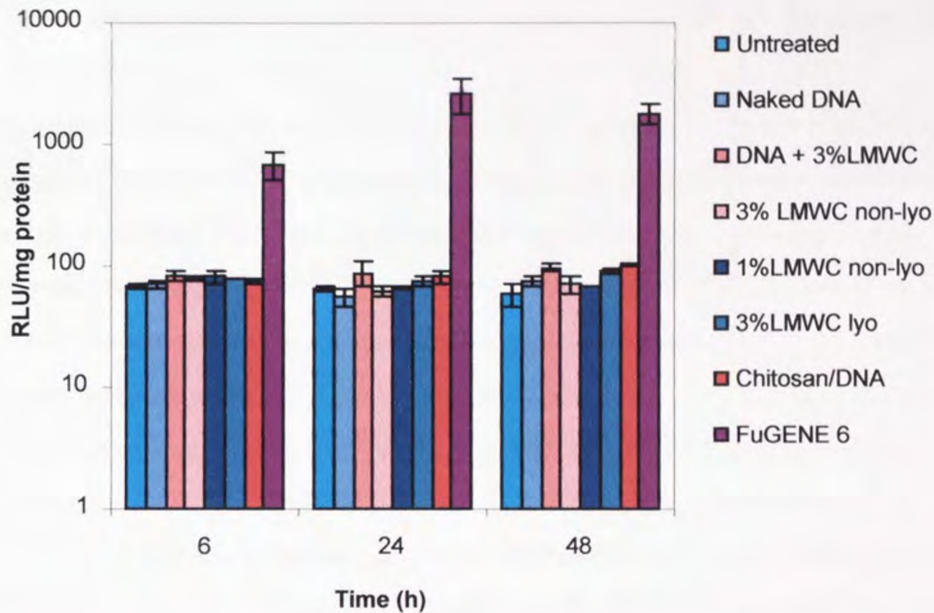


Figure 6.2.1 Luciferase expression in the murine dendritic cell line DC 2.4. Cells were treated with naked luciferase plasmid DNA, DNA + blank PLG/3%LMWC microparticles, DNA-loaded PLG/3%LMWC or PLG/1%LMWC microparticles, DNA-loaded, lyophilised PLG/3%LMWC microparticles, chitosan/DNA complexes or F6/DNA complexes (10μg DNA/well). Control cells were left untreated. Cells were assayed for luciferase expression (method 2.11.2) at 6h, 24h and 48h. The cell numbers in each well were estimated using the BCA protein assay (method 2.11.3).

incubated with the positive control F6/DNA exhibited enhanced luciferase expression compared to cells treated with naked DNA. Expression in cells treated with F6/DNA was 10 fold higher at 6h, 50 fold higher at 24h and 24 fold higher at 48h.

6.2.2 Luciferase expression in primary bone marrow derived dendritic cells

Primary cells are notoriously hard to transfect because the cells are not dividing. The potential of PLG/LMWC microparticles to transfect primary bone marrow derived dendritic cells (BMDCs) was investigated. Primary BMDCs were incubated for up to 2 days with luciferase plasmid DNA formulations, and transfection efficiency was assessed by the luciferase assay (section 2.11.2). Figure 6.2.2 shows the effect of microparticle stabiliser concentration, microparticle lyophilisation and microparticle components on luciferase gene expression. Cells were also transfected with F6/DNA as a positive control. As with the dendritic cell line DC 2.4, neither PLG/LMWC microparticles nor any of the microparticle components were efficient at transfecting primary BMDCs, and luciferase levels were comparable to levels expressed in untreated cells or cells incubated with naked DNA. Even cells treated with the positive control F6/DNA did not express enhanced levels of luciferase. For all treatments, expression levels at each time point were comparable to levels in untreated cells or cells treated with naked DNA.

6.3 *In vivo* evaluation of immune responses in BALB/c mice

6.3.1 Serum IgG responses in mice following immunization with HIV-1 p55 gag DNA.

Stimulation of humoral immunity by DNA-loaded PLG/LMWC microparticles was investigated in a mouse model. The DNA used in this experiment encoded the HIV-1 p55 gag protein. BALB/c mice (n=10) were immunized (i.m.) with DNA formulations (1μg or 10μg DNA dose) at weeks 0 and 4. At week 6 mice were bled through the retroorbital plexus and serum containing the antibodies was collected. HIV-1 gag-specific serum IgG titers were quantified by an ELISA (section 2.13.3). The effect of DNA dose, microparticle stabiliser concentration and microparticle lyophilisation on the induction

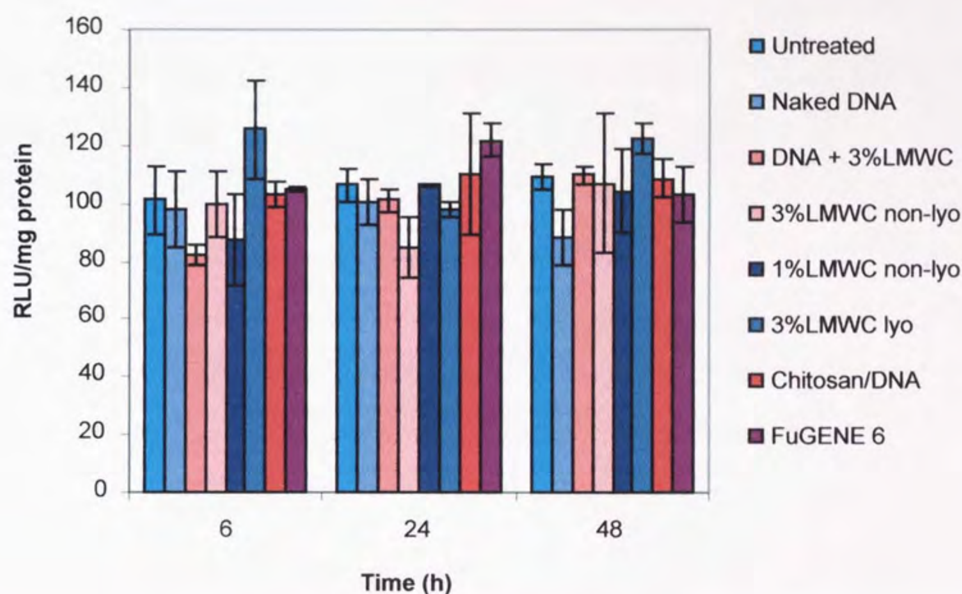


Figure 6.2.2 Luciferase expression in primary bone marrow derived dendritic cells. Cells were treated with naked luciferase plasmid DNA, DNA + blank PLG/3%LMWC microparticles, DNA-loaded PLG/3%LMWC or PLG/1%LMWC microparticles, DNA-loaded lyophilised PLG/3%LMWC microparticles, chitosan/DNA complexes or F6/DNA complexes (10μg DNA/well). Cells were assayed for luciferase expression (method 2.11.2) at 6h, 24h and 48h. Control cells were left untreated. The cell numbers in each well were estimated using the BCA protein assay (method 2.11.3).

Group	Formulation	Route	Mean IgG Titer	Lower IgG Titer	Upper IgG Titer
1	PLG/1%LMWC lyo 1μg	IM	7	6	8
2	PLG/1%LMWC lyo 10μg	IM	10	6	16
3	PLG/1%LMWC non-lyo 1μg	IM	71	39	129
4	PLG/1%LMWC non-lyo 10μg	IM	307	141	667
5	PLG/3%LMWC non-lyophilised 1μg	IM	5	5	5
6	PLG/3%LMWC non-lyo 10μg	IM	24	13	43
7	Naked DNA 1μg	IM	1228	865	1743
8	Naked DNA 10μg	IM	1396	920	2119

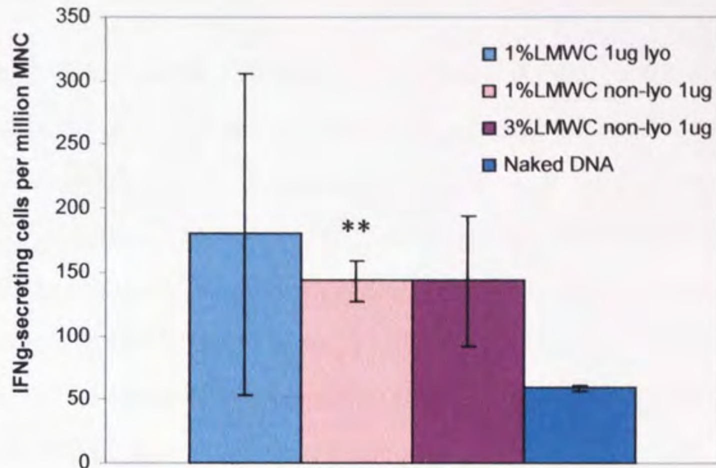
Table 6.3.1. Serum IgG responses in mice following immunisation with p55 gag DNA. BALB/c mice in groups of 10 were immunised with either naked DNA, lyophilised DNA-loaded microparticles stabilised with LMWC (1% or 3% w/v) or non-lyophilised DNA-loaded microparticles stabilised with LMWC (1% or 3% w/v). Each formulation was administered at two doses (1μg and 10μg DNA). Immunisations took place at weeks 0 and 4. HIV-1 p55 gag-specific serum IgG titers were quantified by an ELISA (method 2.13.3).

of serum total IgG titers in mice injected with PLG/LMWC particles is shown in table 6.3.1. At either dose (1μg or 10μg), anti p55 gag IgG titers were significantly higher in animals treated with naked DNA than they were in animals treated with any of the microparticle formulations. The antibody response in mice immunised with lyophilised PLG/1%LMWC microparticles or non-lyophilised PLG/3%LMWC microparticles at either DNA dose was negligible. The only formulation that elicited a small antibody response in the mice was non-lyophilised PLG/1%LMWC microparticles at the 10μg DNA dose. These results suggest that instead of enhancing the antibody response in mice, PLG/LMWC microparticles with adsorbed p55 gag plasmid DNA in some way repress the response.

6.3.2 IFN-γ-secreting cells in the spleen of mice following immunisation with p55 gag DNA.

Stimulation of cellular immunity by DNA-loaded PLG/LMWC microparticles was investigated in a mouse model. BALB/c mice (n=5) were immunised with DNA formulations by a single injection in the tibialis anterior (TA) muscles followed by harvesting of splenocytes at the 3 week time point. The DNA used in this experiment encoded the HIV-1 p55 gag protein and mice were immunised with either a 1μg or 10μg dose. Systemic $CD8^+$ T-cell responses in the spleen were measured by a gag epitope-specific IFN-γ ELISPOT assay (section 2.13.4). IFN-γ is secreted by CTLs ($CD8^+$) following their activation and proliferation. CTLs become activated when they recognise foreign antigen in conjunction with MHC class I on the surface of cells, and receive co-stimulatory signals (IL-2) from T-helper cells. Using the ELISPOT assay, IFN-γ secretion from activated CTLs ($CD8^+$) that specifically recognised p55 gag peptide in conjunction with MHC class I molecule on the surface of spleen cells was measured. The effect of DNA dose, microparticle stabiliser concentration and microparticle lyophilisation on the number of gag-specific IFN-γ-secreting cells in the spleen of mice immunized with PLG/LMWC microparticles is shown in figure 6.3.2. At a 1μg dose (6.3.2A) the number of IFN-γ- secreting cells was higher in animals treated with all three of the microparticle formulations (lyophilised PLG/1%LMWC, non-lyophilised PLG/1%LMWC and non-lyophilised 3%/LMWC) than in animals injected with naked DNA at this dose. However,

A.



B.

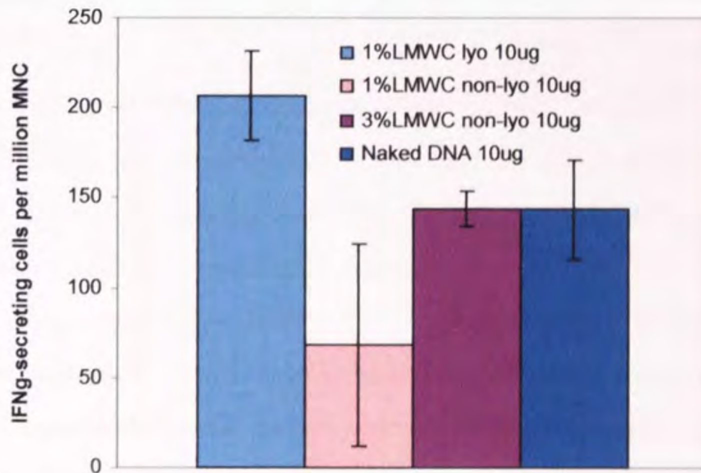


Figure 6.3.2 IFN-γ-secreting cells in the spleen of mice following immunisation with p55 gag DNA formulations. The immunisation protocol for the CTL study involved a single injection in the TA muscles of BALB/c mice ($n = 5$ mice per group) followed by harvesting of splenocytes at the three week time point. Systemic $CD8^+$ T-cell responses were measured by the ELISPOT assay (method 2.13.4). (A) Cells from mice immunised with a 1μg DNA dose. **Denotes p-values <0.01 compared to naked DNA results. (B) Cells from mice immunised with a 10μg DNA dose. The data are presented as the mean number of gag-specific IFN-γ-secreting cells per million mononuclear cells (MNC) ± s.d. of two independent experiments.

the difference was only statistically significant in animals treated with non-lyophilised PLG/1%LMWC microparticles ($p<0.01$), where the number of IFN-γ-secreting cells was approximately 3 fold higher.

At the higher 10μg dose (6.3.2B) only the spleens of mice injected with lyophilised PLG/1%LMWC microparticles exhibited higher numbers of IFN-γ- secreting cells than the spleens of mice injected with naked DNA, although the difference was not statistically significant. Numbers of IFN-γ- secreting cells in mice injected with non-lyophilised PLG/3%LMWC microparticles were comparable to numbers in animals treated with naked DNA, whereas mice injected with non-lyophilised PLG/1%LMWC microparticles had fewer IFN-γ-secreting cells. Overall, these results suggest that DNA-loaded PLG/LMWC microparticles, injected via the i.m. route, are capable of eliciting a cellular immune response in mice. However, this response only appears to be more potent than the response to naked DNA at lower DNA doses.

6.4 Discussion

In the previous chapter it was demonstrated that pCMV-km-Luc DNA-loaded PLG/LMWC microparticles were capable of efficiently transfecting C_2C_{12} myoblast and 293T kidney epithelial non-phagocytic cell lines. However, the PLG/LMWC delivery vector described here was specifically designed to target and introduce genes directly into phagocytic antigen presenting cells (APCs) to allow direct priming of both cytotoxic and helper T cell responses. In this way limitations of APC targeted peptide-based vaccines such as MHC molecule genetic polymorphism and the need to direct the protein-subunit into the endogenous antigen processing pathway for eliciting cytotoxic T cell responses can be avoided. The most potent APCs of the immune system are dendritic cells (DCs), and as such these cells are the most logical target for the development of genetic vaccine therapies (Banchereau & Steinman, 1998). DCs play multiple roles in the induction of potent immune responses within the body. These cells internalise, process and present antigen, migrate to lymphoid tissues, secrete cytokines and express co-stimulatory

molecules required for lymphocyte signalling. These multiple actions result in the activation of helper and cytotoxic T cells, and stimulate the release of antibodies.

Although DCs make ideal targets for the delivery of DNA vaccines, *in vitro* the transfection efficiency of DCs by non-viral methods has been extremely poor (Arthur *et al,* 1997). Physical and chemical methods of transfection such as electroporation, lipofectin and $CaPO_4$ which are used to efficiently transfect a variety of cell types are ineffective at transfecting primary DCs (Melero *et al,* 1999). In the study described here, it was demonstrated that neither DNA-loaded PLG/LMWC microparticles, nor any of the microparticle components were effective at transfecting the DC 2.4 cell line (fig 6.2.1) or primary BMDCs (fig 6.2.2). These results were not completely unexpected as previous studies, while describing efficient transfection of non-phagocytic cell lines by microparticulate DNA carriers, also reported inefficient transfection of professional APCs. Walter *et al* found only a small number of transfected RAW macrophages using DNA-loaded PLG/PEI microparticles compared to high transfection of non-phagocytic cells (Walter *et al,* 2002). Likewise, Erni *et al* who demonstrated efficient transfection of non-phagocytic cell lines using solid lipid microparticles with adsorbed DNA did not detect transfection in a macrophage cell line or in primary macrophages (Erni *et al,* 2002). There are a number of possible explanations for the observed inability of the PLG/LMWC microparticles to transfect DCs. Thiele *et al* studied the effect of size, surface-material and zeta potential of various microparticle formulations on uptake by human-derived macrophages (MPhis) and DCs and concluded that not only were microparticle size and surface charge important factors, but that phagocytosis was also influenced by the character of the bulk or coating material of the particle (Thiele *et al,* 2001). Although PLG/LMWC microparticles were designed to be of a size and charge favourable for phagocytosis by DCs, it is possible that localisation of chitosan at the particle surface may be unfavourable for uptake. Many studies have demonstrated the accumulation of microparticles and/or DNA within endosomal compartments of professional APCs following phagocytosis (Denis-Mize *et al,* 2000; Dupuis *et al,* 2000). If DNA accumulates in the endosomal compartments but is not capable of escaping before DNA degradation occurs, once again transgene expression will be prevented.

Finally, even if the DNA escapes from the endosome intact it must still be able to cross the nuclear membrane and enter the nucleus for gene expression to occur.

Alternatively, it is possible that transfection of DCs by PLG/LMWC microparticles did occur but was not detected by the luciferase assay. Denis-Mize *et al* demonstrated gene expression in bone marrow derived dendritic cells (BMDCs) transfected using PLG/CTAB microparticles by RT-PCR and detection of HIV gag peptide presentation in transfected cells (Denis-Mize *et al*, 2000). This *in vitro* model system used a T cell hybridoma to detect presentation of the HIV-1 gag-p7g epitope by antigen presenting cells. The group showed that DCs pulsed with PLG/CTAB-DNA encoding the HIV gag protein resulted in stimulation of the antigen specific T cell hybridoma to produce IL-2, while equivalent doses of naked plasmid could not. This technique is a sensitive and functionally relevant method for the detection of target antigen expression, processing and presentation in DCs. By comparison, in the present study, transfection of DCs by PLG/LMWC microparticles was evaluated using a luciferase reporter gene assay. This assay is neither as sensitive as the T cell hybridoma assay described above (Denis-Mize *et al* reported that as few as 10 BMDCs pulsed with synthetic p7g peptide could be detected), nor does it give information as to whether encoded antigen is processed and presented in the correct manner. Processing and presentation of encoded antigen are equally if not more important than efficient gene expression for successful DNA vaccination.

When Denis-Mize's group attempted to detect reporter gene activity (β-galactosidase) in transfected BMDCs they failed. In addition, following administration of fluorescently labelled DNA adsorbed to PLG/CTAB microparticles they could not detect fluorescence in the nuclei of the BMDCs. It is therefore quite possible that gene expression did occur in BMDCs transfected with PLG/LMWC microparticles albeit at levels below the sensitivity of the luciferase assay. There is evidence to suggest that the level of antigen expression and presentation in DCs does not have to be high to elicit potent responses *in vivo*. If the enhanced immunity observed in animal models immunised with PLG/CTAB microparticles (Singh *et al*, 2000; O'Hagan *et al*, 2001) is a result of direct DC

transfection, Denis-Mize *et al* estimated that less than 10% of the BMDC cell population expressed and presented antigen mediated by PLG/CTAB-DNA although presentation of the epitope was dose dependent (Denis-Mize *et al,* 2000).

The only other successful attempts to efficiently transfect primary DCs *in vitro* by non-viral methods resulted from the use of either plasmid DNA complexed with the cationic peptide CL22 (Irvine *et al,* 2000), or mannose polyethylenimine DNA complexes. In the latter case uptake occurred as a result of receptor-mediated endocytosis via mannose receptors, which are highly expressed on DCs (Diebold *et al,* 1999).

In the study described here, only F6 complexed with DNA was capable of transfecting the murine DC 2.4 cell line (fig 6.2.1) although these complexes were inefficient at transfecting primary BMDCs (fig. 6.2.2). Even though F6 was used as a positive control in these experiments the inability of the transfection reagent to transfect primary BMDCs was not unexpected. Commercially available lipid based transfection reagents such as F6 and lipofectamine are traditionally used as an effective means of transfecting many cell types *in vitro*. However, transfection of primary DCs by these agents has previously been demonstrated to be ineffective (Denis Mize *et al,* 2000). For a true positive control a recombinant viral vector or intracellular bacteria that can efficiently deliver genes encoding antigen to DCs would have been more appropriate (Specht *et al,* 1997; Song *et al,* 1997; Paglia *et al,* 1998). Denis-Mize *et al* used a recombinant vaccinia virus encoding HIV-p55gag protein as a positive control and demonstrated that T cell hybridoma IL-2 production (as a measure of transfection efficiency in primary BMDCs) in cells treated with PLG/CTAB-DNA was approximately 2% of that observed in cells transfected with the viral vector (Denis-Mize *et al,* 2000).

It is well known that gene transfer is limited to the state of cell proliferation and that transfection of primary BMDCs is relatively inefficient because the cells are not dividing. Previously, using fluorescence *in situ* hybridization (FISH) analysis for the detection of transfected plasmid DNA, it has been reported that in quiescent cells DNA is not transported efficiently into the nucleus (Wilke *et al,* 1996). This is because of the nuclear

membrane barrier. DNA can only reach the nucleus by one of two routes. It can either enter via the nuclear pore complex which has a functional diameter of only 26nm (fig 1.5.4), or it can enter when breakdown of the nuclear barrier occurs during mitosis. Non-dividing cell populations do not enter mitosis and therefore DNA can only enter the nucleus inefficiently via the nuclear pore complex resulting in very low levels of transfection (Hagstrom *et al;* 1997). In comparison, dividing cells are more easily transfected, as during mitosis the nuclear membrane disintegrates, allowing access to the nuclear transcription machinery. Therefore the difference in the ability of F6/DNA to transfect DC 2.4 cells and primary BMDCs could be attributed to the proliferative state of the cells. If the luciferase assay results give a true representation of the transfection efficiency of PLG/LMWC microparticles in DC2.4 cells and BMDCs then it is unlikely that the initial barrier to transgene expression is the nuclear membrane as DC2.4 cells are dividing. It is more probable that the limiting step is either lack of phagocytosis of the microparticles by DCs or inefficient release of DNA from the endosomal compartment leading to DNA degradation.

To date, DNA vaccines have a reputation for being more effective at priming T-cell responses than antibody responses in humans (Wang *et al,* 1998). This is not surprising when you consider how DNA vaccines are processed within the body. If immunisation with a DNA vaccine results in transfection of professional APCs and transgene expression, natural processing of encoded protein within the cell occurs and antigenic peptides are displayed on the cell surface in conjunction with MHC class I or MHC class II molecules. This leads to the activation of $CD4^+$ helper T cells and induction of cytotoxic T lymphocytes ($CD8^+$) and cellular immunity. If non-phagocytic cells are transfected and gene expression occurs, the encoded protein is digested inside the cell as part of the natural process by which a cell continually renews its protein content. Once digested, peptides are displayed on the cell surface in conjunction with MHC class I molecules and, in conjunction with co-stimulation by IL-2 secreted from activated $CD4^+$ cells, will trigger activation of circulating $CD8^+$ cells. By comparison, stimulation of humoral immunity by DNA vaccines requires the release of encoded antigenic peptides into the extracellular environment. In this environment, circulating immature B

lymphocytes can interact with the antigen, and, in conjunction with stimulatory signals from $CD4^+$ helper T cells, differentiate and divide into antibody secreting plasma cells or memory cells.

To determine whether PLG/LMWC microparticles were capable of inducing humoral and/or cellular immunity in mice, animals were immunised with either a 1μg or 10μg DNA dose. The number of DNA doses tested was kept to a minimum to reduce the number of animals used in the study. The PLG/LMWC microparticle DNA delivery system has been designed for potential use in humans therefore, taking into consideration the average body weight of the animal model, the chosen doses aimed to reflect what would be considered a high and a low DNA dose in the average human. In mice the higher (10μg) dose would be equivalent to a 2.8mg dose in the average adult human whereas the lower (1μg) dose would be equivalent to a 280μg dose. The higher dose was tested to determine whether the delivery vector enhanced the potency of the immune response at doses equivalent to the doses of naked plasmid DNA currently required to elicit an immune response in vaccinated humans (Calarota *et al,* 1998; Wang *et al,* 1998; MacGregor *et al,* 1998), and the lower dose was tested to assess if the potency of the vaccine could be maintained at decreased DNA concentrations. Furthermore, by using these doses, the *in vivo* efficacy of the PLG/LMWC microparticles in mice could be directly compared to the efficacy of PLG/CTAB microparticles in mice as equivalent doses were tested (Singh *et al,* 2000). In addition to DNA dose, the affect of the microparticle formulation on the immune response was also investigated. Non-lyophilised microparticle formulations i.e. microparticles in DDW that were produced fresh prior to immunisation were compared with dry, lyophilised microparticles reconstituted in DDW immediately prior to immunisation. It was expected that non-lyophilised DNA-loaded PLG/3%LMWC microparticles would give enhanced immune responses in immunised mice compared to non-lyophilised PLG/1%LMWC and lyophilised PLG/1%LMWC particles because they were of a size capable of targeting professional APCs (6.64μm, table 4.3.4). By comparison, DNA-loaded non-lyophilised and lyophilised PLG/1%LMWC microparticles were >15μm in size (15.9μm, table 4.3.4 and 16.4μm, table 3.4.7 respectively). The lyophilised microparticles were expected to

perform least well *in vivo* not only because of their size but also because non-lyophilised microparticles had previously been shown to be more efficient at transfecting non-phagocytic cell lines *in vitro* in particular myoblast cells (fig 5.3.5). By comparing these formulations it was possible to establish whether microparticle size, lyophilisation and *in vitro* transfection efficiency of non-phagocytic cells could be used to predict the *in vivo* performance of the delivery systems.

DNA-loaded PLG/LMWC microparticle formulations failed to stimulate humoral immunity in mice as demonstrated by the significantly lower HIV-1 p55 gag specific IgG titers compared to levels in mice immunised with naked DNA (fig 6.3.1). One possible explanation for these results is that antigen production was low as a result of ineffective transfection of cells *in vivo* by PLG/LMWC microparticles. This theory is supported by the results of the luciferase assay which suggest the particles are not capable of transfecting DCs *in vitro* (figs 6.2.1 and 6.2.2), although in chapter 5 it was demonstrated that PLG/LMWC microparticles were capable of efficiently transfecting non-phagocytic myoblast cells *in vitro* (fig 5.3.2). A second possible explanation, which would require further investigation, is that transfection of cells and gene expression does occur, but the presence of microparticles within the cell, by some unknown mechanism, suppresses the release of antigenic peptide into the extracellular environment, resulting in the observed inhibition of specific IgG production. In comparison to PLG/LMWC microparticles, immunisation of mice, guinea pigs and rhesus macaques with HIV-1 p55 gag DNA-loaded PLG/CTAB microparticles resulted in enhanced antibody titers compared to animals immunised with naked DNA (O'Hagan *et al,* 2001).

Although PLG/LMWC microparticles appear to be incapable of transfecting DCs *in vitro*, or enhancing antibody responses *in vivo*, enhancement of systemic $CD8^+$ T-cell responses, based on the measurement of intracellular IFN-γ production in spleen cells of immunised mice were observed for some formulations compared to naked DNA (fig 6.3.2). Because the mice were immunised by the i.m. route and $CD8^+$ T-cell activation was measured in the spleen this suggests that DCs presenting HIV-1 p55 gag specific epitope migrated from the site of immunisation to the lymphoid tissue. It is possible that

the observed enhancement in T-cell response was the result of direct transfection of DCs at the injection site followed by expression, processing and presentation of the antigenic peptide. However, since PLG/LMWC microparticles were not detectably capable of transfecting DCs *in vitro* (figs 6.2.1 and 6.2.2) direct transfection of these cells *in vivo* may not be responsible. Instead, activation of T-cell responses may be a result of cross priming of DCs by other non-phagocytic cells. Ulmer *et al* demonstrated that *in vivo* expression of influenza nucleoprotein (NP) by muscle cells was sufficient to induce immune responses in the form of both antibodies and cytotoxic T lymphocytes (CTL) (Ulmer *et al*, 1997). These findings suggest that if PLG/LMWC microparticles were capable of transfecting muscle cells *in vivo* as efficiently as they transfect myoblast cells *in vitro* (fig 5.3.2), this may be sufficient to stimulate the observed response. Although it is possible that myocytes process, and present antigen in association with MHC class I directly to T cells, muscle cells are not considered to be antigen presenting cells. This is because these cells are not thought to express co-stimulatory molecules critical for the induction of long-lived and potent CTL responses (Schwartz, 1992). Antigen is more likely to be transferred from the muscle cell to professional APCs, and this is termed cross priming. The means by which muscle-expressed antigen could be internalized by antigen presenting cells is unclear but may involve phagocytosis, transfer of vesicles (Raposo *et al,* 1996), or heat shock proteins (Suto & Srivastava). Ulmer *et al* demonstrated that injected exogenous NP protein did not induce CTL (Ulmer *et al,* 1996). These findings suggest that cross priming does not occur as a result of exogenous protein released from the muscle cells. *In vivo* studies using PLG/LMWC microparticles support these findings. If cross priming were a result of protein released extracellularly, PLG/LMWC microparticles could potentially elicit an antibody response in mice.

In vivo, PLG/LMWC microparticles were frequently found to be either less effective, or not significantly more effective than naked DNA at eliciting immune responses in mice. However, at the lower dose (1μg DNA), T-cell responses in mice treated with non-lyophilised DNA-loaded PLG/1%LMWC microparticles were 3-fold higher than in mice treated with naked DNA (fig 6.3.2A), and all animals immunised with microparticle formulations at this dose exhibited enhanced T-cell responses. By comparison, at the

higher (10μg) DNA dose, none of the microparticle formulations significantly enhanced T-cell responses compared to naked DNA (fig 6.3.2B). These results suggest that at lower DNA concentrations PLG/LMWC microparticles have an adjuvant effect on T-cell responses and this attribute could be exploited in DNA vaccine production. One of the major problems in developing DNA vaccines for use in humans is that at present, high doses of DNA are required (>1mg) to elicit an immune response (Calarota *et al,* 1998;Wang *et al,* 1998; MacGregor *et al,* 1998). The use of such high doses is not desirable from either a safety or a commercial point of view. Therefore, one of the key parameters in designing new DNA vaccine delivery vectors is to reduce the DNA dose whilst maintaining the potency of the vaccine.

In this work a general lack of correlation between the *in vitro* and the *in vivo* performance of the microparticle formulations was observed. It was expected that larger DNA-loaded microparticles would not perform as well as smaller particles *in vivo* because they would not be efficiently phagocytosed by professional APCs. None of the formulations were capable of eliciting antibody responses in mice. However, of the formulations tested *in vivo* non-lyophilised PLG/1%LMWC elicited the greatest enhancement in T-cell responses compared to naked DNA at a 1μg dose (fig 6.3.2A). This result was unexpected as DNA-loaded, non-lyophilised PLG/1%LMWC microparticles were larger (>15μm) than non-lyophilised PLG/3%LMWC microparticles (approximately 6μm) which performed less well. Since efficient phagocytosis only occurs with microparticles <10μm, this suggests that phagocytosis by APCs may not be the mechanism of action of PLG/LMWC microparticles. Instead, cross priming of DCs by myocytes transfected with smaller (<100nm) DNA/stabiliser complexes released from the particle surface may account for the observed response. However, if this were the case non-lyophilised 3%PLG/LMWC microparticles would still be expected to perform better *in vivo* as these particles were more effective at transfecting myoblasts *in vitro* than PLG/1%LMWC microparticles. One possible explanation for the observed disparity is that different plasmids were used for the *in vitro* and *in vivo* studies. For the *in vitro* experiments a luciferase reporter plasmid was used whereas *in vivo* the plasmid used encoded the HIV-1 p55 gag protein. Previously it has been demonstrated with other delivery systems that the

type of plasmid not only affects *in vitro* DNA loading and release characteristics but also the immune response *in vivo* (Briones *et al,* 2001). Furthermore, in the present study although PLG microparticles with different chitosan contents had different adsorption efficiencies and release rates for DNA, the magnitude of the *in vivo* T-cell response did not seem to be greatly affected by higher rates of DNA release. In fact PLG/1%LMWC microparticles which released the greatest quantity of DNA (fig 4.3.5) performed the best at the 1μg DNA dose (fig. 6.3.2A). This finding suggests that potentially the quantity of chitosan in the formulation could be reduced further without loss of efficacy thus minimising toxicological concerns. Finally, although *in vitro* lyophilised microparticles were not capable of transfecting non-phagocytic cells (fig. 5.3.5), *in vivo* T-cell responses in mice immunised with lyopophilised PLG/LMWC microparticles were comparable to the responses in mice immunized with non-lyophilised microparticle formulations. Indeed at the 10μg DNA dose this formulation performed the best (fig 6.3.2B). These findings highlight the frequently encountered problem of establishing meaningful correlations between *in vitro* and *in vivo* performance of DNA delivery systems.

In summary, PLG/LMWC microparticles were ineffective at transfecting either the dendritic cell line DC 2.4 or primary BMDCs *in vitro. In vivo*, mice immunised with PLG/LMWC microparticles exhibited lower serum IgG titers than mice immunised with naked DNA. However, at the lower DNA dose (1μg) an enhancement in T-cell responses was observed in mice immunised with microparticle formulations compared to animals immunised with naked DNA. In mice immunised with DNA-loaded non-lyophilised PLG/1%LMWC this enhancement was statistically significant ($p<0.01$). Findings suggest that T-cell responses may occur as a result of cross priming of DCs by non-phagocytic cells, particularly muscle cells, rather than by direct transfection and priming of professional antigen presenting cells.

7. General discussion and conclusions

In an ideal world a vaccine would be safe, induce a potent immune response in the recipient after only one-dose, inexpensive to manufacture and easy to administer. In addition, booster shots would not be required. Unfortunately at present no vaccine meets all these requirements. However, DNA vaccines are relatively economical to produce, safe to use and are capable of eliciting both cellular and humoral immunity, and as such meet many of the above criteria. Currently the greatest hurdle to using these vaccines for clinical use in humans is that multiple injections of large quantities (>1mg) of DNA are required to elicit an immune reaction and often humoral immunity is not stimulated (Calarota *et al,* 1998; Wang *et al,* 1998; MacGregor *et al,* 1998). Therefore, the development of safe delivery technologies for clinical use that enhance the magnitude of the immune response to DNA vaccines with lower and fewer doses is ongoing (Singh & O'Hagan,1999; Dubensky *et al,* 2000) with particular emphasis on developing systems that effectively boost the humoral response. To enhance DNA vaccine potency the design of vectors that specifically target professional APCs in particular DCs has become a particular focus as these cells are known to be very important in stimulating both cellular and humoral immunity (Banchereau & Steinman, 1998). By designing APC-transfecting nucleic acid based vaccines the limitations of peptide-based vaccines imposed by genetic polymorphism in MHC epitope selection can be eliminated. Furthermore, DNA-vaccines that directly transfect professional APCs reduce the need to direct protein-subunit vaccines into the endogenous antigen processing pathway for eliciting cytotoxic T cell responses.

The overall objective of the research presented in this thesis was to develop a non-viral, cationic microparticulate DNA vaccine delivery vector capable of targeting professional APCs based on a system previously described by Singh *et al.* Singh's group designed cationic cetyltrimethyl ammonium bromide (CTAB) surfactant stabilised PLG microparticles with surface adsorbed DNA (Singh *et al,* 2000). The PLG/CTAB microparticles were capable of transfecting DCs *in vitro* and enhanced cellular and humoral immune responses in mice, guinea pigs and non-human primates' *in vivo* (Singh

et al, 2000; O'Hagan *et al,* 2000). This novel vaccine delivery technology could therefore potentially stimulate both cellular and humoral immunity in human recipients and reduce the DNA dose required for effective immunisation. However, cationic surfactant stabilised microparticles have not been comprehensively evaluated toxicologically in humans and therefore their safety for long term use is questionable. In this study the replacement of CTAB with a more toxicologically acceptable substitute was attempted, with the emphasis on maintaining the efficacy of the formulation. A variety of cationic systems were evaluated using the cationic polymers PVP-PDAEMA and chitosan as alternatives to the surfactant CTAB.

Out of the formulations investigated PLG microparticles stabilised with low molecular weight chitosan (PLG/LMWC) showed the greatest potential as a safer but comparable DNA vaccine delivery system to PLG/CTAB microparticles. Both PLG and chitosan are considered to be biodegradable, biocompatible and non-toxic polymers (Hirano *et al,* 1990; Chandy & Sharma, 1990; Struszczyk *et al,* 1991; Brine *et al,* 1992; Anderson Shire, 1997) and the lack of toxicity of chitosan in short term cell cultures was confirmed experimentally here. In addition, the particles displayed physicochemical characteristics which were expected to help overcome some of the biological barriers to efficient transgene expression. These barriers include passage across the cell membrane, degradation of DNA within the cells lysosomes and crossing the nuclear membrane. It was envisaged that the cationic nature of the microparticles would promote electrostatic interaction with the negatively charged cell surface proteoglycans and could enable efficient internalisation into target cells (Mislick & Baldeschwieler, 1996). In addition, chitosan has been reported to have a membrane destabilising effect (Fang *et al,* 2001; Yang *et al,* 2002) which may enhance particle uptake. The size of the microparticles (approx 3-4μm) was also selected to encourage passive targeting of professional APCs and allow for exploitation of the cells phagocytic uptake mechanism. Furthermore, if like PEI the polycation chitosan acts like a 'proton sponge' resulting in endosomal swelling and rupture (Haensler & Szoka, 1993; Boussif *et al,* 1995; Remy *et al,* 1998; Pollard *et al,* 1998), the inclusion of chitosan in the delivery system could enhance escape from

endosomal or phagosomal cell compartments prior to fusion with the acidic lysosome preventing degradation of the DNA.

Although PLG/LMWC microparticles were designed in such a way as to favour targeting of professional APCs, *in vitro* it was observed that although the particles were efficient at transfecting non-phagocytic cells, levels of transfection in DCs were not detectable. Since the microparticles were too large to be taken up whole by non-phagocytic cells two possible hypotheses have been suggested. Transfection by DNA-loaded PLG/LMWC microparticles was either occurring as a result of particles docking on the cell surface resulting in a localised increase in DNA concentration and enhanced transfection levels or, alternatively, nanometer sized chitosan/DNA complexes which could be taken up by endocytosis or pinocytosis were released from the particle surface leading to cell transfection. The latter theory is supported by evidence that efficient transfection of non-phagocytic cells *in vitro* can be achieved using chitosan/DNA complexes (Vankatesh & Smith, 1997; Erbacher *et al,* 1998; Sato *et al,* 2001; Mao *et al,* 2001).

From the present study it became apparent that the *in vitro* transfection efficiency of PLG/LMWC microparticles in non-phagocytic cells was linked to both the cell type, and the viability of treated cells. In general, 293T cells were not only transfected more efficiently by PLG/LMWC microparticles than C_2C_{12} myobalst cells but the viability of these cells remained higher over the incubation period. These findings were unusual as frequently high transfection efficiency by gene delivery systems is associated with increased cytotoxicity. Corsi *et al* demonstrated lower transfection efficiency of DNA-chitosan nanoparticles in cell lines where viability remained high (human osteosarcoma (MG63) or mesenchymal stem (MSC) cells). By comparison lower cell viability in human embryonic kidney (HEK 293) cells correlated with increased transfection efficiency (Corsi *et al,* 2003). Similarly, in the present study an inverse relationship between cell viability and transfection efficiency was observed in C_2C_{12} myoblast cells treated with PLG/LMWC microparticles.

The difficulty of predicting the *in vivo* behaviour of a DNA vaccine delivery system from the *in vitro* data was highlighted in this study. *In vitro* the incorporation of chitosan in the PLG microparticles could be varied resulting in formulations with different DNA loading and release characteristics and different transfection efficiencies of non-phagocytic cells. However, *in vivo,* no correlation could be drawn between the respective performances of the PLG/LMWC microparticle formulations and *in vitro* DNA release and transfection data. Similarly, Singh *et al.* reported that the performance of PLG/CTAB microparticle formulations *in vivo* were not affected by the concentration of CTAB stabiliser, and that particles with low CTAB loading levels and high *in vitro* DNA release performed as well as particles that contained more CTAB but had lower *in vitro* DNA release (Singh *et al,* 2003). The behaviour of lyophilised PLG/LMWC microparticles *in vivo* was also unexpected based on *in vitro* data. *In vitro* these particles were incapable of transfecting any of the cells tested. However, *in vivo* at the 10μg DNA dose systemic T-cell responses were higher than for other PLG/LMWC formulations. These findings suggest that size may not be a critical factor in immune stimulation by PLG/LMWC microparticles as lyophilised particles were >15μm in diameter and would not be efficiently phagocytosed by professional APCs. By comparison Singh *et al* demonstrated that for PLG/CTAB microparticles the potency of the immune response in mice was dependent on their size and that smaller microparticles stimulated a greater response (Singh *et al,* 2000).

Although *in vitro* PLG/LMWC microparticles share characteristics in common with the PLG/CTAB system described by Singh *et al.* such as positive surface charge, micrometer size distribution and comparable DNA release profiles, the two systems behave quite differently *in vivo*. Unlike PLG/CTAB particles DNA-loaded PLG/LMWC microparticles were incapable of eliciting a humoral immune response in immunised mice. In fact, the antibody response to DNA-loaded PLG/LMWC microparticle formulations was lower than in mice immunised with naked DNA. Clearly further investigation is required to establish why such seemingly similar delivery systems behave so differently *in vivo* and by what mechanism PLG/LMWC microparticles suppress the production of antibodies in mice. It is suggested here that the chitosan component of the formulation may be involved. Either the chitosan prevents the particles from transfecting DCs thus limiting

the potency of the antibody response, or, alternatively, transfection of DCs does occur but the chitosan either prevents correct processing and presentation of the peptide by DCs, or prevents release of protein into the extracellular environment from transfected cells. However, a number of reports have demonstrated the production of antibodies *in vivo* following topical, oral or intranasal immunisation with chitosan/DNA complexes or DNA chitosan nanoparticles suggesting chitosan alone does not suppress antibody production (Roy *et al,* 1999; Cui & Mumper, 2001; Kumar *et al,* 2002).

Systemic T-cell responses in mice immunised with DNA formulated on PLG/LMWC microparticles at the lower dose tested (1µg) were enhanced compared to responses in mice immunised with naked DNA. This led to the conclusion that antigen presentation by DCs must be occurring following immunisation. However, because the luciferase assay used in this study may not have been sensitive enough to detect DC transfection, further research is required to establish whether DCs are being directly transfected by microparticles or whether cross-priming of DCs is occuring. If direct transfection of DCs is occurring this could be established using the sensitive *in vitro* H-2K^d-restricted T-cell hybridoma assay described by Denis-Mize *et al* (Denis-Mize *et al,* 2000). Only very low transfection levels of DCs may be required to elicit an immune response as efficient antigen presentation and recognition can compensate for inefficient transfection (Fynan *et al,* 1993). However, from the evidence available in this study the mechanism of action of the systemic T-cell response to PLG/LMWC microparticles appears to be cross priming of DCs by myocytes at the sight of injection (Fu *et al,* 1997). This theory is supported by the fact that DNA-loaded PLG/LMWC microparticles were capable of efficiently transfecting non-phagocytic cells including myoblasts *in vitro.* In mice at the higher DNA dose tested (10µg) the systemic T-cell response to most DNA-loaded microparticle formulations was equivalent to naked DNA. These findings suggest there may be a ceiling of T-cell responsiveness. This theory was first put forward by O'Hagan *et al* who observed that in mice immunised with 10µg DNA formulated on PLG/CTAB microparticles equivalent CD8$^+$ T-cell responses were seen with naked DNA (O'Hagan *et al,* 2001). If at higher DNA doses the immune response levels off then a more relevant indicator of DNA vaccine potency may be enhancement of response at low doses. To date

CTL responses in humans have only been reliably seen with high doses of DNA (>1mg) so delivery systems which are capable of reducing the dose whilst maintaining potency are particularly desirable.

One of the biggest advantages of using a microparticle vector as a carrier for a DNA vaccine is the flexibility of the system. There are a variety of ways in which the response to the vaccine could be enhanced. Bioactive components such as adjuvants or protein antigens to stimulate humoral immunity could be entrapped within microparticles with surface adsorbed DNA. Alternatively, a separate population of microparticles with entrapped or adsorbed adjuvants could be co-administered with the DNA vaccine to further enhance the levels of immunity. Singh *et al* demonstrated significantly enhanced immune responses in mice immunised with PLG/CTAB microparticles with the addition of the adjuvant aluminium phosphate compared to mice immunised with microparticles alone (Singh *et al,* 2000). Mixed populations of microparticles could also be produced, with each population carrying a plasmid encoding a gene from a different pathogen. Studies have also demonstrated that cationic microparticles may facilitate the development of combination vaccines whereby two plasmids encoding different antigens could be adsorbed to the same population of particles. The formulation of plasmids encoding either the HIV gag or env proteins with PLG/CTAB microparticles resulted in loss of the hyporesponsiveness to the env component previously observed in guinea pigs immunised with naked DNA preparations of the two plasmids (O'Hagan *et al,* 2001). Because microparticle systems are not restricted by the size of the plasmid that can be delivered, it may also be possible to produce single plasmids which encode genes for several pathogens or for different strains of a virus. This approach may be particularly relevant for immunising against diseases such as influenza and HIV where the subtype of the virus changes regularly. Likewise, the plasmid could be modified to contain genes encoding adjuvants. Miyahira *et al* recently demonstrated that the RANK L gene encoding the ligand to receptor activator of NF-κβ implicated in T-cell priming by DCs co-administered with a *Trypanasoma cruzi* gene markedly enhanced the induction of *Trypanasoma* Ag-specific $CD8^+$ T-cells and improved the DNA vaccine efficacy in mice (Miyahira *et al,* 2003).

The results presented in this thesis show that DNA-loaded PLG/LMWC microparticles are an effective system for transfection of non-phagocytic cells *in vitro.* By contrast, the particles appear to be ineffective at targeting and transfecting professional APCs. *In vivo* the particles do not elicit humoral immunity in mice although at lower DNA doses systemic T-cell responses are enhanced compared to naked DNA. PLG/LMWC microparticles therefore although potentially safer for human use, demonstrate some significant disadvantages over PLG/CTAB microparticles as a delivery system for DNA vaccines. Further research is necessary to clarify how the particles transfect non-phagocytic cells, whether they directly transfect DCs, why antibody suppression occurs *in vivo* and how the particles induce systemic T-cell activation. However, this system does offer potential in vaccine delivery as PLG/LMWC microparticles can enhance T-cell responses at reduced DNA doses. To develop this delivery system further a wider range of chitosans and chitosan derivatives could be investigated as stabilisers for the PLG particles. Chitosan derivatives such as deoxycholic acid modified-chitoasn (Kim *et al,* 2001; Lee *et al,* 1998) and trimethyl chitosan oligomers (TMO) (Thanou *et al,* 2001) have been used in gene delivery research to enhance transfection efficiency, whilst the derivative trimethyl chitosan chloride (TMC) has been demonstrated to be soluble at neutral and basic pH (Thanou *et al,* 2001). In addition, chitosan is amenable to ligand modification and conjugates have been exploited for targeted gene trafficking. Galactosylated chitosan-graft-dextran-DNA complexes have been shown to efficiently transfect Chang liver cells expressing asioglycoprotein *in vitro* indicating a specific interaction of the galactose ligands bound to chitosan with this receptor (Park *et al,* 2000). Trimethylated chitosan polymers, bearing antennary galactose residues specifically targeted and transfected Hep-G2 cells expressing the galactose receptor and transferrin-KNOB protein conjugated chitosan vectors (Mao *et al,* 2001) have also been used to target cells *in vitro.* It may therefore be feasible to produce chitosan with a conjugated ligand that would aid targeting of the microparticles to professional APCs. For example, mannose receptors are over expressed on DCs and Diebold *et al* previously demonstrated efficient transfection of DCs by mannose PEI/DNA (Diebold *et al,* 1999).

The poor humoral immune response to PLG/LMWC microparticles could be redressed by incorporating protein antigen or vaccine adjuvants into the formulation or alternatively PLG/LMWC vaccine could be used as a mixed modality or prime-boost vaccination. This system involves initial vaccination by one type of vaccine followed by boosting with a different type of vaccine. Using this approach promising preclinical results have been obtained by immunising first with DNA then boosting with a vaccinia or adenovirus vector encoding the same antigen, or with a recombinant protein version of the same antigen that the DNA vaccine encoded.

Although the production of a single dose, non-viral DNA vaccine still seems distant the cationic microparticle DNA delivery technology described here could be used to complement other vaccine systems and aid the future production of safer, more effective and longer lasting vaccines.

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