Development of Microparticulate DNA Vaccines for Pulmonary Delivery

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

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June 2007
Declaration of Plagiarism and Copyright

This thesis describes research conducted in the School of Pharmacy, University of London between 19 June 2003 and 19 June 2006 under the supervision of Professor Oya Alpar and Doctor Frank Koppenhagen (3M) and Doctor Chris Blatchford (3M).

I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

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Abstract

DNA vaccines have emerged to be an alternative to conventional vaccines due to their increased safety compared with recombinant protein vaccines and live/attenuated vaccines. Pulmonary delivery of vaccines could offer additional advantages, because from an immunological point of view, there is a large presence of macrophages for antigen/DNA uptake and it also part of the mucosal immune system, which is important for effective immune protection. Encapsulating the DNA into a particulate delivery system would be advantageous for protection against degrading nucleases.

The aim of this project was to produce microparticles encapsulating plasmid DNA with a high loading efficiency, appropriate size suitable for pulmonary delivery and with the maintenance of plasmid DNA conformation.

Microparticles were produced using poly(DL-lactide-co-glycolide) as the polymer due to its well-known biodegradable and biocompatibility characteristics, and polyvinyl alcohol (PVA) as the main stabiliser. Plasmid DNA encoding for luciferase protein was used as a model plasmid for studies. Water-in-oil-in-water (W/O/W) was used as the method for making particles.

Factorial experimental design was used to rapidly optimize a formulation that had the desired properties. Further studies examined the effect of adding various excipients (0.1M NaHCO₃, 1% m/m Na₂HPO₄) on the loading, diameters, morphology and charge on the microparticles.

Analytical (including TGA, NMR and DSC) and cell culture experiments - using a human alveolar cell line (A549) and mouse macrophage/monocyte cell line (J774A.1) - were carried out to analyse their physical and biological properties.

The factorial experimental design aided in choosing a platform formulation, with particle diameters of 2-5 μm and loading of 66-72% m/m. Particles without added excipients had low plasmid DNA loading and the DNA had lost most of its original conformation. However, addition of buffers into the aqueous phases, particularly 1% m/v NaH₂PO₄, reduced the loss of conformation and enhanced loading efficiency.

Physicochemical studies gave mixed results on the formulations tested in terms of thermal stability, DCM and PVA levels and performance as a dry powder. Results demonstrated that microparticles made without buffer were more thermally stable, had lower residual DCM and PVA levels in contrast to those made with 1% m/v Na₂HPO₄ and 0.1M NaHCO₃. The microparticles did not aerosolise well as a dry powder - even with addition of surfactants such as lecithin and DPPC - with diameters between 69-95 μm being measured, which is in contrast to the diameters measured in a ‘wet’ state (2-5 μm).

Cell culture studies demonstrated that entrapped DNA (which was extracted out from the particles and delivered to cells using a commercial agent) was still biologically active, but the A549 cell line was much easier to transfect than the J774A.1 cells. However, the microparticles themselves were poor delivery vehicles of DNA in these models.

Overall, these experiments demonstrated the range of factors that need to be considered when trying to create suitable microparticulate carriers for pulmonary delivery of DNA vaccines.
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<th>Description</th>
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<tbody>
<tr>
<td>16HBE14o-</td>
<td>human bronchial epithelial cell</td>
</tr>
<tr>
<td>293T</td>
<td>human kidney epithelial cell line</td>
</tr>
<tr>
<td>A549</td>
<td>human alveolar epithelium cell line</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BCG</td>
<td>bacilli Calmette- Guérin</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer, Emmett, Teller</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>Calu-3</td>
<td>human sub-bronchial gland cell line</td>
</tr>
<tr>
<td>CFC</td>
<td>chlorofluorcarbon</td>
</tr>
<tr>
<td>CpG</td>
<td>cytidine-phosphate-guanosine motifs</td>
</tr>
<tr>
<td>COS-7</td>
<td>monkey kidney fibroblast</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane (methylene chloride)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DofE</td>
<td>Design of Experiment</td>
</tr>
<tr>
<td>DPI</td>
<td>dry powder inhaler</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>DPTA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>ds</td>
<td>double-stranded</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPI</td>
<td>Expanded programme of immunization</td>
</tr>
<tr>
<td>EU</td>
<td>endotoxin unit</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FID</td>
<td>flame-ionized detector</td>
</tr>
<tr>
<td>GRAS</td>
<td>generally regarded as safe</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney epithelial cell line</td>
</tr>
<tr>
<td>HeLa</td>
<td>human cervix epithelial cell line</td>
</tr>
<tr>
<td>HFA</td>
<td>hydrofluoroalkane</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGC</td>
<td>inverse gas chromatography</td>
</tr>
<tr>
<td>IHNV</td>
<td>Infectious Haematopoietic Necrosis Virus</td>
</tr>
<tr>
<td>i.m</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>J774A.1</td>
<td>mouse monocyte/macrophage cell line</td>
</tr>
<tr>
<td>kcps</td>
<td>kilo counts per second</td>
</tr>
<tr>
<td>KSCN</td>
<td>potassium thiocyanate</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LD_{50}</td>
<td>lethal dose, 50%</td>
</tr>
<tr>
<td>M</td>
<td>Mole</td>
</tr>
<tr>
<td>mA</td>
<td>milliamps</td>
</tr>
<tr>
<td>MANOVA</td>
<td>multiple analysis of variance</td>
</tr>
<tr>
<td>MDI</td>
<td>metered-dose inhaler</td>
</tr>
<tr>
<td>mM</td>
<td>millimole</td>
</tr>
<tr>
<td>m/m</td>
<td>mass per mass</td>
</tr>
<tr>
<td>mV</td>
<td>millivolt</td>
</tr>
<tr>
<td>m/v</td>
<td>mass per volume</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MVD</td>
<td>mean volume diameter</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium hydrogen carbonate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>sodium phosphate (dibasic)</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>sodium sulphate</td>
</tr>
<tr>
<td>NaBr</td>
<td>sodium bromide</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NaClO₄</td>
<td>sodium perchlorate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
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</table>
NaSCN  sodium thiocyanate
NIH 3T3  mouse embryo fibroblast cell line
NMR  nuclear magnetic resonance
o/c  open-circular
OFAT  one-factor-at-a-time
OLA  oligolactic acid
OVA  ova serum albumin
PAA  poly (aspartic acid)
PBS  phosphate buffered saline
pCMV  Cytomegalovirus promoter
PCS  photon correlation spectroscopy
PEG  polyethylene glycol
PEI  polyethylenimine
PLGA  poly(dl-lactic acid-co-glycolide)
PLG  poly glycolide
PLL  poly-l-lysine
pMDI  pressurised metered-dose inhaler
psi  pounds per square inch
PVA  Poly (vinyl alcohol)
PVP  Poly (vinyl pyrrolidone)
RLU  relative light units
RNA  ribonucleic acid
RPM  revolutions per minute
s  second(s)
s.c  subcutaneous
s/c  supercoiled
SDS  sodium dodecyl sulphate
SEM  scanning electron microscopy
TB  tuberculosis
TE  tris-EDTA
TBE  tris-borate-EDTA
Tg  glass transition temperature
TGA  thermogravimetric analysis
Tris  tris (hydroxymethyl) aminomethane
UV  ultra violet
V   volts
v/v volume per volume
w₁/o/w₂ water-in-oil-in-water
WHO World Health Organization
WNV West Nile Virus
XPS x-ray photoelectron spectroscopy
Chapter 1

General Introduction
Pulmonary delivery of microparticulate DNA vaccines is a new area in research and is a challenging topic for discussion. It covers many areas including, vaccines in general, DNA vaccines specifically, the pulmonary system and methods to deliver vaccines. A brief overview of each subject will be given below.

1.1. Vaccines

Success: Vaccines have eradicated smallpox and reduced mortality and morbidity of many other infectious diseases.

Challenges: There are still no vaccines for diseases such as malaria or human immunodeficiency virus (HIV), and there is a need for better vaccines for diseases such as tuberculosis (TB) and hepatitis B.

There is no doubt that vaccines have been one of the major success stories in scientific history. Over 2 million deaths are currently averted through immunization each year and this could increase to four to five million by the year 2015 (WHO, 2006). However, as mentioned above, there are still many diseases for which there are no vaccines available, or are not optimal.

1.1.1. Traditional Approaches to Vaccination

Traditionally vaccines are developed using 4 main methods, which are summarized as follows (Bloom, 1989; Roitt et al., 1998):

1) Live/attenuated vaccines (e.g. bacilli Calmette-Guérin (BCG), smallpox) – were developed by trial and error and those that are used today have advantages in that they are inexpensive to produce. Disadvantages occur through the risk of the vaccine reverting to full virulence, extreme immune reactions and the need for cold-chain transport and storage.

2) Killed vaccines (e.g. polio, rabies) – these are the simplest and cheapest to prepare and usually induce an immune response in all individuals that receive them. However, production methods can lead to varied strengths of the vaccines, and careful monitoring is required to ensure that all the microorganisms have been killed. As with live/attenuated vaccines, there is also the risk of extreme immune reactions to these types of vaccines.
3) Sub-unit vaccines (e.g. hepatitis B, *Haemophilus influenza* b) – these are products of a small part of the microorganism, usually the surface antigens that the immune system sees first and responds to. These are chemically defined and are produced in a reproducible manner. However, several injections are usually required before long-term immunity is provided.

4) Inactivated toxins/toxoids (e.g. tetanus toxoid, diphtheria toxoid) – these are based on the exotoxins produced from an organism, and are produced in the same manner as sub-unit vaccines. They also need several injections before long-term immunity is produced.

Although successful so far, inherent risks of these types of vaccines - including adverse reactions to the vaccine or its components, especially for the young or immunocompromised, or the lack of long-term immunity for inactivated pathogens, as they are not as immunogenic as ‘live’ versions and do not induce a cytotoxic (cellular) immune response (Gregoriadis, 1998) - mean that improvements need to be made.

With no vaccines currently available for diseases such as malaria or HIV; and in this modern age, with the added threat of bioterrorism with agents such as anthrax and botulinum toxin (Garmory et al., 2005), there is an urgent need to develop new methods of vaccination.

**1.1.2. Future Vaccines**

Vaccines ideally should have an impact on a global scale, and so far, this has only been achieved with smallpox and polio. This was due to the World Health Organisation’s (WHO) expanded programme of immunisation (EPI). Set up in 1974, building on the success of smallpox eradication, the WHO set their sights on vaccinating children all over the world against other diseases. The six diseases chosen, based on disease burden and trusted vaccine history, were TB, diphtheria, neonatal tetanus, whooping cough, polio and measles - yellow fever and hepatitis B added later (Bland and Clements, 1998). Today, to be considered
for inclusion into the EPI any new or improved vaccine should ideally have the following attributes (Bloom, 1989):

1) Inexpensive;
2) Must be extremely effective, offering immune protection in 90-100% of recipients;
3) Should produce lifetime immunity;
4) Heat-stable and need not have cold-chain requirements;
5) Should only require one-shot, or fit in with the schedule of other vaccines;
6) Simple to give and ideally via a non-invasive route;
7) Should be able to be given to neonates or as close to birth as possible (i.e. not be affected by maternal antibodies).

No longer is it just a case of producing a vaccine that works, economics is a factor too, and in the next few sections are methods that are being used to address the above points.

1.2. Mucosal versus Systemic Immunity

Success: Most vaccines administered today give excellent immune protection.

Challenges: Systemically administered vaccines however, do not offer mucosal immunity.

The respiratory, gastrointestinal and urogenital tracts are lined with a mucosal layer, which are the primary sites of interaction between the pathogen and its environment (Dietrich et al., 2003). For many pathogens, such as 
*Vibrio cholerae*, *Escherichia coli*, rotaviruses, influenza virus and HIV, the mucosal surface is their first point of entry, and vaccines that are delivered by the traditional intramuscular (i.m) or subcutaneous (s.c) route induce strong systemic responses but generally no mucosal immunity (McCluskie and Davis, 1999; Holmgren et al., 2003). By contrast, if a vaccine is administered to a mucosal surface, both systemic and mucosal immunity is induced (Jones et al., 1997b; Kaneko et al., 2000; Wikingsson and Sjoholm, 2002; He et al., 2005).
The mucosa at the respiratory, gastrointestinal and urogenital tracts share similar characteristics, such as the M-cells, which constantly sample the environment, and together they make up the common mucosal immune system. It is beyond the scope of this introduction to describe the full details of the immunology of the mucosal immune system, but interested readers are directed to a number of excellent reviews (Nugent et al., 1998; McNeela and Mills, 2001; Ogra et al., 2001; Brandtzaeg, In Press 2006). However, it is important to note that different areas of the mucosa, although separate (i.e. the lung is spatially separate from the gut), once one area has been exposed to an antigen and an immune response mounted, this response can be conferred to other areas of the mucosa (Ruedl et al., 1996; Challacombe et al., 1997; Zuercher et al., 2002). Therefore, a single vaccine dose could potentially elicit a robust immune response against an antigen at local mucosal sites, distant mucosal sites and systemically.

Mucosal immunization can also offer the advantage of targeting a broader range of recipients, including the elderly, as unlike the systemic immune system, the mucosa-associated lymphoid system does not undergo an age-related decline (Szewczuk et al., 1981; Walker, 1994; Nugent et al., 1998). It has also been discussed that mucosal immunization could be one method of improving neonatal immunization (Mahon, 2001), as mucosal immunization is less affected by the presence of maternal antibodies (Walker, 1994).

Additional incentives for using a non-parenteral method of immunization include the reduction in needle-stick injuries, minimal training of personnel to administer the vaccine, improved patient compliance (Holmgren et al., 2003) and, as it is a product that is not going to enter the blood stream directly, there is no need for the vaccine to be sterile (Johansen et al., 2000).
1.3. Pulmonary Delivery of Vaccines

*Success:* Local delivery of drugs is already established. The new field of systemic delivery has been proven with the first inhalable insulin available on the market since January 2006.

*Challenges:* There have been few studies that take advantage of the pulmonary route for vaccine delivery.

Although the nasal (Gizurarson et al., 1998; Alpar et al., 2001; Illum, 2003) and oral routes (Wikingsson and Sjoholm, 2002; Carcaboso et al., 2003; Howard et al., 2004) have been investigated and used as portals for vaccine delivery, the lung holds particular attractions.

Historically, lung delivery has been mainly used for the local delivery of drugs for treatment of diseases and conditions such as asthma, respiratory distress syndrome and chronic obstructive pulmonary disease (Labiris and Dolovich, 2003a). However, with its large surface area (between 80-140m²), thin absorptive alveolar epithelium (0.1-0.2 μm), and the avoidance of first pass metabolism, the lung is also an appealing organ to target for systemic drug delivery (Yu and Chien, 1997; Scheuch et al., 2006). And in January 2006, this was achieved when the first inhalable insulin was approved by the Food and Drug Administration in the USA, and by the European Commission (Pfizer/Nektar, 2006a, b).

As mentioned in the previous section, advantages of targeting vaccines to the lung include the possibility of inducing mucosal immunity (at local and distant sites) and systemic responses as well. Pulmonary vaccines would be non-invasive and easy to administer and this will be advantageous as it has been postulated that in the future, an infant will have received 20 to 25 vaccine doses by injection by the ages of 12-18 months (Ogra et al., 2001; Cook, 2006).

The immunological mechanisms of defense in the lung are well described, and therefore designing vaccines to exploit these mechanisms is theoretically straightforward.
1.3.1. Immunology of the Lung

The respiratory system is essentially composed of two major regions; the conducting airways and the respiratory region (Figure 1.1), and each area has distinct properties for immunological defense.

Figure 1.1 Diagram of the conducting airways and respiratory region. The conducting zone is made up of the nasal cavity, pharynx, larynx, trachea, bronchi and bronchioles. It is not involved in gas-exchange, but serves to filter and humidify the inhaled air, and to warm it to body temperature. The respiratory zone is where gas-exchange occurs and consists of the respiratory bronchioles and alveoli. Adapted from Yu and Chien, 1997.
1.3.1.1. Immunology of the Conducting Airways

The conducting airways are the first sites of contact with inhaled antigens and this area is protected by the lymphoid tissues known as the Waldeyer’s ring, which include the adenoids and tonsils (Nugent et al., 1998). Non-specific forms of defense include the mucociliary escalator, the cough reflex and the presence of circulating macrophages (Poulter, 1997). The conducting airways are lined with ciliated epithelium, interspersed with goblet cells and submucosal glands. Mucus secreted by the goblet cells and submucosal glands forms a layer over the cells and entraps insoluble inhaled particles, which gets swept up the trachea by the movement of the cilia, and is then either coughed out or swallowed and destroyed by the acid in the stomach (Yu and Chien, 1997; Labiris and Dolovich, 2003a).

The mucus also contains numerous chemicals that have antimicrobial properties, such as lysozyme, lactoferrin, uric acid, peroxidase and defensins (Fokkens and Scheeren, 2000). If the mucus layer is breached, the innate immune system, comprising neutrophils, macrophages and natural killer cells, is activated. These cells have receptors that recognize conserved bacterial or viral products (cell-wall constituents, DNA motifs) and they phagocytose bacteria and release proteolytic enzymes and immuno-modulatory compounds that recruit other monocytes to assist in the killing and digestion of bacteria or virus-infected cells (Lohmann-Matthes et al., 1994; Fokkens and Scheeren, 2000; Lambrecht et al., 2001).

As well as innate immunity, adaptive immunity is also required for building up immunological memory, so that if a virus or bacteria infects again, a more rapid and efficient immune response can be mounted. This is dependent on the priming of both T and B cells (Fokkens and Scheeren, 2000). T cells can only respond to antigen that has been processed and presented by antigen presenting cells (APCs), and in the lung, this takes place in the Waldeyer’s ring (Fokkens and Scheeren, 2000). Dendritic cells (DCs) are specialized APCs with two unique characteristics that aid in building up immunological memory: they have the greatest phagocytic ability, but when exposed to inflammatory stimuli, they lose
their capacity for phagocytosis and become potent stimulators of naive T lymphocytes (Massard et al., 1996; Nicod et al., 2000). The specific function of DCs in the lung has been reviewed extensively (Lambrecht et al., 1996; Nicod et al., 2000; Lambrecht et al., 2001), and therefore will not be discussed here. However, it is important to note that these cells are essential in determining whether an immune response is mounted or not, and are crucial in controlling the extent of inflammatory responses, which is particularly important in an organ such as the lung. There is a constant movement of DCs from the airways to the draining lymph nodes, and they continually sample the environment for antigens. They are normally suppressed from becoming mature DCs by macrophages, to avoid inadvertent damage, but if exposed to triggers, such as heat-shock proteins, lipopolysaccharide and toll-like receptors from microorganisms, or released antigens from damaged cells, their activation is rapid (Lambrecht et al., 2001).

Immunoglobulin A (IgA) is the dominant immunoglobulin that is secreted in mucosal fluids in the body, as opposed to IgG, which is dominant in serum (Poulter, 1997). Secretory IgA has a close relationship between the mucosal lymphoid tissue, which produces IgA, and the epithelium, which transports IgA within the mucosal lumen. Here, IgA is thought to inhibit the binding of bacteria to the epithelium, neutralizes viruses, as well as inducing the loss of bacterial plasmids that encode for adherence or antibacterial resistance, and interference with enzymes needed by the pathogen for growth factors and invasion. IgA provides a first-line of defense, and prevents the development of potentially damaging inflammatory responses (Pilette et al., 2001).

1.3.1.2. Immunology of the Alveolar Region

Once the airways branch down to the respiratory bronchioles, the structure and function of the cells change from conduction and conditioning of the inhaled air, to gas exchange (Yu and Chien, 1997). Here, the alveolar macrophages and the release of surfactant are the mechanisms of non-specific defense (Poulter, 1997).

The alveolar surface is mainly composed of a single layer of squamous epithelial cells – Type I alveolar cells. These form a number of alveoli as a continuous
sheet and they meet at tight junctions with a gap of about 1 nm. This junction is crucial in preventing the movement of macromolecules e.g. albumin, into the alveoli, and for preserving the oncotic pressure to avoid pulmonary oedema. Despite this, the junctions still allow passage of macrophages and small molecules (Yu and Chien, 1997). Another type of cell, Type II alveolar cells, are scattered among the Type I cells and are responsible for the production of surfactant, and differentiate into Type I cells if they are damaged. Surfactant (composed of lipids (~90%) and surfactant-specific proteins) is predominantly produced by Type II cells in the terminal airways and has two main roles. It lowers the surface tension in the alveoli, thereby allowing ease of expansion of the sacs (Haagsman and Diemel, 2001), and acts as a non-specific barrier against microorganisms, and also has a direct microbicidal activity and can promote phagocytosis and microbicidal activity in alveolar macrophages (Poulter, 1997; Yu and Chien, 1997).

Alveolar macrophages are plentiful in the lung (with a ratio of 1:8 with respect to Type I cells) and are considered to be the most important lung phagocytes (Suarez and Hickey, 2000). This is reflected in the fact that alveolar macrophages make up 85% of the phagocytic cells in the alveoli, whereas DC only make up 1% (Nicod et al., 2000). They pass freely from the circulation through the interstitial space and through the gaps between alveolar epithelial cells to lie in the extracellular lining of the alveolar surface. They are very active in combating infection and scavenging foreign bodies such as bacteria and dust particles (Yu and Chien, 1997). The constant exposure of these phagocytes to the environment means that they contain a high level of microbicidal enzymes (e.g. lysozyme), one of the highest compared with other macrophages in the body (Bowden, 1984). Alveolar macrophages are poor APCs, and might even suppress T-cell proliferation; this may help in avoiding unwanted or excessive inflammatory responses (Massard et al., 1996; Nicod et al., 2000). However, alveolar macrophages can produce a wide range of immuno-modulatory mediators that attract other cells to the lung, including DCs, which can then mount a specific immune response through activation of T cells (Nicod et al., 2000). Further details on the activities of alveolar macrophages and dendritic cells can be found in the following reviews (Lohmann-Matthes et al., 1994; Lambrecht et al., 1996;
As DCs are the most potent of APCs and are implicated in the control and direction of mucosal immunity (Banchereau and Steinman, 1998), it would be prudent to design mucosal vaccines for the lung to target these APCs to ensure that a robust immune response is stimulated.

1.3.2. Previous Studies on Delivering Vaccines to the Lung

The feasibility of using the lung as a portal for delivery of vaccines has been reviewed previously (Roth et al., 2003; Laube, 2005). These two papers outline previous trials of aerosol vaccination on humans against anthrax, tetanus and botulinum toxoids, measles and plague. These took place mainly in Eastern Europe and the former Soviet Union.

In the West, trials have mainly concentrated on aerosolised measles vaccine (Kim et al., 2004; Wong-Chew et al., 2006), and this is now a major project with the WHO, the aim of which is to develop and license a measles aerosol vaccine for children aged 12-59 months, for routine vaccination, and 9 months to 18 years, for mass vaccination, (Henao-Restrepo and WHO, 2006).

In terms of economics, it has been calculated that in a trial in which 8,565,230 children were immunized with the measles vaccine, the cost of syringes and needles alone was US$2,057,753 (63% of the total budget), and it took the involvement of 10,527 centres and 30 days to complete. By contrast, it was estimated that if aerosol immunisation was used, then it would cost $300,000 and take one day to complete (Sabin, 1991, 1992).

Today, new technologies and an increased understanding of how infectious diseases interact with the immune system have added to the knowledge for the development of more effective vaccines. One of the most recent developments in pulmonary drug delivery is the possibility of delivering DNA vaccines, which can either be for prophylactic purposes in the case of providing immunity from infectious diseases, or for therapeutic purposes, in the case of gene therapy, e.g. in treatment of cystic fibrosis.
1.4. DNA Vaccines

Success: DNA vaccines are now available on the market for veterinary use.

Challenges: There is still no DNA vaccine for humans.

DNA immunization involves the inoculation of a plasmid encoding for microbial or viral proteins in the case for prophylaxis, or for proteins that are redundant due to a defective gene in a patient in the case of gene therapy. Although there is much similarity in the methods used for each type of therapy, the main focus in this project is towards prophylactic treatment.

In 1990 Wolf et al., (1990) demonstrated that the direct injection of a plasmid DNA encoding for a foreign gene into muscle cells of a mouse could lead to transcription of the proteins. At the end of the paper, the authors suggested that this work could be used as an alternative approach to vaccine development. Since then, the field of DNA vaccines has exploded (Figure 1.2).

![Figure 1.2. Expansion of 'DNA vaccine' research 1990-2005. *The number of hits obtained when the search term 'DNA vaccine' was entered into the search engine PubMed, limiting searches to one year - beginning Jan 01, ending Dec 31 for each year. Please note that searches may include hits for recombinant proteins made from using foreign DNA in bacteria.](image-url)
The advantages of immunization with plasmid DNA include less complicated and higher yields in production, easier quality control, more rapid creation and testing of new vectors, heat stability, less risk of recombination and no risk of inadvertent infection (Davis et al., 1996; Tighe et al., 1998; Gurunathan et al., 2000a). For most viral and bacterial infections, primary protection is mediated by a humoral response (production of antibodies). For intracellular infections, e.g. TB and *Leishmania major*, protection is mediated by cellular (cytotoxic) immunity; and for some diseases, e.g. HIV and malaria, both humoral and cellular responses are likely to be required (Gurunathan et al., 2000a).

The cellular immune response consists of two pathways, depending on the mode of antigen presentation. Antigens that have been taken up by the APC from the external environment (e.g. from killed/inactivated pathogens or recombinant protein) are processed by the APC to form major histocompatibility complex class II molecules (MHC II) on their cell surface. MHC II stimulates CD4+ (T-helper) cells, which, depending on the cytokine milieu, mount an antibody or cytotoxic response (Gurunathan et al., 2000a).

CD4+ T-helper cells have two distinct profiles of cytokine production, Th1 and Th2. Cytokines such as IFNγ, TNFβ and IL-12, and Th1 cells mediate cytotoxic, inflammatory and delayed hypersensitivity reactions. Whereas, Th2 cells and the presence of IL-4, IL-5 and IL-13 are involved in antibody production (Roitt et al., 1998; Gurunathan et al., 2000a).

Antigen that is produced and processed endogenously present MHC I class molecules that are recognized by CD8+ (T-lymphocyte) cells, which then mount a cytotoxic response (Gurunathan et al., 2000a).

The ability of DNA vaccination to steer an immune response to a particular direction is attractive, and it has been suggested that for infectious diseases, the ability of DNA vaccines to enhance Th1 responses might be useful for preventing intracellular infections requiring Th1 immunity, or modifying ongoing immune responses to optimize intracellular killing (Gurunathan et al., 2000a).
This use of DNA vaccines to control progress of disease has been demonstrated in a number of infectious diseases including TB (Zhu et al., 2005), HIV (MacGregor et al., 2005) and hepatitis B (Thermet et al., 2003). In terms of the very young and old, DNA vaccines could also have the potential to steer the immune response to a direction that would offer more protection (Sarzotti et al., 1997; Meyer, 2001; Garvy, 2004). For example, a cytotoxic T-cell response was induced in neonatal mice, when injected with plasmid DNA, which protected them from developing a neurological disease on infection with a virus (Sarzotti et al., 1997); and a booster injection of a DNA vaccine into aging mice offered greater protection against TB challenge compared with non-boosted mice (Goter-Robinson et al., 2006).

The field of DNA vaccines was given significant encouragement when two DNA vaccines were licensed in 2005 – one against West Nile Virus in horses (CDC, 2005), and one against Infectious Haematopoietic Necrosis Virus (IHNV) in salmon (Vical, 2005). Their success in humans, however, remains to be seen.

1.4.1. Basic Aspects of DNA Vaccines

Most DNA vaccines involve the delivery of a modified plasmid that contains a gene that encodes for a protein. Once a host cell has taken up the plasmid, the cell expresses the protein and this can serve as an antigen and thus stimulate an immune response as a consequence. Figure 1.3. illustrates the main components that make up a basic DNA vector. A vector has to contain two main components, the first is involved in the propagation of the plasmid in the microbial host (usually the bacteria Escherichia coli), and the second is involved in driving the transcription of the protein once taken up in the eukaryote host (Glenting and Wessels, 2005).
Figure 1.3. Schematic diagram of a basic DNA vector. For DNA vaccines, a vector includes basic elements such as a strong bacterial origin of replication (pUC origin), an antibiotic selection marker (KanR); a eukaryotic promoter (pCMV), an intron and multiple cloning sites (MCS) for antigen insertion, and a polyadenylation signal sequence (polyA). Adapted from (Ertl and Thomsen, 2003)

An additional factor that has been discovered regarding DNA vectors is that they may be inherently immunogenic. This is due to the presence of unmethylated CpG (cytidine-phosphate-guanosine) motifs in bacterial sequences. These are present in bacterial DNA in high frequencies but not so in mammalian cells. Unmethylated CpG motifs are recognized by mammalian immune cells, i.e. B cells, via Toll-like receptors (TLR9 in particular), and these directly stimulate APCs, leading to the host mounting firstly an innate and then antigen-specific immune response (van Uden and Raz, 1999; Klinman et al., 2004). Optimization of CpG sequences can therefore be used as an extra tool in designing more immunogenic DNA vaccines (Hartmann et al., 2000; Klinman et al., 2004).

1.4.2. How DNA Vaccines Work

The specific methods as to how DNA vaccines work have yet to be elucidated, however three theories have been put forward and reviewed, a summary of which
is illustrated in Figure 1.4 (Gurunathan et al., 2000b; Coombes and Mahony, 2001).

It was originally thought that non-bone-marrow derived cells themselves could take up antigen and induce a cellular immune response, however, it was shown that this was not the case. Dendritic cells were discovered to be the cells that were crucial in the priming of immune responses following DNA vaccination. And, depending on where the DNA is taken up, a different immune response is generated. Either the dendritic cells can be transfected directly by the DNA vaccine, or the muscle cells can take up the DNA and then transfer the antigen to an APC - termed cross-priming (Gurunathan et al., 2000b; Coombes and Mahony, 2001).

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**Figure 1.4. Possible mechanisms of antigen presentation to naive T-cells following DNA vaccination.** (A) Direct transfection of somatic cells, e.g. myocytes and keratinocytes; (B) Direct transfection of antigen presenting cells (APCS) and/or dendrite cells (DCs); (C) Cross-priming, in which the antigen produced in somatic cells is transferred to DC either as soluble antigen via macropinocytosis, or by phagocytosis of apoptotic cell bodies. Redrawn from Coombes and Mahony (2001).
1.4.3. Safety of DNA Vaccines

It is important to define that this project is for developing DNA vaccines and not gene therapy. For gene therapy, the aim is to provide a permanent replacement of the defective gene, whereas for DNA vaccines, only a transient transcription of the gene is necessary. One of the major causes of resistance for the concept of DNA vaccinations is the risk of the DNA being irreversibly integrated into the host genome (Wang et al., 2004). Studies so far have demonstrated that this risk is minimal.

Initial studies show that DNA vaccines are generally well tolerated at doses up to 2.5 mg given over three or more injections via i.m or s.c injection (Donnelly et al., 2003) and that the risk of plasmid DNA integrating into host is well below that of the spontaneous rate of gene-inactivating mutations (Wang et al., 2004). These results were confirmed in a number of studies that investigated the biodistribution and toxicology of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome and West Nile Virus in mice and rabbits (Sheets et al., 2006a; Sheets et al., 2006b). The studies found that delivery of DNA vaccines (up to 2 mg) by Biojector or needle and syringe led to a restricted pattern of biodistribution (confined to the site of injection) and clearance within 2 months, regardless of the promoter in the backbone or the gene being expressed (Sheets et al., 2006b). For DNA doses up to 8 mg per immunization, mild to moderate reactions were observed, which were localized at the site of injection, and were self-limiting (Sheets et al., 2006a). These studies allowed these vaccine candidates to be put forward into human clinical trials.

1.5. Delivery of DNA Vaccines

Success: Microparticles have been shown to act as an adjuvant as well as protect proteins/peptides/DNA from degradation in vivo.

Challenges: A commercial microparticulate DNA vaccine has yet to be developed for humans.

There have been many methods used to deliver DNA into the body and a summary of advantages and disadvantages is given in Table 1.1.
Table 1.1. A general outline of advantages and disadvantages of viral and non-viral vectors. All viral vectors share the disadvantage of safety concerns arising from the generation of replication-competent virus during manufacturing or after administration to patients. QC = Quality Control. Adapted from Mountain, A. (2000).

<table>
<thead>
<tr>
<th>Vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>Very high transfection efficiency ex vivo &amp; in vivo</td>
<td>Repeat dosing ineffective owing to strong immune responses; insert-size limit of 7.5 kb; manufacture, storage, QC moderately difficult; short duration of expression</td>
</tr>
<tr>
<td></td>
<td>Transfects proliferating &amp; non-proliferating cells</td>
<td></td>
</tr>
<tr>
<td>Retrovirus</td>
<td>Fairly prolonged expression</td>
<td>Low transfection efficiency in vivo; insert-size limit of 8 kb; transfects only proliferating cells; safety concern of insertional mutagenesis; manufacture, storage, QC extremely difficult</td>
</tr>
<tr>
<td></td>
<td>High transfection efficiency ex vivo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low immunogenicity</td>
<td></td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Transfects proliferating &amp; non-proliferating cells</td>
<td>Safety concerns from immunodeficiency origins; manufacturing, storage, QC extremely difficult; insert-size limit of 8 kb</td>
</tr>
<tr>
<td></td>
<td>Transfects haematopoietic cells</td>
<td></td>
</tr>
<tr>
<td>Adeno-associated Virus (AAV)</td>
<td>Efficiently transfects a wide variety of cells in vivo</td>
<td>Insert-size limit of 4.5 kb; manufacture, QC very difficult; safety concern of insertional mutagenesis; repeat dosing affected by neutralising antibody responses</td>
</tr>
<tr>
<td></td>
<td>Very prolonged expression in vivo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low immunogenicity</td>
<td></td>
</tr>
<tr>
<td>Naked DNA</td>
<td>Manufacturing, storage, QC simple &amp; cheap</td>
<td>Very short duration of expression in most tissues</td>
</tr>
<tr>
<td></td>
<td>Very low immunogenicity</td>
<td>Very inefficient transfection ex vivo &amp; in vivo</td>
</tr>
<tr>
<td></td>
<td>Very good safety profile</td>
<td></td>
</tr>
<tr>
<td>Cationic Lipids</td>
<td>Relatively simple manufacturing, storage, QC</td>
<td>Inefficient transfection in vivo</td>
</tr>
<tr>
<td></td>
<td>Efficient transfection ex vivo</td>
<td>Very short duration of expression</td>
</tr>
<tr>
<td></td>
<td>Low immunogenicity; good safety profile</td>
<td></td>
</tr>
<tr>
<td>Biodegradable microparticles</td>
<td>Relatively simple manufacturing, storage, QC</td>
<td>Inefficient transfection in vivo</td>
</tr>
<tr>
<td></td>
<td>Efficient transfection ex vivo</td>
<td>Very short duration of expression</td>
</tr>
<tr>
<td></td>
<td>Low immunogenicity; good safety profile</td>
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</tbody>
</table>
Interested readers are also referred to these excellent reviews (Nugent et al., 1998; Mountain, 2000; Alpar et al., 2005; Patil et al., 2005).

Despite the great potential of DNA vaccines, the main hurdle that needs to be overcome is the extremely low transfection efficiencies of cells \textit{in vivo} and thus poor immune responses as a result. Naked DNA has often been used for immunization, however, once \textit{in vivo}, naked DNA degrades within 48h (Vanniasinkam et al., 2006), and because of its large size (up to 2,000 kDa or more) and negative charge, is not able to enter cells efficiently (Perez et al., 2001; Nishikawa et al., 2005). Methods are therefore needed to enhance its half-life \textit{in vivo} and to aid its delivery into cells. Two main classes of delivery methods for DNA have been used, viral and non-viral, and this project aims to develop a non-viral method.

Encapsulation of DNA into a delivery vehicle offers several advantages, it protects the DNA from degradation once administered into the body and the material that the delivery vehicle is manufactured from can be manipulated to enhance release kinetics that can mimic conventional regimens of vaccination, thereby offering the chance of a 'one-shot' vaccine (Langer et al., 1997).

Liposomes have been tested as potential drug delivery vehicles due to their ability to be manufactured with phospholipids endogenous to the lung, and their high capacity for entrapping both hydrophilic and hydrophobic drugs (Zeng et al., 1995). Cationic lipids/phospholipids/liposomes especially have been investigated intensively due to their higher efficiency of gene delivery compared with other non-viral DNA delivery systems (for an outline refer to Ledley, 1996). Although this may be the case, there have also been studies demonstrating the limitations of cationic liposomes for DNA delivery such as toxicity in macrophages (Filion and Phillips, 1998), and so the greater stability and safety of biodegradable polymeric microparticles \textit{in vitro} and \textit{in vivo} means that these may be more suitable, especially to an organ such as the lung (Zeng et al., 1995).
1.5.1. Methods of Microparticle Preparation

Many techniques used to produce microparticles have been reviewed and therefore will not be discussed here. Interested readers are referred to the following: (O'Donnell and McGinity, 1997; Jain, 2000; Lengsfeld et al., 2002).

The most popular method of producing microparticles includes water-in-oil-in-water (w₁/o/w₂) double emulsion solvent evaporation (Figure 1.5). Multiple emulsions involve the dispersion of one immiscible liquid into another to form droplets. In w₁/o/w₂ double emulsions, the primary emulsion consists of an aqueous phase (w₁) that is dispersed into an oil solution (e.g. polymer dissolved in solvent). Formation of the emulsions is usually by mechanical means such as homogenization or sonication, and the drug to be entrapped is usually dissolved either in the oil or aqueous phase, but is most commonly dissolved in the aqueous phase. This primary emulsion is then dispersed into second aqueous phase (w₂) to form the double emulsion (Florence and Whitehill, 1982; O'Donnell and McGinity, 1997).

Figure 1.5. Schematic diagram of w₁/o/w₂ double emulsion solvent evaporation method. (1) The primary emulsion (w₁) involves dispersing an aqueous solution (with drug dissolved) into an oil solution (with polymer dissolved). (2) The secondary emulsion (w₂) disperses the emulsion from the first step into a secondary aqueous phase. (3) Once the secondary emulsion is completed, the emulsion is left to evaporate off the solvent and to allow the polymer to precipitate out and form the microparticles. (4) The final step involves freeze-drying of the microparticles into a dry powder. In this thesis, w₁ and w₂ represent the primary and secondary aqueous phases.
To stabilize the emulsions made, addition of an emulsifier is added into the aqueous phases. Various ionic and non-ionic surfactants are available that can be used, however non-ionic surfactants are preferred because of their lower toxicity and lower interaction with ionic compounds (Florence and Whitehill, 1982). Once the double emulsion has been made, the microparticles are formed through the precipitation of the polymer out of the oil phase, and the concurrent removal of the solvent, usually by simple evaporation at room temperature.

In terms of product stability, microparticles that are made are usually dried, usually by freeze-drying. Keeping the microparticles as a dry powder has many advantages. Drying lowers the moisture content as well as reducing molecular movement, which lowers the rate of degradation and therefore, enhances stability (Middaugh et al., 1998). In this way, a stable vaccine can be developed, which should help in the development of a product that would fulfil the requirements of the WHO's EPI.

1.5.2. DNA Entrapment into Microparticles

Delivery of DNA by biodegradable microparticles has been a fairly recent event. Entrapment of DNA into liposomes has been mentioned as long ago as 1978 (Hoffman et al., 1978), whereby the entrapment of DNA into liposomes was used for mechanistic studies of membrane interactions.

The first example of the w₁/o/w₂ double emulsion solvent evaporation being used as a technique to entrap water soluble drugs was in 1988, when Ogawa et al developed the technique to entrap leuprolide acetate into polylactic acid (PLA) microparticles (Ogawa et al., 1988). Since then, it took almost another 10 years before the technique was used to entrap plasmid DNA into biodegradable microparticles (Jones et al., 1997a; Mathiowitz et al., 1997; Jones et al., 1998). As DNA is an extremely hydrophilic molecule (Walter et al., 1999), w₁/o/w₂ double emulsion solvent evaporation would be the most suitable method to use to prepare microparticles with a high loading of DNA.
1.5.2.1 Materials used for Microparticle Production

Although many polymers have been used for manufacturing biodegradable microparticles that encapsulate DNA, e.g. polyphosphoesters, (Zhao et al., 2003), chitosan, (Borchard, 2001) and polyacryl starch (Wikingsson and Sjoholm, 2002), poly(lactic acid-co-glycolic acid) (PLGA) will be used as the material to manufacture microparticles in this particular study, due to its known safety as a biomaterial and extensive research on its biodegradability characteristics (Ignatius and Claes, 1996; Shive and Anderson, 1997; Viswanathan et al., 2001; Wang et al., 2002). It is a widely available and comes in different molecular weights, and differing lactic acid: glycolide ratios, so altering characteristics of the microparticle will be more easily obtainable. The protective and adjuvant effects of entrapping antigens and DNA into PLGA microparticles have been demonstrated in a number of studies: (Thomasin et al., 1996; Jones et al., 1997b; Audran et al., 2003; Carcaboso et al., 2003; He et al., 2005).

Dichloromethane (DCM) will be the solvent used to dissolve the polymer because of its high solvency power, relative inertness and low boiling temperature (40°C) (ECSA, 1995) – compared with other solvents that have been used for making microparticles, including acetone (56.2°C), chloroform (61°C) and ethyl acetate (77°C) (Bain et al., 1999; Soppimath and Aminabhavi, 2002). The low boiling point of DCM will allow rapid evaporation of the solvent once the double emulsion is made. This will aid in minimizing potential toxic effects of residual solvent.

Poly (vinyl alcohol), will be used as an emulsifier because of its known properties as a stabilizer of emulsions (Jeffery et al., 1991, 1993; Prabha and Labhasetwar, 2004), and low toxicity (DeMerlis and Schoneker, 2003).

It is possible that once the stability and methodologies for microencapsulating DNA have been optimized for a model DNA plasmid, then it should be applicable to other DNA molecules as they generally have very similar chemical and physical properties (Walter et al., 1999). However, it is important to maintain the tertiary structure of the plasmid, as different conformations (supercoiled, open-circular and linear) have different release and stability characteristics, i.e. supercoiled DNA is more bioactive than linear DNA (Hao et al., 2000).
The inclusion of additives such as NaHCO₃ (Walter et al., 1999), PVA/poly (vinyl pyrrolidone) (PVP) (Perez et al., 2001) and sucrose (Cherng et al., 1999) have been used to improve DNA encapsulation and stability during the manufacturing process with varying success. Mechanistically, the introduction of pauses during the primary emulsification step has been demonstrated to increase encapsulation efficiency (Tinsley-Bown et al., 2000) and also lowering the temperature of the primary emulsion has led to high encapsulation efficiencies and increased stability of the DNA (Ando et al., 2001). Uses of some of these techniques will be evaluated in the thesis.

1.5.3. Delivery of Microparticles to the Lung

Delivery of drugs to the lung depends on a number of factors, including, rate of inspiration, where the drug is deposited in the lung (conducting or respiratory region) and the rate at which the particle dissolves (Mobley and Hochhaus, 2001). Molecules are absorbed much more rapidly in the alveolar region as a result of higher permeability, large surface area and lack of mucociliary cells (Zeng et al., 1995). It is therefore the main area that is targeted when considering delivery of drugs that have systemic effects to the lung. For vaccine delivery, it would also be ideal to target this area to ensure maximum residence time for the microparticles for macrophage, DC and/or epithelium uptake.

For humans, the ideal particle size for deposition into the deep region of the lung is approximately 2-3 microns for slow, deep inhalation and 1-2 microns for faster inhalation rates (Wu-Pong and Byron, 1996). Therefore, for vaccines, diameters of 1-3 microns should be developed for optimum delivery.

A number of studies have demonstrated that pulmonary delivery of vaccines show enhanced or at least equivalent immune responses compared with other routes for a number of infectious disease including, plague (Eyles et al., 2000), TB (Bivas-Benita et al., 2004) and hepatitis B (Lombry et al., 2004). This, therefore, confirms the potential of researching this area as an alternative route for delivery of vaccines.

The aims of this thesis are outlined in the next section.
1.6. Overall Project Aims

The overall aim of the project was to combine all the advantages of DNA vaccines, microparticles and pulmonary delivery to produce a formulation that will be delivered easily and also give a robust immune response.

The project is divided into 5 main sections:

**Phase I. Formulation and characterization of microparticles encapsulating DNA and optimization of parameters**

Poly (DL-lactide-co-gлcolide) (PLGA) was the polymer of choice for the manufacture of microparticles because of its known safety and biodegradability properties. The main aim was high efficiency of DNA encapsulation without causing denaturing of the DNA during the manufacturing process and to produce particles of appropriate diameter that would be suitable for delivery into the lung. Experimental design was used to optimize loading and diameters of the particles, and buffers were used to protect the conformation of the DNA. To assess these parameters, methods such as agarose gel electrophoresis, Picogreen® and laser diffraction were used.

**Phase II. Physicochemical studies on optimized formulations**

The main advantage of producing microparticles is that they are more stable, once freeze-dried, than other delivery systems. Once the particles were made, analysis of the formulation with regards to water content and thermal stability was made to ensure that stability of the polymer was maintained. Other aspects such as the residual content of organic solvent and emulsifier were also investigated as these will affect the stability and to ensure that they were below accepted regulatory levels.

**Phase III. Cell culture studies**

As the aim of delivery is for the lung and are for vaccine use, initial cell culture studies used a lung alveolar cell line (A549) and a monocyte/macrophage cell line (J774A.1) to assess the uptake of particles and gene expression. It was important to ensure that the microparticles were not toxic to the cells and that they were efficient vehicles for delivery of DNA.
Phase IV. Dry powder testing of formulations
The successful formulations would hopefully be used in a dry powder inhaler, so the microparticles were analyzed as a dry powder to see if they still dispersed into diameters that were suitable for delivery into the lung.

Phase V. Further Formulation Optimization
From the above investigations, the main formulations that were developed did not meet all the requirements that were needed to move onto in vivo testing. Therefore, further optimizations of the above formulations were carried out. These included using alternative stabilizers and the use of a condensing agent to increase DNA loading into the microparticles.

This thesis outlines the process involved in developing novel formulations and the characterizations involved to ensure that they are suitable in terms of the biological effects produced and stability as a pharmaceutical product. Not many publications look at both aspects at the same time, and this work demonstrates the difficulties and challenges encountered.
Chapter 2

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

2.1. Chemicals and Reagents

Unless specified, all chemicals and reagents were of analytical grade and purchased from VWR International (Lutterworth, UK) or Sigma-Aldrich (Poole, UK).

2.1.1. Microparticles

Poly(DL-lactide-co-glycolic acid) (PLGA) of ratio 50:50 DL-lactic acid and glycolic acid was purchased from Lakeshore Biomaterials (previously Alkermes) (Birmingham, AL, USA). This polymer is approximately 58.8 kDa, has an inherent viscosity of 0.38 - 0.48 g/l and has a complete degradation time of approximately 3-4 weeks (Lakeshore, 2006). Poly (vinyl alcohol), (PVA), 13-23 kDa, 87-89% hydrolysed was purchased from Sigma-Aldrich (Poole, UK). Dichloromethane (DCM) and D-Mannitol were from VWR International (Lutterworth, UK), as were the freeze-drying vials (5 ml and 10 ml) and 20 mm rubber stoppers.

2.1.2. Molecular Biology

All chemicals for making up bacterial growth medium and agar plates were purchased from Oxoid Ltd (Basingstoke, UK), with the exception of sodium chloride which was from VWR International (Lutterworth, UK) and D-glucose, which was from Sigma-Aldrich (Poole, UK).

The bacterial E-coli strain DH5-α from Invitrogen (Paisley, UK) was used for transformation. The vector pCMVβ-Gal was obtained from Clontech (CA, USA) and pGL3-Control from Promega (Southampton, UK). Restriction enzymes (Not I, Xba I and Nco I) and T4 DNA-ligase were purchased either from Promega (Southampton, UK) or New England Biolabs (Hitchin, UK). The plasmid gWiz Luciferase was from Aldevron (Fargo, ND, USA).
Agarose and the Molecular Probes Quant-iT™ PicoGreen® dsDNA Assay Kit were obtained from Invitrogen (Paisley, UK). Ethidium bromide was purchased from VWR International (Lutterworth, UK). The molecular weight marker Lambda DNA/Hind III and 6x loading dye solution were from Fermentas (York, UK).

All water used in experiments was distilled and de-ionized using an Option 4 Water Purifier USF ELGA (ELGA Labwater, High Wycombe, UK).

### 2.2. Production of Microparticles

Specific formulations are given in the relevant chapters. All microparticles were made using the water-in-oil-in-water (W/O/W) double emulsion solvent evaporation technique. An outline of the method is given below for an example of a formulation.

**2.2.1. Water-in-oil-in-water (w/o/w) double emulsion solvent evaporation**

300 mg of PLGA was dissolved in 5 ml of DCM. An internal aqueous solution containing 200 μg DNA (200 μl of a 1 mg/ml solution) and 500 μl 10% m/v PVA was emulsified into the polymer/solvent mix using an Ultraturrax T25 homogenizer (Janke & Kunkel IKA Labortechnik, Germany), with a 1 cm diameter probe. The primary emulsion was homogenized for 2 min at a speed of 24,000 rpm. This primary emulsion was then added drop-wise and emulsified into a second aqueous phase containing 2.5% m/v PVA, with a Silverson L4RT homogenizer (Silverson, UK) equipped with a 2.5 cm diameter probe. The secondary emulsion was homogenized for 5 min at a speed of 9,500 rpm. After which, a magnetic bar was added into the resulting w/o/w mixture and this was left to stir at room temperature for about 3-4 h on a magnetic stirrer plate for the solvent to evaporate and the polymer to precipitate and form the microparticles. After the solvent had evaporated, the microparticles were washed 2-3 times with water and centrifuged at 15,000 rpm for 15 min (Beckman J2-21 Hi-Speed centrifuge with JA-20 rotor, Beckman) before being freeze-dried (see section 2.2.2). The washing step removes any residual PVA from the formulation. All preparative solutions were either kept at 4°C overnight before being homogenized. Homogenization of all emulsions was carried out on ice.
2.2.2. Freeze-drying of Microparticles

Freeze-drying or lyophilization is widely used for pharmaceuticals to improve the stability and long-term storage of labile drugs, especially protein drugs. They not only have advantage of better stability, but also improve handling, i.e. in shipping/transport and actual handling (Tang and Pikal, 2004). Here, washed particles were re-suspended into approximately 1 ml of water and dispensed into freeze-drying vials. The samples were then kept at -20°C. The frozen product was then dried by using a Virtis Advantage freeze-dryer (Virtis, USA) using the parameters set out in Table 2.1.

Table 2.1. Freeze-drying cycle for Virtis Advantage freeze-dryer.

<table>
<thead>
<tr>
<th>Drying Stage</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Pressure (psi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermal Stage</td>
<td>-20</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>Primary Drying Stage</td>
<td>-20</td>
<td>800</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>800</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>800</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>800</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>-20</td>
<td>800</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>-38</td>
<td>320</td>
<td>200</td>
</tr>
<tr>
<td>Secondary Drying Stage</td>
<td>5</td>
<td>640</td>
<td>50</td>
</tr>
</tbody>
</table>

Once the particles were dried, the vials were capped with 20 mm rubber stoppers and then stored in a dessicator at room temperature.

2.3. Characterisation of Microparticles

2.3.1 Analysis of Diameters Using Laser Diffraction

The mean diameter and diameter distributions of the microparticles were analysed via laser diffraction using a Mastersizer (Malvern Instruments, UK), equipped with a 5mW Helium-Neon laser (632.8 nm). The machine measures particles with a diameter range of between 0.02 μm to 2,000 μm (depending on the type of dispersion unit used), and uses Mie theory to calculate the diameter. Mastersizer-S v2.18, was the software used to collect and analyse the data.

Approximately 2.5 mg of dried particles was re-suspended into 1.0 ml of water. The resulting solution was briefly vortexed (5 s) and sonicated (5-10 min). This suspension was left for 10 min at room temperature to allow any heat generated from the sonication step to dissipate. The sample was analysed at an obscuration of 10-20% using the following parameters:
Hardware Setup:
300RF lens – 0.5-90μm; beam width 14.3nm; Magnetic Cell Stirrer MS7, with the
stirrer rate set at the 3rd or 4th demarcation.

Presentation:
3OHD (Standard wet) = 1.5295 (refractive index of sample), 0.1000 (absorption/
imaginary index) in 1.33 (refractive index of water).

Samples were analysed in triplicate. The mean volume diameter (MVD) (D 4,3), and
the distributions D[v 0.1], D[v 0.5] and D[v 0.9] were recorded. Where MVD is the
mean of the diameters of spheres having the same volume as the microparticles; D[v,
0.1] is the point at which the diameters are cut off at 10% of the distribution; D[v, 0.5]
is the volume median diameters and D[v, 0.9] is the point at which the diameters are
cut off at 90% of the distribution. The width of the distribution can be calculated
(termed Span) by the following equation: Span = D[v, 0.9] – D[v, 0.1] ÷ D[v, 0.5].
The span has no units, but gives an idea of whether the distribution is narrow or wide.
An example of a report output is shown in Figure 2.1.

Figure 2.1. An example of a report output using a Mastersizer. A Mastersizer
(Malvern, UK) was used to measure the diameters of microparticles. The mean
volume diameter (MVD) (D 4,3), and the D[v 0.1], D[v 0.5] and D[v 0.9] were
recorded.
2.3.2. Analysis of Diameters Using Photon Correlation Spectroscopy

Microparticles were analysed via photon correlation spectroscopy (PCS) using a ZetaMaster (Malvern Instruments, UK), equipped with a 2mW Helium-Neon laser (633 nm). The machine measures particles with a diameter range of between 2 nm to 3 µm and uses dynamic light scattering of the particles by Brownian motion to calculate the diameters. The lower detection limit for measurement is determined by the power of the laser, and the upper detection limit is determined by the density of the microparticle. Larger particles tend to sediment and therefore cancel out the movement caused by Brownian motion. PCS v1.52, was the software used to collect and analyse the data. The “Automatic” algorithm was used to calculate the diameters. It combines monomodal, non-negative least square fit (NNLS) and CONTIN (a continuous version of NNLS) methods of calculation and is suggested to be a more accurate algorithm to use than using them individually. Size is expressed as the Z-average (Z-ave) in nm (a unique expression of particle size particular to the software analysis), with polydispersity as an indication of how narrow or wide the distribution of the diameters are. A polydispersity of less than 0.100 indicates a narrow distribution, and above 0.500, a wide distribution, indicating a very polydisperse sample. It is suggested by the manufacturer that Z-ave diameter compares well with mean diameters measured using other hardware, but only for monodisperse systems. If the system is polydisperse, then it is more difficult to directly compare mean diameters of the same sample measured from different machines. This is due to the unique way the PCS software derives the mean diameters compared to other techniques used by other machines. Specifics of method used in this thesis are outlined below.

Approximately 2.5 mg of dried particles was re-suspended into 5.0 ml of water (previously filtered through a 0.22 µm filter). The suspension was sonicated briefly and left for 10 min to allow for any heat generated from sonication to dissipate before decanting the sample into a clear-sided acrylic cuvette for analysis. Samples were ensured that they were above 20,000 counts per second and were read on an automatic mode of 10 readings per analysis.
Measurements using this machine were carried out only once due to the length of time needed to perform one measurement (approximately 20 min or more depending on the sample) and the number of samples to be measured. The Z-ave (nm) and the polydispersity of the samples were recorded (Figure 2.2).

![Figure 2.2](image.png)

**Figure 2.2.** An example of a report output from using a ZetaMaster. A ZetaMaster (Malvern, UK) was used to analyse the diameters of microparticles. The Z-ave (nm) and the polydispersity of the samples were recorded.

### 2.3.3. Analysis of Zeta Potential using Laser Doppler Electrophoresis

Zeta potential is an important measurement for dispersion characteristics such as dispersion stability, flocculation, viscosity and film forming characteristics. It is not a direct measurement of surface charge as the composition of the medium used to disperse and measure the samples can affect the zeta potential result. The method works by analysing the changes in the Doppler effect caused by the movement of the particles between a pair of oppositely charged electrodes. The electrophoretic mobility of the particles is converted to a zeta potential by the software (PCS v.1.52) using the Smoluchowski or Huckle approximation. A zeta potential reading which is greater than +30 mV or -30 mV is an indication that the samples are electrostatically stable in that dispersion medium. Specifics of the method used are presented below.
Approximately 2.5 mg of dried particles was re-suspended into 1.0 ml of 5% m/v D-Mannitol (VWR International, UK). The suspension was then sonicated for 10 min before being diluted 1:50 (with 5% m/v D-mannitol) for analysis on a Malvern Zetasizer (Malvern, UK). Readings were carried out at count rates were between 2,000 and 2,500 kcps.

D-Mannitol was chosen as the medium for dispersing the microparticles in because it was suitable for giving a medium that was at the pH and isotonic level that could mimic in vivo situations without having the excess salt level that PBS had, which would interfere with the conductivity of the cell and give a false result.

Samples were analysed in triplicate. The zeta potential (mV) of the samples was recorded (Figure 2.3).

Figure 2.3. An example of a report output from using a ZetaMaster. A ZetaMaster (Malvern, UK) to analyse the surface charge of the particles. The zeta potential (mV) was recorded.

For the above three techniques (laser diffraction, PCS and laser doppler electrophoresis) further information with regards to theory and practical applications can be found on the manufacturer’s website, www.malvern.co.uk.
2.3.4. Scanning Electron Microscopy (SEM) Analysis

Scanning electron microscopy was used to analyse the size and morphology of the microparticles produced. Dried particles were mounted onto an SEM stub with double-sided carbon impregnated discs using a fine hair paintbrush. Samples were then sputter-coated with gold using an Emitech K550 Sputter Coater for 2 min at 30 mA. Samples were viewed under a Phillips/FEI XL30 scanning electron microscope.

2.4. Analysis of DNA Integrity and Encapsulation Efficiency from Microparticles

2.4.1. DNA Extraction from Microparticles

The method used to extract DNA from the microparticles was adapted from Barman et al., 2000 (Barman et al., 2000).

10 mg of particles were weighed out into a 1.5 ml centrifuge tube. 200 µl Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added and the mixture was vortexed briefly. 500 µl chloroform was added and again, the sample was vortexed. The suspension was shaken horizontally in a 37°C incubator for 90 min. After 90 min, the mixture was centrifuged at 14,000 rpm for 10 min. The aqueous layer (approximately 150 µl or less to avoid contamination from the interface) was taken off and put into a fresh 1.5 ml centrifuge tube. The samples were analysed via agarose gel electrophoresis (for integrity and quantification) and PicoGreen® (for quantification). UV was not chosen as a quantification method for this project due to the high-throughput nature and small volumes that were involved.

2.4.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out to observe the different conformations of the plasmid DNA and also to quantify the amount using densitometry (see section 2.4.3).

0.7-0.8% m/v of agarose was added into tris-borate-EDTA (TBE) buffer (0.089 M Tris base; 0.089 M boric acid; 0.002 M EDTA, final pH 8.3). The agarose was dissolved by heating up the solution to 80°C under magnetic stirring until the mixture turned clear. The agarose was then left to cool down to approximately 40-50°C, to
which ethidium bromide was added (to a final concentration of 0.5 µg/ml). The agarose was then poured into an electrophoresis gel rig (BioRad, UK) and left to set. Samples for electrophoresis were prepared by the addition of 6x loading dye before being loaded into each well of the gel. A standard DNA marker was also run alongside the samples – Lamda DNA/Hind III. Electrophoresis was carried out at 65-80V for 1-2 h depending on the size of the gel being run. Agarose gels were viewed under a UV transilluminator using a Syngene GeneGenius Bio Imaging System (Syngene, UK). Images of the gel were captured using the software GeneSnap v.6.00(m) (Syngene, UK).

2.4.3. Quantification of DNA using Densitometry

Quantification of plasmid DNA was performed using the software GeneTools v.3.00(h) (Syngene, UK). The method is based on the fact that when ethidium bromide intercalates with DNA, it will fluoresce under UV illumination, and the intensity of fluorescence correlates with the amount of DNA present (Freeman et al., 1990). The software converts this fluorescence into a numerical ‘volume’, which can be used like the integration number of a peak on a high-performance liquid chromatography (HPLC) trace for example. The numerical volumes are recorded and used to calculate the amount of DNA in a sample in comparison to standards that were run on the same agarose gel. Preliminary studies showed that the minimum amount of DNA detectable for accurate analysis was 15 µg/ml (or 150 ng per well) and the maximum was 100 µg/ml (or 1000 ng per well) (Figure 2.4).

The supercoiled content of the plasmid DNA in a sample was also calculated. This was done by expressing the ‘volume’ of the supercoiled band as a percentage of the total ‘volumes’ of the bands present.
Figure 2.4. Measurement of DNA concentration using densitometry. Image on the left shows an example of what is seen using GeneTools (Syngene, UK) to analyse an agarose gel. The graph on the right shows a standard curve generated. From this, it showed that the minimum amount of DNA detectable for accurate analysis was 15 μg/ml (or 150 ng per well) and the maximum was 100 μg/ml (or 1000 ng per well).

2.4.4. Quantification of DNA using Quant-iT™ PicoGreen® dsDNA Assay Kit

Picogreen® (Molecular Probes, USA) is an ultrasensitive dye that can detect as little as 250 pg/ml double-stranded (ds) DNA (i.e. there is minimal fluorescence when the dye binds to single-stranded DNA or with RNA). Its sensitivity and the ability to carry out the assay in a 96-well plate format, made it ideal for the high throughput nature of the analysis that was required for this project.

The analysis was carried out according to the manufacturer’s instructions (Probes, 2005). It was ensured that the samples were diluted to within the working range of the kit (250 pg/ml – 2 μg/ml) and that the same DNA used for encapsulation into the particles was used for the standard. Dilutions were made with Tris-EDTA buffer (pH 8.0). Samples were analysed in black 96-well plates (Fisher Scientific International) and read at excitation 485 nm/emission 535 nm using a Wallac Victor2™ 1420 Multilabel Counter (PerkinElmer Wallac, UK). The software Wallac 1420 Workstation v.2.00.0.10 was used to collect the data. Concentrations of DNA in samples were calculated from a standard curve that was assayed in the same plate. An alternative standard curve was used instead of the one recommended by the manufacturer’s protocol, this was to even out the spread of points of the curve to allow for a more accurate calculation of unknown concentrations of DNA from the samples (Table 2.2).
Table 2.2. Volumes of Tris-EDTA buffer and stock DNA used to make the standard curve.

<table>
<thead>
<tr>
<th>Volume (µl) of TE</th>
<th>Volume (µl) of 2 µg/ml DNA Stock</th>
<th>Volume (µl) of Diluted Quant-iT™ PicoGreen® Reagent</th>
<th>Final DNA Concentration (ng/ml) in Quant-iT™ Picogreen® Assay*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>950</td>
<td>50</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>900</td>
<td>100</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>800</td>
<td>200</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>200</td>
<td>800</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>0</td>
<td>1000</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

*As only 50 µl of the sample is added into each well.

2.4.5. Definitions of Loading Efficiency and Encapsulation Efficiency

For clarity, the definitions of terms used in this project for loading efficiency and encapsulation efficiency are outlined below:

Definition of Theoretical DNA Loading (% m/m)

\[
100 \times \frac{\text{DNA used for Encapsulation (mg)}}{\text{Microparticle Weight (mg)}}
\]

Definition of Encapsulation Efficiency (% m/m)

\[
100 \times \frac{\text{Encapsulated DNA (µg)}}{\text{DNA Used for Encapsulation (µg)}} = \frac{\text{Actual DNA content (µg)}}{\text{Theoretical DNA content (µg)}}
\]

2.5. Production of DNA

Standard molecular biology techniques were used to propagate DNA and to prepare long-term stocks. Protocols were carried out according to/or modified from Sambrook and Russell (Sambrook and Russell, 2000). All materials and solutions used were sterilised, to remove degradation enzymes (e.g. deoxyribonuclease I and ribonuclease) either by autoclaving or filtering solutions through a 0.22 µm filter.
2.5.1. Type of plasmid DNA used

Reporter gene plasmids were chosen as a model plasmid DNA to be encapsulated. This is so that for future work, analysis of distribution and gene expression in cells can be investigated more easily as there are commercially available kits for analysis.

pCMV-Luciferase gene was an amalgamation of two different vectors; the backbone was from pCMV-β Gal (Clontech, USA) and the luciferase gene was from the pGL3-Control (Promega, USA) (Figure 2.5). The β-galactosidase gene was removed via restriction enzyme digestion as was the luciferase gene from the pGL3 control vector. The backbone of pCMV and luciferase gene was then ligated together to form the complete construct. The resulting plasmid was then used for further studies.

The plasmid DNA, gWiz-luciferase, from Aldevron (USA) was used once the optical parameters of making microspheres had been produced (Figure 2.6). To avoid any adverse effects from residual bacterial components, which can be a contaminant when producing the plasmid DNA from bacteria, it was ensured that the endotoxin levels of all plasmids were below accepted levels (Table 2.3).

![Figure 2.6. Plasmid vector map of gWiz-luciferase. Taken from Aldevron, USA (www.aldevron.com).](image-url)
Figure 2.5. Cloning of pCMV-luciferase plasmid. The backbone was digested from pCMVβ (Clontech, USA) using Not I enzyme (Promega, USA) and ligated to the luciferase portion digested from the pGL3-Control vector (Promega, USA) using the Ncol and XbaI enzymes (Promega, USA). The adapted plasmid was called pCMV-luciferase. Images adapted from Clontech (www.clontech.com) and Promega (www.promega.com) respectively.
Table 2.3. Summary of details of the plasmids used. EU/mg DNA indicates the level of endotoxin units present in the DNA. The levels indicated for pCMV-luciferase refer to the levels quoted by the manufacturer after preparing a large batch of the plasmid DNA (Qiagen, USA. See section 2.5.3). The levels for gWiz-luciferase are quoted from the manufacturer (Aldevron, USA).

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Reporter Gene</th>
<th>Company</th>
<th>Base pairs (bp)</th>
<th>EU/ mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV-luciferase</td>
<td>Luciferase</td>
<td>Promega and Clontech</td>
<td>~5130</td>
<td>&lt; 100</td>
</tr>
<tr>
<td>gWiz-luciferase</td>
<td>Luciferase</td>
<td>Aldveron</td>
<td>6732</td>
<td>&lt; 100</td>
</tr>
</tbody>
</table>

2.5.2. Production of Plasmid DNA

All chemicals for making up bacterial growth medium and agar plates were purchased from Oxoid (UK). With the exception of sodium chloride, which was purchased from VWR International and D-glucose, which was purchased from Sigma (UK).

The bacterial strain, DH5α (Invitrogen, UK), containing plasmid of interest was streaked out onto Luria-Betani (LB) agar plates supplemented with 100 µg/ml ampicillin (Sigma, UK) and grown in an incubator at 37°C for 15 h. Once colonies were visible to the naked eye, these plates were kept at 4°C for storage for no longer than one month. Long-term stocks were prepared as below:

A single colony picked from a freshly grown LB agar plate and was inoculated into 5 ml LB medium containing 100 µg/ml ampicillin. This was grown in a shaking incubator at 37°C, 255 rpm until late log phase of growth was reached. 0.85 ml of the culture was aliquoted into a sterile 1.5 ml centrifuge tube and this was mixed with 0.15 ml sterile 30% v/v glycerol. These were then stored at -70°C.

3.5.3. Large-Scale Production of Plasmid DNA

The Qiagen EndoFree® Plasmid Giga Kit was used to prepare large amounts of high purity plasmid DNA. Combining the methods of alkaline lysis and ion-exchange chromatography, up to 10 mg of DNA can be produced and the endotoxin levels reduced to less than 0.1 EU/ µg DNA. Method was carried out according to the manufacturer’s instructions (Qiagen, 2005).
Plasmid DNA was re-suspended into a suitable volume of Tris-EDTA buffer pH 8.0, aliquoted out and kept at -20°C for long-term storage. Purity and quantity were analysed using UV spectroscopy (GeneSpec I, Naka Instruments) and agarose gel electrophoresis.

For UV spectroscopy, a ratio of 1.8 ($A_{260}$ nm/$A_{280}$ nm) and a purity of 100% was accepted. Anything less meant that there was protein and/or RNA contamination. If that was the case, the DNA was subjected to an additional purification step (see section 2.5.4) to remove these.

2.5.4. Purification of DNA with Ethanol

To remove contaminants from the DNA, the DNA was subjected to precipitation and washing step with salt and ethanol.

A one-tenth volume of sterile 3M sodium acetate, pH 5.2 (Sigma, UK) was added to the DNA solution together with 2x volume of ice-cold absolute ethanol (VWR International). The solution was mixed and incubated at -20°C for 2-16 hours. The precipitated DNA was centrifuged at 14,000 rpm for 10 min. The supernatant was taken off carefully and the pellet was rinsed with ice-cold 70% v/v ethanol. This was centrifuged at 14,000 rpm for 10 min and the supernatant removed. The pellet was air dried at room temperature before being re-suspended in an appropriate volume of sterile Tris-EDTA buffer (pH 8.0).
Chapter 3

The Use of Factorial Design to Optimize Microparticle Formulation Parameters
3.1. Introduction

As mentioned in the main introduction, one of the most popular methods of producing microparticles includes water-in-oil-in-water (w/o/w) double emulsion solvent evaporation. This method has many parameters that can be manipulated to improve or alter particle characteristics, such as temperature at which microparticles are made (Yang et al., 2000a), addition of additives such as salt (Pistel and Kissel, 2000) and technique of solvent removal (Jeyanthia et al., 1996) to name but a few.

For the most part, analysis of the effect of different factors on the microparticle characteristics, e.g. size and loading efficiency, have been carried out on an empirical basis, where one factor has been changed at a time. This is an effective method to look at the important factors that influence the parameters that are of interest, but it is often time consuming and may not pick up the effect of interactions between factors.

An alternative approach to observe different factors is the use of experimental design. Design of experiment (DoE) is already extensively used in the industrial sector to rapidly screen for an optimal formulation (e.g. cake mixtures!). Academic research is now turning to this as means of optimizing formulations, as evidenced by the increase in recent publications (O'Hara and Hickey, 2000; Hedberg et al., 2004; Lopez-Hurtado, 2005). As a rapid and economical means of determining the optimum formulation, it is also ideal for assessing the robustness and reproducibility of methods used, as stated by Lewis et al.:

"Experimental design can be defined as the strategy for setting up experiments in such a manner that the information required is obtained as efficiently and precisely as possible." (Lewis et al., 1999).

3.1.2. Background to Factorial Design

There are many books and reviews available on the background and theory of Factorial Design and Design of Experiments (DoE). With a multitude of models and designs that can be utilized, only a brief outline of the design used in this thesis is written. It is to be mentioned that the bulk of the ideas come from 2 main sources:
Experimental Design (Industrial DOE) in Electronic Statistics Textbook (StatSoft, 2006) and Statistics for Experimenters: Design, Innovation and Discovery (Box et al., 2005). The Handbook that accompanies the Minitab software is also an excellent source of information (Minitab, 2003).

In this chapter, Fractional Factorial Design at Two-Levels \([2^{4-1}]\) was used. An outline of the idea and design is first presented.

3.1.2.1. Basic Idea

For most cases, factors (variables), affecting the process are investigated at two levels, for instance, the amount of solvent used in the oil phase is set at a high and a low level, to see if there are any changes to the end product (response). Conventionally, all different factors would be tested out in all available combinations, but this becomes problematic when the number of experiments to be investigated is increased exponentially. For example, a study of 7 factors will require 128 \((2^7)\) experimental runs, 10 factors would need 1024 runs and so on. If time and resources are precious, then fractional factorial designs can be used to gain the important information in an efficient manner.

3.1.2.2. Generating the Design

A technical description of how fractional factorial designs are constructed is beyond the scope of this introduction. Further information can be found by referring to the above references. In general, for each level the higher and lower variable are assigned a plus (higher) or minus (lower) sign; a logical configuration of experiments (Run Number) is then constructed as demonstrated in Table 3.1.
Table 3.1. An example of a full experimental design with 3 variables. When the experiment is carried out, the ‘runs’ are randomized to avoid any bias and avoid results that may occur through chance or error.

<table>
<thead>
<tr>
<th>Run Number</th>
<th>Coded Design</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
</tr>
</tbody>
</table>

A, B and C are the variables to be investigated, e.g. volume of solvent, mass of polymer. A decision also has to be made as to what the ‘responses’ of the experiment are to be, for example these could be the size of particles, loading efficiency of drug in particles etc. The relationship between the variable and the response will then be computed and analysed in a statistical manner. In fractional factorial design, such a clear configuration cannot be observed as not all experiments are carried out.

3.1.2.3. Design Resolution

One main consequence of not carrying out all the experiments, as would be done so in a full factorial design, is that not all the effects are calculated. In a fractional factorial design, there are certain effects that are said to be ‘confounded’ with other effects. Confounded effects cannot be attributed to a single factor interaction and are said to be aliased with other factors and interactions. Designs of resolution III, IV and V are particularly important. Their definitions according to Minitab (2003) are as follows:

- Resolution III – no main effect is aliased with any other main effect. However, main effects area aliased with two-factor interactions and two-factor interactions are aliased with each other.

- Resolution IV – no main effect is aliased with any other main effect of two-factor interaction. Two-factor interactions are aliased with each other.

- Resolution IV – no main effect of two-factor interaction is aliased with any other main effect or two factor interaction. Two-factor interactions are aliased with three-factor interactions.
3.1.2.4. Centre Points

Centre points are added to a design to investigate the possibility of curvature in the response of a factor. If only two-levels were investigated (i.e. higher and lower) it may not be true that the response follows a linear relationship. Centre points are usually set at the mid-point value of the higher and lower values selected for the factor/variable to be investigated, for example, if the high level of solvent was 500 ml and low level of solvent was selected at 100 ml, then the centre point would be 300 ml. After the experimental runs have been performed, statistical analysis is used to see if the centre point is significantly different to the other points; if it is, then it can be assumed that a non-linear relationship is present. If curvature is discovered, then depending on what the DofE is for, then further experiments can be carried out. In the experimental design, centre points are assigned as zero, the levels are assigned as +1 (higher level) and -1 (lower level).

3.1.2.5. Analysis of Results

Once all the results of the responses from the variables to be investigated have been obtained, these are usually entered into a statistical software package and the relationship between variable and effect on response is calculated using analysis of variance (ANOVA). An example of the results computed by Minitab is displayed in Figure 3.1.

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect</th>
<th>Coef</th>
<th>SE Coef</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>4.890</td>
<td>0.05884</td>
<td>83.12</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>7.214</td>
<td>3.607</td>
<td>0.05884</td>
<td>61.31</td>
<td>0.000</td>
</tr>
<tr>
<td>PVA</td>
<td>-2.915</td>
<td>-1.458</td>
<td>0.05884</td>
<td>-24.77</td>
<td>0.002</td>
</tr>
<tr>
<td>DCM</td>
<td>-7.593</td>
<td>-3.796</td>
<td>0.05884</td>
<td>-64.52</td>
<td>0.000</td>
</tr>
<tr>
<td>Polymer</td>
<td>2.862</td>
<td>1.431</td>
<td>0.05884</td>
<td>24.32</td>
<td>0.002</td>
</tr>
<tr>
<td>Water*PVA</td>
<td>-1.914</td>
<td>-0.957</td>
<td>0.05884</td>
<td>-16.26</td>
<td>0.004</td>
</tr>
<tr>
<td>Water*DCM</td>
<td>-7.892</td>
<td>-3.946</td>
<td>0.05884</td>
<td>-67.07</td>
<td>0.000</td>
</tr>
<tr>
<td>Water*Polymer</td>
<td>2.269</td>
<td>1.135</td>
<td>0.05884</td>
<td>19.28</td>
<td>0.003</td>
</tr>
<tr>
<td>Ct Pt</td>
<td>-3.711</td>
<td>0.11267</td>
<td>-32.94</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.1. Example of the output displayed by the software Minitab v.1.4. Output is produced after factorial ANOVA of results obtained from performing the experimental design.
For most software, P-values associated with the null hypothesis are set at the 5 and 1% level of probability. Major effects of a variable on a response are analysed according to the P-value, if the P-value is less than or equal to the set value (e.g. 0.05), then it can be concluded that the effect is significant and vice versa. The strength of the effect can also be evaluated, the higher the value, the greater the effect on the response. The sign of the effect determines which factor setting results in a higher response setting. A positive sign indicates that the high factor setting results in a higher response than the low setting. A negative sign indicates that the low factor setting results in a higher response than the high setting.

3.1.2.6. Pareto chart of effects

The Pareto chart of effects is often an effective tool for communicating the results of an experiment, an example of which is shown in Figure 3.2. In a Pareto chart, the ANOVA effect estimates are displayed with the largest effect at the top and lowest effect at the bottom. The magnitude of each effect is represented by a column; the larger the effect, the longer the column. Any column that extends beyond the reference line (P-value) indicates which effects are significant.

Figure 3.2. An example of a Pareto chart. This is displayed using the software Minitab v.1.4 (Minitab, USA) after factorial analysis of results obtained from performing the experimental design. The chart displays the factor(s) that have the largest effect at the top and the factor(s) that have the least effect at the bottom. Any bar extending across the reference line (in this case 4.30) is considered statistically significant.
3.1.2.7. Main effects plot

The main effects plot visualises the effect of the factors on the response and to compare the relative strength of the effects. Minitab plots the response means for each factor level then connects the points for each factor. If the line is parallel to the horizontal line, there is no main effect present. The greater the slope of the line, the stronger the effect, an example is displayed in Figure 3.3.

![Main Effects Plot](image)

**Figure 3.3. An example of a main effects plot.** This is displayed using the software Minitab v.1.4 (Minitab, USA) after factorial analysis of results obtained from performing the experimental design. The axis for each chart plot the levels of the variables (water, PVA, DCM and polymer) and the slope of the line gives and indication of how the factor affects the response (particle size). For example, for ‘water’ as the level of water increases, so the size of the particle increases. The steeper the slope, the greater the effect.

3.1.2.8. Interaction plots

A visual look at the effect of interactions is demonstrated in Figure 3.4. This allows the user to see at a glance which interactions are significant. Interpretation of interaction plots are similar to that of main effects plot, if the lines are parallel to each other then there is no interaction. If the lines cross, then there may be interaction. In general, all results need to be interpreted in relation with each other, a main effects plot or interaction plot may show an important effect, but it may not be significant.
Figure 3.4. An example of an interaction plot. This is displayed using the software Minitab v.1.4 (Minitab, USA) after factorial analysis of results obtained from performing the experimental design. The axis for each chart plot the levels of the variables (water, PVA, DCM and polymer) and the slope of the line gives and indication of how the factor affects the response (particle size). An interaction plot gives a visual assessment of any interactions between the variables which affect the response. Parallel lines indicate no interactions e.g. water and PVA; intersecting lines indicate an interaction e.g. polymer and PVA.

3.1.2.9. Summary

The ability to study the impact of large number of factors on a process with relative efficiency means that fractional factorial designs are now the norm in industrial experiments. Despite this though, there are disadvantages to their efficiency in that certain relationships such as curvature can be missed and higher order (greater than two-way) interactions can be missed. It is also important to note that, with all statistical analysis, care must be taken to interpret the results with the knowledge of the technique. A statistically significant result may not be important and vice versa.

3.2. Aims

At the beginning of the project there were problems with reproducibility of microparticles made and it was taking too much time to do one-factor-at-a-time (OFAT) method of optimizing formulation. A list was made of the key items that would affect formulations (Table 3.2) and four factors were to be identified to be studied further.
Table 3.2. Factors influencing microparticle formation in w₁/o/w₂ double emulsion solvent evaporation technique.

<table>
<thead>
<tr>
<th>Step</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Emulsion</strong></td>
<td>Concentration of polymer</td>
</tr>
<tr>
<td></td>
<td>Concentration of PVA</td>
</tr>
<tr>
<td></td>
<td>Type of polymer</td>
</tr>
<tr>
<td></td>
<td>Type of PVA</td>
</tr>
<tr>
<td></td>
<td>Mixer type, speed, time</td>
</tr>
<tr>
<td></td>
<td>Concentration of DNA</td>
</tr>
<tr>
<td></td>
<td>Type of DNA</td>
</tr>
<tr>
<td></td>
<td>Concentration/type of additives</td>
</tr>
<tr>
<td></td>
<td><strong>Temperature</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Volume of water</strong></td>
</tr>
<tr>
<td><strong>Secondary Emulsion</strong></td>
<td>Volume of water</td>
</tr>
<tr>
<td></td>
<td>Concentration of PVA</td>
</tr>
<tr>
<td></td>
<td>Beaker type</td>
</tr>
<tr>
<td></td>
<td>Mixer type, speed, time</td>
</tr>
<tr>
<td></td>
<td><strong>Temperature</strong></td>
</tr>
<tr>
<td></td>
<td>Addition of primary emulsion</td>
</tr>
<tr>
<td><strong>Evaporation Phase</strong></td>
<td>Time</td>
</tr>
<tr>
<td></td>
<td>Stirrer speed</td>
</tr>
<tr>
<td></td>
<td><strong>Temperature</strong></td>
</tr>
<tr>
<td></td>
<td>Evaporation area (beaker type)</td>
</tr>
<tr>
<td><strong>Washing Phase</strong></td>
<td>Centrifuge force</td>
</tr>
<tr>
<td></td>
<td>Centrifuge time</td>
</tr>
<tr>
<td></td>
<td><strong>Volume of wash</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Number of washes</strong></td>
</tr>
<tr>
<td><strong>Freezing</strong></td>
<td>Temperature</td>
</tr>
<tr>
<td></td>
<td>Time</td>
</tr>
<tr>
<td></td>
<td><strong>Volume</strong></td>
</tr>
</tbody>
</table>

The text in bold were parameters that were considered to have a large influence on the formulations, the ones in italics were parameters that were difficult to control and so were not chosen to be studied further. A fractional factorial experiment was then designed based on previous knowledge of particle making.

The aim of the design was to produce particles in a reproducible manner, and to observe which parameters had the largest influence on the particles with regards to the responses to be assessed.
3.3. Materials and Methods

For materials and methods used for making and characterising microparticles using DofE, please refer to Chapter 2.

3.3.1. Fractional Factorial Design

The software, Minitab v1.4 (Minitab, USA) was used to generate the design. A half-fraction with IV Resolution, $2^{4-1}$ design was selected. The experiment was designed as one block and 3 centre points were added. The four factors (X) chosen are listed on Table 3.3. and the design generated in Table 3.4.

Table 3.3. Variables and Level Values for $2^{4-1}$ Fractional Factorial Design.

<table>
<thead>
<tr>
<th>Code</th>
<th>Variable</th>
<th>Low Level (-1)</th>
<th>Centre Point (0)</th>
<th>High Level (+1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁</td>
<td>Internal Aqueous Volume (ml)</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
</tr>
<tr>
<td>X₂</td>
<td>PVA Concentration (% m/v)</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>X₃</td>
<td>DCM Volume (ml)</td>
<td>2.5</td>
<td>5.0</td>
<td>7.5</td>
</tr>
<tr>
<td>X₄</td>
<td>Polymer (mg)</td>
<td>100</td>
<td>300</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 3.4. Run Parameters for $2^{4-1}$ Fractional Factorial Design. Std Order = Standard or Yates Order, where the + and - are ordered in a logical fashion. This is more noticeable in a full factorial design, less so in a fractional design as not all the combinations are shown. Experiments are carried out in the ‘Run order’ generated by the software to ensure randomisation.

<table>
<thead>
<tr>
<th>Std Order</th>
<th>Run Order</th>
<th>X₁</th>
<th>X₂</th>
<th>X₃</th>
<th>X₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>-1</td>
<td>-1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>+1</td>
<td>+1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>-1</td>
<td>+1</td>
<td>-1</td>
<td>+1</td>
</tr>
</tbody>
</table>

For each ‘run’, the volumes and masses of material were used according to the DofE. Other parameters involved were kept at fixed values to avoid any other factors influencing the experimental responses. Fixed parameters are set out below:
- External Aqueous Phase was 40 ml of 2.5% m/v PVA.
- Primary emulsion was 24,000 rpm, 2 min, using Ultraturrax homogenizer in small 10 ml glass vials.
- Secondary emulsion was 9,000 rpm, 5 min using Silverson L4RT homogenizer in tall 200 ml glass beakers.
- Solvent evaporated overnight at room temperature.
- Particles washed (in distilled water) and centrifuged two times at 15,000 rpm for 15 min (Sorvall Dupont Ultracentrifuge).

Two experiments were carried out:

- Particles made without DNA (instead of DNA, the equivalent volume of TE pH 8.0 buffer was added). For these, the responses (Y) to be observed were chosen to be, recovery of particles, size of particles and zeta potential.
- Particles with DNA encapsulated. Mass of pCMV-luciferase DNA added to DNA-loaded particles was kept constant at 200 μg (200 μl of 1mg/ml stock solution). For these, the responses (Y) were recovery of particles, size, zeta potential and encapsulation efficiency.

For each type of experiment, only one batch of particles was made. This was because there was already a control for replication by the inclusion of centre points, and also because this was a preliminary screening experiment.

3.4. Results
3.4.1. Characterisation of Particles
3.4.2. General comments

All formulations produced particles. Raw data of the responses are shown below in Table 3.5. for empty particles and Table 3.6. for DNA-loaded particles. It can be seen that there is a wide range in diameters and morphology of particles produced (Tables 3.5 and 3.6; Figure 3.5). Because the diameters of the microparticles produced were unknown, they were sized using both PCS and laser diffraction techniques. It can be clearly seen that there is a large disparity between the diameters measured using either PCS or laser diffraction, and that for these microparticles, laser diffraction was the more accurate measurement, as they correlated well with the SEM images.
Table 3.5. Data for the responses analysed for each variable for empty particles.

<table>
<thead>
<tr>
<th>Run No</th>
<th>Particle Yield (m/m)</th>
<th>Z-Ave (nm) (Polydispersity)</th>
<th>MVD (µm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>81.1</td>
<td>635.7 (0.40)</td>
<td>2.60 ± 2.357</td>
<td>-24.8 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>80.0</td>
<td>1337.7 (0.38)</td>
<td>2.35 ± 1.815</td>
<td>-26.2 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>72.1</td>
<td>603.9 (0.38)</td>
<td>2.11 ± 2.145</td>
<td>-25.2 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>76.0</td>
<td>616.1 (0.24)</td>
<td>18.89 ± 10.286</td>
<td>-26.1 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>87.3</td>
<td>906.3 (0.31)</td>
<td>2.42 ± 1.807</td>
<td>-25.1 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>84.5</td>
<td>896.8 (1.00)</td>
<td>1.95 ± 1.178</td>
<td>-26.0 ± 0.4</td>
</tr>
<tr>
<td>7</td>
<td>87.1</td>
<td>1035.0 (0.40)</td>
<td>2.04 ± 1.652</td>
<td>-24.2 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>79.5</td>
<td>1141.3 (0.42)</td>
<td>2.09 ± 1.570</td>
<td>-23.7 ± 0.6</td>
</tr>
<tr>
<td>9</td>
<td>83.5</td>
<td>1361.3 (0.50)</td>
<td>2.25 ± 1.763</td>
<td>-23.9 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>83.5</td>
<td>929.2 (0.35)</td>
<td>2.30 ± 1.730</td>
<td>-26.9 ± 0.6</td>
</tr>
<tr>
<td>11</td>
<td>75.8</td>
<td>929.2 (0.35)</td>
<td>10.20 ± 5.196</td>
<td>-24.2 ± 0.3</td>
</tr>
</tbody>
</table>

Table 3.6. Data for the responses analysed for each variable for DNA-loaded particles.

<table>
<thead>
<tr>
<th>Run No</th>
<th>Particle Yield (m/m)</th>
<th>Z-Ave nm (Polydispersity)</th>
<th>MVD (µm)</th>
<th>Zeta Potential (mV)</th>
<th>Encapsulation Efficiency (m/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65.9</td>
<td>664.0 (0.29)</td>
<td>2.20 ± 2.228</td>
<td>-20.6 ± 1.0</td>
<td>5.27</td>
</tr>
<tr>
<td>2</td>
<td>68.2</td>
<td>2694.6 (0.58)</td>
<td>6.39 ± 4.311</td>
<td>-22.2 ± 1.0</td>
<td>77.77</td>
</tr>
<tr>
<td>3</td>
<td>59.4</td>
<td>760.2 (0.45)</td>
<td>5.49 ± 10.411</td>
<td>-20.6 ± 1.0</td>
<td>5.05</td>
</tr>
<tr>
<td>4</td>
<td>57.0</td>
<td>1260.1 (0.41)</td>
<td>40.16 ± 20.025</td>
<td>-21.1 ± 1.0</td>
<td>43.52</td>
</tr>
<tr>
<td>5</td>
<td>77.4</td>
<td>1042.6 (0.40)</td>
<td>3.09 ± 2.389</td>
<td>-21.7 ± 1.0</td>
<td>38.79</td>
</tr>
<tr>
<td>6</td>
<td>77.7</td>
<td>1472.5 (0.42)</td>
<td>2.70 ± 1.864</td>
<td>-21.1 ± 1.0</td>
<td>66.60</td>
</tr>
<tr>
<td>7</td>
<td>83.7</td>
<td>2438.4 (0.69)</td>
<td>5.14 ± 2.578</td>
<td>-22.1 ± 1.0</td>
<td>65.57</td>
</tr>
<tr>
<td>8</td>
<td>84.7</td>
<td>1822.1 (0.37)</td>
<td>3.96 ± 2.269</td>
<td>-21.0 ± 0.1</td>
<td>68.63</td>
</tr>
<tr>
<td>9</td>
<td>81.9</td>
<td>1445.1 (0.42)</td>
<td>3.92 ± 2.407</td>
<td>-21.0 ± 0.5</td>
<td>72.29</td>
</tr>
<tr>
<td>10</td>
<td>83.7</td>
<td>945.0 (0.62)</td>
<td>7.33 ± 9.389</td>
<td>-21.2 ± 1.0</td>
<td>45.12</td>
</tr>
<tr>
<td>11</td>
<td>70.6</td>
<td>3138.6 (0.84)</td>
<td>16.67 ± 9.301</td>
<td>-21.9 ± 1.0</td>
<td>30.06</td>
</tr>
</tbody>
</table>

Figure 3.5. A sample of scanning electron microscope pictures of DNA-loaded DoE particles. These illustrate the range in size and morphology of the particles produced. A = Run 1, B = Run 4, C = Run 9. Empty and loaded particles showed similar morphology (not shown).
DNA that was extracted from the microparticles demonstrated that the process had altered the plasmid conformation to varying degrees, i.e. supercoiled to open-circular to linear forms are observed (Figure 3.6). Most formulations had lost the supercoiled conformation, indicating that the process was damaging the DNA. There did not seem to be a correlation between the loading efficiency (Table 3.6) and the amount of supercoiled DNA preserved.

Figure 3.6. Agarose electrophoresis gel analysis of the DNA extracted for the DofE particles. Positive = Blank particles spiked with equivalent input DNA and extracted in the same manner as loaded particles. The ‘input DNA’ (lanes 25-27) on the gel have not been through the extraction process, and therefore is a control to show that the extraction process does not affect the DNA conformation.

3.4.3. Statistical Analysis of Particles

The hypothesis for the statistical analyses is:

- **Null** (\(N_0\)) hypothesis is that the factor has no effect on response.
- **Alternative** (\(A_0\)) hypothesis is that the factor has an effect on the response.

3.4.3.1. Blank Particles

Factorial ANOVA showed that all factors gave a statistically significant change in particle size, and there are some significant interactions (Figure 3.7). The presence of curvature in the response complicated the analysis and made the interaction plot difficult to interpret. However, it was clear that high water volume in general leads to larger particle size, and that high DCM volume results in low particle size. For the other
responses analysed such as zeta potential and recovery of particles, there were no significant effects observed.

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>7.214</td>
<td>0.000</td>
</tr>
<tr>
<td>PVA</td>
<td>-2.915</td>
<td>0.002</td>
</tr>
<tr>
<td>DCM</td>
<td>-7.593</td>
<td>0.000</td>
</tr>
<tr>
<td>Polymer</td>
<td>2.862</td>
<td>0.002</td>
</tr>
<tr>
<td>Water*PVA</td>
<td>-1.914</td>
<td>0.004</td>
</tr>
<tr>
<td>Water*DCM</td>
<td>-7.892</td>
<td>0.000</td>
</tr>
<tr>
<td>Water*Polymer</td>
<td>2.269</td>
<td>0.003</td>
</tr>
<tr>
<td>Ct Pt</td>
<td></td>
<td>0.001</td>
</tr>
</tbody>
</table>

Figure 3.7. Pareto chart, main effects plot and interaction plot for response analysed ‘size’ for empty particles. All factors gave significant effects, with the volume of water and volume of DCM having the largest effect on the particle size.

3.4.3.2. Loaded Particles

Results for DNA-loaded particles were very similar to the blank particles with regards to size. The effects and trends were the same, that is, all factors gave a statistically significant change in particle size, and there were some significant interactions (Figure 3.8).
Table 3.8: Pareto Chart of the Standardized Effects (response is particle size, α = .05)

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>5.502</td>
<td>0.004</td>
</tr>
<tr>
<td>PVA</td>
<td>-6.591</td>
<td>0.003</td>
</tr>
<tr>
<td>DCM</td>
<td>-15.248</td>
<td>0.001</td>
</tr>
<tr>
<td>Polymer</td>
<td>14.685</td>
<td>0.001</td>
</tr>
<tr>
<td>Water*PVA</td>
<td>-14.140</td>
<td>0.001</td>
</tr>
<tr>
<td>Water*DCM</td>
<td>-5.669</td>
<td>0.004</td>
</tr>
<tr>
<td>Water*Polymer</td>
<td>6.329</td>
<td>0.003</td>
</tr>
<tr>
<td>Ctp</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.8. Pareto chart, main effects plot and interaction plot for response analysed ‘size’ for DNA-loaded particles. All factors gave significant effects, with the volume of water and volume of DCM having the largest effect on the particle size.

For zeta potential, there were no significant effects. For recovery of particles, there was some degree of an effect with PVA significantly affected the recovery with more PVA giving slightly higher recovery (results not shown). The question was whether this was of any practicable difference. For the encapsulation efficiency of DNA, there were significant interactions between inner aqueous volume and PVA (Figure 3.9).
Figure 3.9. Pareto chart, main effects plot and interaction plot for response analysed ‘loading efficiency’ for DNA-loaded particles. There is a significant interaction between volume of water and PVA.

3.4.3.3. Statistical analysis using all the data – Blank and DNA-loaded

Analysis of the responses, size, zeta potential and recovery was performed using all the data attained. Note that this was done on coded units, so on the charts, instead of the actual numerical levels being displayed, the low values are -1, centre point becomes 0, and the high values becomes +1, as outlined in the design in Table 3.4.

For the microparticle size, the analysis was performed a number of times to discard the non-significant factors. Following this, the factors that were statistically significant were, DCM, polymer, water, and some interactions (water with PVA and with DCM, and loading of DNA with polymer) (Figure 3.10). Significant curvature was observed – i.e. on the main effects plot, the centre point does not sit within the line which is drawn between the low and high levels of the variables.
Figure 3.10. Pareto chart, main effects plot and interaction plot for response analysed ‘size’ for DNA-loaded and empty particles.

There was a significant effect of loading on zeta potential; with loaded particles being less negatively charged. For the recovery (yield) of microparticles, analyses indicated that loading and PVA had an effect on recovery. The addition of DNA resulted in lower recovery and an increase in PVA led to higher a recovery.

3.4.3.4. Further remarks

The centre point is usually used in DoE for two reasons: 1. to measure the curvature of the responses, and 2. as they are repeated several times, they give an indication of the repeatability of the test result. In this case, there was significant curvature for most of the parameters tested; with the centre point sometimes giving the most optimal result and in other cases it looked like there was some sort of a limit to the analysis. For example, the centre point and one of the levels of the variables would behave the same, but the other level gave a different result. This indicated that the model on occasion did not fit the data very well. Despite this, there was a good agreement between the results obtained for blank and DNA-loaded particles. This result was encouraging as it gave confidence that the technique used to manufacture the particles was reproducible.
3.4.3.5. Response Optimization

Another function of Minitab programme is that on the basis of the analyses of the results obtained from the fractional factorial design, it can give an optimized formulation based on the responses that are desired of the formulation. The parameters that are required are entered into the programme (Table 3.7) and the programme will offer a ‘Global Solution’. For this project, the diameter of the microparticles, the encapsulation efficiency and the yield of the microparticles were of most importance, so these were selected to be optimized.

Table 3.7. Response Optimizer. The encapsulation efficiency, size and recovery of the microparticles were selected as the most important parameters to optimize. For details of the response optimizer, please refer to the main text.

<table>
<thead>
<tr>
<th>Response</th>
<th>Goal</th>
<th>Lower</th>
<th>Target</th>
<th>Upper</th>
<th>Weight</th>
<th>Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Encapsulated</td>
<td>Maximum</td>
<td>60</td>
<td>80</td>
<td>80</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Size</td>
<td>Target</td>
<td>0.7</td>
<td>2.7</td>
<td>4.7</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Recovery</td>
<td>Maximum</td>
<td>60</td>
<td>80</td>
<td>80</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

For each response, the ‘goal’ is chosen according to what is most desired (Minitab, 2003):

- Minimum = the desirability is one for all response values less than or equal to the target and decreases for response values greater than the target.

- Maximum = the desirability increases as response values increase from the lower bound to the target, and the desirability is one for all values at or above the target.

- Target = the desirability is one at the target and decreases the more the response deviates from the target in either direction.

- Lower = smallest acceptable response value
- Target = the most desirable response value
- Upper = the highest acceptable response value

- Weight = determines how the desirability is distributed over the interval between the lower (or upper) bound and target.

- Importance = determines the relative importance of the multiple response variables.
After analysing all factors, the global solution is set out below:

**Global Solution**

- **Water** = 0.5 ml
- **PVA** = 10.0 % m/v
- **DCM** = 5.0 ml
- **Polymer** = 300.0 mg

It was a coincidence, but the 'global solution' offered by the software programme also happened to be the parameters used for the centre point in the experimental design.

### 3.5. Discussion

The DofE was able to give a rapid assessment of reproducibility and effect of variables on responses in an economical and rational manner. Out of interest, results obtained from this experimental design agreed with many previous studies carried out on an OFAT basis of investigation.

#### 3.5.1. Particle size

Factors that were statistically significant were, DCM, polymer, water, and some interactions (water with PVA and with DCM, and loading with polymer). Increasing water volume lead to an increase in size of the particles, whilst increasing the amount of PVA lead to a decrease in size. Similarly, increasing the volume of DCM decreases size and increasing polymer increases size of particles. This has been attributed to the change in viscosity of the different phases and the differences in phase distributions. The increase in polymer concentration increased the viscosity of the oil phase, which decreased the distribution of the oil phase and therefore the formation of larger microspheres occurs (Liu and Deng, 2002). Also, the more viscous polymer solution is more difficult to be broken up into smaller droplets at the same input power of mixing (Yang et al., 2000b). The addition of stabilisers such as PVA can enhance the stability of the w1/o/w2 emulsion, therefore increasing the amount of PVA can lead to smaller particles (Liu and Deng, 2002). In general, regardless of processing technique employed to make DNA loaded particles, the following trends seem to hold; decrease in particle size results from increases in the polymer matrix molecular weight, increases in extraction rate, or decreasing emulsion micelle size or mixing scale length (Lengsfeld et al., 2002).
3.5.2. Zeta potential

There was a significant effect of loading on zeta potential. Particles encapsulating DNA were less negatively charged. This has not been investigated much in the literature, but in this case it was probably more an experimental anomaly. The zeta potentials of blank and DNA-loaded particles were measured on different days. Unfortunately, the equipment used to measure the zeta potential is subject to slight fluctuations on a day-to-day basis, and as the differences, although consistent, between blank and DNA-loaded microparticles was so minimal, it would be more realistic to ignore this 'significant' result. One method to test if this relationship is true is to measure all the samples together at one time, or mix up some loaded and empty particles and then measure to avoid the daily variability.

3.5.3. Recovery

Loading and PVA had an effect on recovery. DNA-loading resulted in lower recovery, with higher PVA leading to higher recovery. It is unclear why the addition of DNA would lead to a lower yield, but as with the result obtained with zeta potential, this 'significant' result can probably be discounted. The differences in yield, is not significant in a practical sense. For the result of additional PVA leading to higher yields, this is probably due to the extra mass of PVA added. PVA is known to bind irreversibly to the microparticle surface (Lee et al., 1999) and this could contribute to the 'yield'.

3.5.4. Encapsulation Efficiency

For the encapsulation efficiency of DNA, there were significant interactions between inner aqueous volume and PVA. Increasing water volume lead to a decrease in loading efficiency; increasing PVA, decreased loading and increasing DCM lead to decrease in loading. This is all related to size of particles, in general the smaller the size of particles, the lower the loading efficiency. The surface area of larger particles is smaller, which reduces the transferring chance of DNA (Liu and Deng, 2002). Also, the increased polymer concentration of the oil phase was equal to the decrease of the organic solvent volume, for with the increased precipitation rate of polymer from organic solvent was obtained. Therefore, the loading efficiency of the microspheres increased with increasing concentration of polymer in oil phase (Liu and Deng 2002). Although Yang et al., (2000) did their experiments with BSA, the trends observed were similar. The
volume ratio of oil to internal water had less effect on encapsulation efficiency and this was in agreement with results obtained in this project, as it showed that there was no interaction involved. They also suggested that high polymer concentration causes a solidification of the microspheres, which inhibits diffusion of the BSA towards the external phase. This might be the reason for the relatively high encapsulation efficiency. This could be the explanation for the DofE observation that an increase in polymer concentration results in increased loading efficiency. In some cases the results obtained from the DofE disagreed with other published results. Jeffrey et al., (1993) showed that an increase in the internal aqueous volume increased the entrapment of OVA in their PLGA particles. This is opposite to what has been observed with the DNA particles where an increase in water volume decreased the entrapment efficiency.

3.6. Conclusions and Future Work

The use of experimental design allowed a rapid and economical process for selecting the best formulation for manufacturing microparticles with suitable characteristics for encapsulating plasmid DNA and also allowed assessment of reproducibility of the method used to make the particles. Certain relationships between the factors and responses were elucidated and the results of the experimental design also correlated well with results from previous publications that used OFAT methods of analysis of relationships between factors in particle manufacture methods. The results obtained also highlighted the importance of interpreting ‘statistically significant’ findings according the knowledge of the techniques used to make the microparticles as well as the techniques used to analyse the responses.

The software used for experimental design, also allowed analysis and prediction of a formulation that was optimal for the parameters required. The ‘global solution’ that was predicted, also happened to be the formulation for the centre points for the design, and so this formulation was chosen as a platform for further development.
Chapter 4

The Use of Buffers to Enhance DNA Stability During Microparticle Preparation
4.1. Introduction

The experimental design presented a formulation that had a suitable diameter, yield and encapsulation efficiency for pulmonary delivery of DNA vaccines. The next step of the process now involved improving the 'stability' of the DNA that was entrapped. DNA that was extracted from the microparticles was mainly in the open-circular and linear conformation, i.e. it was not in the same conformation as the original, unprocessed form. To understand how DNA is stabilized, and why it is important to maintain its conformation, a brief explanation is given below.

4.1.2. Different conformations of plasmid DNA

DNA is a polymer made up of sugars (2'-deoxyribose), 4 different bases – double-ring purines (adenine and guanine) and single ring pyrimidines (thymine and cytosine) and phosphate groups that are attached via the sugar. Double-stranded DNA consists of two polynucleotides joined together by phosphodiester bonds through the phosphate groups and the bases of the DNA interact through hydrogen bonds (Figure 4.1).

![Figure 4.1. Two versions of the DNA double-helix structure.](image)

Left = Simplified model
Right = Space-filled model. Taken from (Brown, 1996)

Much of the DNA that is used in recombinant DNA technology is based on plasmid DNA. Plasmid DNA is a circular piece of DNA that is found in bacterial and some other types of cell (Brown, 1996). As circular plasmid DNA is very
large (often larger than the bacteria itself), enzymes such as DNA topoisomerases cause the DNA to wind around itself so that it takes on a more compact form (Brown, 1996). Therefore, in general, plasmid DNA can take on 3 different conformations, supercoiled (closed-circular, Form I), open-circular (nicked, relaxed, Form II) and linear DNA (Form III) (Middaugh et al., 1998). The different forms are dependent on whether there is breakage in the original supercoiled conformation. If there is a cut in supercoiled DNA, it unravels out into its open circular form, if there is another cut in this DNA directly opposite the first cut, the open circular form opens up again into a linear piece of DNA (Middaugh et al., 1998).

These 3 different forms of DNA can be readily separated on the basis of their size by means of agarose gel electrophoresis (Levy et al., 2000). Supercoiled DNA being most compact moves fastest through the gel matrix compared to open-circular and linear (Figure 4.2).

![Figure 4.2. Schematic diagram of the movement of different conformations of plasmid DNA through an agarose gel.](image)

It is generally accepted that for genetic transfection of foreign DNA into cells (be it prokaryotic or eukaryotic), the supercoiled form of plasmid DNA offers the highest efficiency of transfection (Adami et al., 1998; Hao et al., 2000).
4.1.3. Factors that degrade DNA

Although the conversion of supercoiled plasmid DNA to open-circular and linear forms are considered as damage to the molecule, on the whole, the double-stranded DNA (dsDNA) is still intact and therefore functional, albeit the supercoiled form is the most efficient (Adami et al., 1998; Hao et al., 2000). DNA can be seriously damaged in that the bonds between the molecules that keep the double-helix structure can break and lead to fractions of DNA being formed, this can cause the DNA to be completely redundant functionally. This type of damage can be caused by free-radical oxidation or through depurination and β-elimination of the bases of the DNA and lead to cleavage of the phosphodiester backbone (Middaugh et al., 1998).

Many researchers have investigated different parameters that degrade DNA during microparticle preparation. Exposure of plasmid DNA to pH ≤ 3 resulted in rapid loss of transfectivity of HEK293 cells (Walter et al., 1999). Large deviations of pH from normal have been shown to contribute to the breakdown of DNA in aqueous solution by the way of hydrolysis. Although this process is slow, it can be accelerated if the pH is below 7.5 or above 9.0 (Lentz et al., 2005).

Lengsfeld and Anchordoquy (2002) looked at the effects of molecular weight of the plasmid DNA, strain rate, particle size, flexibility, ionic strength, gas-liquid interfaces and turbulence on the fluid flow degradation of supercoiled DNA. They found that plasmid DNA smaller than 5 kb were not very sensitive to shear-induced degradation and that strain rate rather than shear stress is more indicative of plasmid DNA damage. Gas-liquid interfaces and flexibility had unclear effects on plasmid degradation. Although, Levy et al., (1999) found that air-liquid interfaces caused damage to DNA, the discrepancy to Lengsfeld and Anchordoquy's findings could be due to the different sizes of plasmid DNA investigated. Levy's plasmid DNA's ranged from 13 – 29kb; Lengsfeld and Anchordoquy did not specify specific sizes of plasmid DNA investigated.
Shear-stress has been cited as the main cause of plasmid DNA degradation in other research as well, Lentz et al., (2005), observed this as the case for jet nebulisation of DNA polyplexes and the process of spray-drying also causes supercoiled DNA to change to open-circular form (Kuo, 2003; Jilek et al., 2004). Shear sensitivity is dependent on size, with increasing sensitivity with increasing molecular weight of DNA, but independent of plasmid concentration (Levy et al., 1999).

For all researchers that are developing plasmid DNA vaccines, protection of the DNA from shear stress (caused mainly by the methods to entrap DNA into polymeric microparticles) and other damage is of paramount importance and in the section below some methods that have been utilized are outlined.

4.1.4. Methods to prevent DNA degradation

There have been two main methods of protecting DNA from damage – one is to reduce the DNA hydrodynamic size and the other is to use buffers in the aqueous phase in which the DNA is in. The buffers may act as chelators to scavenge any free radicals or to stabilise the pH to which the DNA is exposed.

Addition of buffers such as NaHCO₃ (Walter et al., 1999) and simple salts such as NaCl (Lengsfeld and Anchordoquy, 2002) have been shown to protect DNA against shear-stress and pH-related degradation. They may work through a variety of methods; increases in ionic strength of the solution that the DNA is in has been shown to alter the supercoiled form of the plasmid DNA (Lyubchenko and Shlyakhtenko, 1997) – the negative charge of the phosphate backbone can be neutralised by the buffers and thus reduce the hydrodynamic diameter of the DNA. As the plasmid DNA is smaller, it will be less susceptible to shear. Acidic pH is known to accelerate the degradation of DNA, increasing the pH that the DNA is in will protect it from depurination. It has been suggested that increasing the pH of a formulation to pH 8.5 greatly enhances the stability of DNA in solution at room temperature (Middaugh et al., 1998).
Plasmid DNA can be condensed directly using cations that will neutralise the charge on the phosphate backbone and cause it to collapse into an extremely compact molecule and thus protect the DNA from shear-induced damage. Such cations include poly-L-lysine (PLL), polyethylenimine (PEI), spermidine and polyethylene glycol (PEG) (Lengsfeld and Anchordoquy, 2002; Kuo, 2003).

Chelators such as ethylenediaminetetraacetic acid (EDTA) can protect DNA from free-radical damage, and also by scavenging trace metals (e.g. Mg$^{2+}$) that can be used by enzymes, such as DNase, that degrade DNA (Lentz et al., 2005). If EDTA binds to iron, the complex formed is an effective generator of hydroxyl radicals. Chelators effective against Fe$^{2+}$ include Desferal, diethylenetriaminepentaacetic acid (DPTA), inositol hexaphosphosphate and ethylenediaminebis(o-hydroxyphenylacetic acid) (Middaugh et al., 1998).

Other solutions for reducing shear-induced damage include freezing the primary DNA emulsion during w/o/w$_2$ double-emulsion solvent evaporation preparation of microspheres by immersing it into liquid nitrogen before proceeding onto the secondary stage of emulsion. The frozen state of the primary emulsion means that there is zero shear and hence the DNA is protected (Ando et al., 1999). Introduction of pauses during emulsification of DNA into polymer also helped in reducing damage and increasing encapsulation efficiency (Tinsley-Bown et al., 2000).

4.2. Aims

From the literature, the inclusion of NaHCO$_3$ has been used to protect DNA from degradation through shear-forces (Walter et al., 1999; Li et al., 2003). With this in mind another buffer suitable for in vitro/in vivo use was sourced for a comparative study. The following were thus selected:

1. Sodium Bicarbonate (NaHCO$_3$) – pH = 8.3 for freshly prepared 0.1M aqueous solution; alkalinity increases on standing, agitation and heating. GRAS listed (Crowe et al., 2003).
2. Sodium Phosphate, Dibasic (\(\text{Na}_2\text{HPO}_4\)) – \(\text{pH} = 9.1\) for 1% w/v aqueous solution of anhydrous material. Very soluble in water. GRAS listed (Crowe et al., 2003).

As well as investigating the protective effects of the addition of buffers, further analytical experiments were also carried out to elucidate how and to what degree do buffers offer protection, these included:

1. At which point in the manufacturing process is the DNA degrading?
2. Is it pH that is more important for DNA conservation? or
3. Is it salt concentration that is more important?

4.3. Materials and Methods

The salts, \(\text{NaHCO}_3\) and \(\text{Na}_2\text{HPO}_4\) were from Sigma (Poole, UK). For the list of companies from where the other materials were purchased from, please refer to Chapter 2.

4.3.1. Plasmid DNA Stability

Plasmid DNA in various solutions (with or without buffer and with or without PVA) was homogenized at 24,000 rpm with an Ultraturrax T25 homogenizer. After homogenization, the samples were then run on an agarose gel (Chapter 2, Section 2.4.2). For DNA that was homogenized in the presence of polymer, the DCM was allowed to evaporate off, before the DNA was extracted out (Chapter 2, Section 2.41.) from the resulting polymer film. The extracted DNA was then run on an agarose gel as normal.

4.3.2. Microparticle Preparation

4.3.2.1. Different buffers

A summary of the microparticle formulations made with 0.1M \(\text{NaHCO}_3\) and 1% m/v \(\text{Na}_2\text{HPO}_4\) are outlined in Table 4.1. The DNA used in these preparations was gWiz-luciferase at a stock solution of 1 mg/ml.
Table 4.1. Outline of formulations made. *Internal phase volume was 0.5 ml and included 10% m/v PVA; this was made up using water or relevant buffers. ^External phase was 40.0 ml and included 2.5% m/v PVA; this was made up using water or relevant buffers.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>PLGA 50:50 DL 4A (mg)</th>
<th>DNA (µg)</th>
<th>Internal Phase*</th>
<th>External Phase^</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300</td>
<td>None</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>200</td>
<td>Water</td>
<td>Water</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>None</td>
<td>0.1M NaHCO₃</td>
<td>0.1M NaHCO₃</td>
</tr>
<tr>
<td>4</td>
<td>300</td>
<td>200</td>
<td>0.1M NaHCO₃</td>
<td>0.1M NaHCO₃</td>
</tr>
<tr>
<td>5</td>
<td>300</td>
<td>None</td>
<td>1% m/v Na₂HPO₄</td>
<td>1% m/v Na₂HPO₄</td>
</tr>
<tr>
<td>6</td>
<td>300</td>
<td>200</td>
<td>1% m/v Na₂HPO₄</td>
<td>1% m/v Na₂HPO₄</td>
</tr>
</tbody>
</table>

4.3.2.2. Different pH

The effect of different pH on the particle characteristics was investigated. Different pH were obtained through different ratios of monosodium phosphate (anhydrous) and trisodium phosphate dodecahydrate using the pH calculator from the following website: http://members.nuvox.net/~on.jwclymer/phos.html (accessed 10 May 2005).

As a 1% m/v solution of Na₂HPO₄ is 70 mM, other salt concentrations were based on this molarity. The different pH solutions were then used to make up the internal and external PVA solutions (Table 4.2).

Table 4.2. Percentage m/v of monosodium phosphate and trisodium phosphate used to make up the different pH solutions.

<table>
<thead>
<tr>
<th>pH</th>
<th>Monosodium Phosphate % m/v</th>
<th>Trisodium Phosphate % m/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.8349</td>
<td>0.0160</td>
</tr>
<tr>
<td>7</td>
<td>0.5976</td>
<td>0.7673</td>
</tr>
<tr>
<td>9</td>
<td>0.4229</td>
<td>1.3206</td>
</tr>
<tr>
<td>11</td>
<td>0.4008</td>
<td>1.3904</td>
</tr>
<tr>
<td>12</td>
<td>0.0724</td>
<td>2.4299</td>
</tr>
</tbody>
</table>

4.3.2.3. Different salt concentrations

For the effect of different salt concentrations on the plasmid DNA, 3% m/v and 0.02% m/v Na₂HPO₄ were made and compared to those made with 1% m/v Na₂HPO₄. These different salt concentration solutions were then used to make up the internal and external PVA solutions.

- 90 -
For all formulations, microparticles were prepared as outlined in Chapter 2, Section 2.2.

4.3.3. Microparticle Characterisation

For details of characterizing the microparticles with regards to size, zeta potential and loading efficiency, please refer to Chapter 2, Sections 2.3.1, 2.3.3 and 2.4 accordingly.

4.4. Results

4.4.1. Point of DNA degradation during microparticle preparation

Studies were conducted to observe which events contributed to the degradation of the DNA. Was it due to shear stress alone? Or was it through some other factors? Figures 4.3 and 4.4 demonstrated that DNA was not affected visibly using agarose gel electrophoresis when it was homogenized in the presence of PVA or DCM. All DNA was in the same conformation as untreated (control) DNA, and in these cases, the presence of buffer did not confer any added protection.

![Figure 4.3. Agarose gel image of plasmid DNA homogenized at 24,000rpm for 2 minutes in buffered or non-buffered solution and with or without PVA.](image)

![Figure 4.4. Agarose gel image of plasmid DNA homogenized at 24,000rpm for 2 minutes in presence of dichloromethane (DCM). In buffered or non-buffered solution and with and without PVA.](image)
When the plasmid DNA was homogenized as normal, in the presence of polymer, it was seen that the DNA was ‘degraded’ to its open-circular and linear conformation after the primary emulsification step when no buffer was added (Figure 4.5). This process was irreversible, and the appearance of streaking underneath the bands of DNA was indicative of further degradation to the DNA. All of this was prevented when the microparticles were prepared in the presence of either 0.1M NaHCO₃ or 1% m/v Na₂HPO₄ (Figure 4.5). The DNA maintained the supercoiled conformation and there was no streaking underneath the bands. So, it seemed that the presence of polymer expedited the degradation of plasmid DNA during homogenization, and that the presence of buffers prevented this.

![Figure 4.5. Agarose gel electrophoresis pictures of the effect of different stages of preparation of microparticles on the stability of plasmid DNA.](image)

When making the microparticles, a sample of the formulation was taken after each stage of the process (as indicated in the legend) and extracted out and run on an agarose gel. The DNA for each formulation is therefore has the same original DNA, and the figure depicts at which stage of the process the DNA is degraded.

### 4.4.2. Addition of 0.1M NaHCO₃ or 1% m/v Na₂HPO₄ to the aqueous phases

As demonstrated above, the addition of buffers in the aqueous phases aided in preserving the supercoiled conformation of the plasmid DNA during the various stages of the microparticle preparation. All the formulations had similar yields and zeta potentials, but the encapsulation efficiencies were notably different.
between microparticles made without buffer and those made with buffer (Table 4.3). Although the buffered particles showed a similar degree of encapsulation efficiency, the diameters of Na₂HPO₄ particles were slightly smaller and with a narrower distribution compared to NaHCO₃ microparticles (Table 4.4).

**Table 4.3. Particle yield, encapsulation efficiency and zeta potential of particles made with and without buffer.** For the particle yield, this is the mean ± s.d. of three batches of particles made. For the encapsulation efficiency, 2 samples from each batch made were analysed and therefore is the mean ± s.d. of 6 readings in total. For the zeta potential, each batch of particles made was measured three times, therefore is the mean ± s.d. of 9 readings.

<table>
<thead>
<tr>
<th>Formulation*</th>
<th>% Particle Yield</th>
<th>% Encapsulation Efficiency</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76.01 ± 1.17</td>
<td>n/a</td>
<td>-23.11 ± 2.48</td>
</tr>
<tr>
<td>2</td>
<td>81.09 ± 0.87</td>
<td>32.77 ± 12.96</td>
<td>-22.90 ± 2.47</td>
</tr>
<tr>
<td>3</td>
<td>87.86 ± 3.55</td>
<td>n/a</td>
<td>-23.51 ± 2.31</td>
</tr>
<tr>
<td>4</td>
<td>85.48 ± 0.99</td>
<td>40.72 ± 4.32</td>
<td>-24.58 ± 1.58</td>
</tr>
<tr>
<td>5</td>
<td>79.79 ± 2.62</td>
<td>n/a</td>
<td>-24.52 ± 2.35</td>
</tr>
<tr>
<td>6</td>
<td>86.48 ± 2.34</td>
<td>45.65 ± 5.27</td>
<td>-23.70 ± 2.16</td>
</tr>
</tbody>
</table>

*For details please refer to Table 4.1

**Table 4.4. Size distributions of particles made with and without buffer.** Three batches of particles were made and each batch was measured 3 times. Mean ± s.d. of 9 readings.

<table>
<thead>
<tr>
<th>Formulation*</th>
<th>MVD µm</th>
<th>D[v10] µm</th>
<th>D[v50] µm</th>
<th>D[v90] µm</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.86 ± 0.15</td>
<td>0.73 ± 0.04</td>
<td>1.50 ± 0.14</td>
<td>3.45 ± 0.34</td>
<td>1.81 ± 0.19</td>
</tr>
<tr>
<td>2</td>
<td>2.23 ± 0.14</td>
<td>0.79 ± 0.04</td>
<td>1.80 ± 0.16</td>
<td>4.29 ± 0.20</td>
<td>1.96 ± 0.10</td>
</tr>
<tr>
<td>3</td>
<td>4.56 ± 0.29</td>
<td>1.78 ± 0.08</td>
<td>4.21 ± 0.34</td>
<td>8.01 ± 0.48</td>
<td>1.48 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>4.45 ± 0.12</td>
<td>1.78 ± 0.16</td>
<td>4.14 ± 0.13</td>
<td>7.68 ± 0.40</td>
<td>1.42 ± 0.12</td>
</tr>
<tr>
<td>5</td>
<td>2.88 ± 0.02</td>
<td>1.32 ± 0.10</td>
<td>2.66 ± 0.07</td>
<td>4.77 ± 0.10</td>
<td>1.30 ± 0.11</td>
</tr>
<tr>
<td>6</td>
<td>2.96 ± 0.21</td>
<td>1.31 ± 0.22</td>
<td>2.69 ± 0.28</td>
<td>4.99 ± 0.14</td>
<td>1.41 ± 0.18</td>
</tr>
</tbody>
</table>

*For details please refer to Table 4.1

A more detailed analysis of the conformations of the plasmid DNA from the different formulations demonstrated that the DNA extracted out from microparticles made with 0.1M NaHCO₃ had a slightly higher amount of supercoiled DNA (66.14 ± 3.37 %) compared to that of 1% Na₂HPO₄ (61.06 ± 3.83 %) (Figure 4.6).
The morphologies of the microparticles made also demonstrated marked differences between those made with buffer and those without buffer (Figure 4.7). Addition of buffer into the aqueous phases led to microparticles having a very porous structure, which influenced the diameters of the particles. A further study was carried out where the emulsions were taken out at different stages of the manufacturing process to observe when the porosity occurred. Morphologies of all particles were formed after the secondary emulsion and during the evaporation process (Figure 4.8). It was hypothesised that the salt from the buffer particles would dissolve out during the washing process by the way of concentration gradient and leave the porous structures behind, but it has been demonstrated here that this is not the case, as the morphologies are formed before the washing step.
4.4.3. Effect of different pH on plasmid DNA and particle parameters

To try and elucidate the effect of pH on the parameters of the microparticles, a range of phosphate buffered solutions were made at 70 mM concentrations and at pH ranging from 5-12. The zeta potential of these particles was not of interest, therefore it was not measured. There was a varied effect on the yield of the particles, but no clear correlation between pH and yield was apparent (Figure 4.9). This was not observed for the encapsulation efficiency and degree of supercoiled content of the plasmid DNA (Figure 4.9), where there was a correlation between the pH and amount of DNA entrapped and supercoiled content. Figure 4.9 demonstrates clearly the relationship between pH, entrapped DNA and supercoiled content; for the pH ranges 7-12, there is a linear decrease.

Figure 4.8. SEM images of the morphology of microparticles at different stages of w₁/o/w₂ double emulsion solvent evaporation process.
in the encapsulation efficiency. But, for the supercoiled content, there is a linear increase between the pH ranges of 5-11. What is clearly deduced is that it is at pH 9 where the best compromise is achieved for encapsulation efficiency and degree of supercoiled content; this also happened to be the pH of the buffer solution 1% m/v Na₂HPO₄.

<table>
<thead>
<tr>
<th>pH</th>
<th>% Particle Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>50.66 ± 0.24</td>
</tr>
<tr>
<td>7</td>
<td>38.25 ± 5.69</td>
</tr>
<tr>
<td>9</td>
<td>44.36 ± 0.88</td>
</tr>
<tr>
<td>11</td>
<td>66.41 ± 0.79</td>
</tr>
<tr>
<td>12</td>
<td>70.17 ± 0.79</td>
</tr>
</tbody>
</table>

Figure 4.9. Graphical representation of % encapsulation efficiency (♦) and % supercoiled content (■) vs pH. For the encapsulation efficiency, 2 samples from each batch made was analysed and therefore is the mean ± s.d. of 4 readings in total. Supercoiled content of control DNA was 78.56 ± 0.08. For the particle yield (inset), this is the mean ± s.d. of two batches of particles made.

There was also a similar trend observed for the diameters of the microparticles, an increase in the pH led to an increase in the diameters (Table 4.5). A plot of the D[v,50] versus pH also seemed to indicate that pH 9 was the limit to achieving a diameter of approximately 2 µm before there was a sharp increase (Figure 4.10). The diameters of the microparticles were confirmed by SEM images, which also showed that pH also affected the degree of porosity (Figure 4.11). At pH 5, the particles were smooth and spherical, for pH's 7 and 9 there was a degree of porosity but also intense aggregation of the microparticles, which were decreased at pHs 11 and 12.
Table 4.5. Size distributions of particles made with different pH buffers. Two batches of particles were made and sizes are mean ± s.d of 3 readings from each batch.

<table>
<thead>
<tr>
<th>pH</th>
<th>MVD (μm)</th>
<th>D[v,0.1] (μm)</th>
<th>D[v,0.5] (μm)</th>
<th>D[v,0.9] (μm)</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.06 ± 0.29</td>
<td>0.70 ± 0.03</td>
<td>1.38 ± 0.13</td>
<td>3.89 ± 0.22</td>
<td>2.32 ± 0.08</td>
</tr>
<tr>
<td>7</td>
<td>2.68 ± 0.15</td>
<td>0.94 ± 0.02</td>
<td>2.10 ± 0.04</td>
<td>5.31 ± 0.59</td>
<td>2.08 ± 0.25</td>
</tr>
<tr>
<td>9</td>
<td>2.48 ± 0.19</td>
<td>0.89 ± 0.11</td>
<td>1.96 ± 0.17</td>
<td>4.65 ± 0.15</td>
<td>1.93 ± 0.15</td>
</tr>
<tr>
<td>11</td>
<td>3.58 ± 0.12</td>
<td>1.59 ± 0.21</td>
<td>3.21 ± 0.16</td>
<td>6.05 ± 0.01</td>
<td>1.39 ± 0.14</td>
</tr>
<tr>
<td>12</td>
<td>5.18 ± 0.09</td>
<td>2.57 ± 0.09</td>
<td>4.78 ± 0.09</td>
<td>8.34 ± 0.07</td>
<td>1.21 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 4.10. Graphical representation of the D[v,50] vs pH. Mean ± s.d of two batches of particles, each measured 3 times. Therefore, total of 6 readings.

Figure 4.11. SEM pictures of microparticles made with different pH. (a) pH 5; (b) pH 7; (c) pH 9; (d) pH 11 and (e) pH 12.
4.4.4. Effect of different salt concentration on plasmid DNA and particle parameters

The effect of different salt concentrations on the microparticle characteristics were less dramatic than that of pH, although this may be because the range of values tested was fewer. It is to be noted that 5% m/v Na$_2$HPO$_4$ was tested as well, but the PVA did not dissolve in this solution, therefore no microparticles were made. The microparticles made with 3% and 0.02% Na$_2$HPO$_4$ demonstrated very different characteristics compared to 1% Na$_2$HPO$_4$. The yields and encapsulation efficiencies were much lower (Table 4.6), and the diameters were also larger (Table 4.7), although the supercoiled content and the D$_{[v,50]}$ for microparticles made with 1% and 3% Na$_2$HPO$_4$ were similar. It was more difficult to decipher any correlations here, but again, it did seem that 1% Na$_2$HPO$_4$ was the optimum concentration of salt to use in the aqueous phases. This is also probably related to the pH of the solutions, the pH of 3% and 0.02% Na$_2$HPO$_4$ were measured to be 8.98 and 8.82 respectively; whereas for 1% Na$_2$HPO$_4$ it was 9.28. It is unclear why there was a difference between 3% and 1%, but it may explain the results obtained when taking into account the relationships observed in the previous section.

Table 4.6. A summary of results of particles made with different buffer concentrations. For the particle yield, this is the mean ± s.d. of two batches of particles made. For the encapsulation efficiency, 2 samples from each batch made was analysed and therefore is the mean ± s.d. of 4 readings in total. Supercoiled content of control DNA = 77.84 ± 0.11.

<table>
<thead>
<tr>
<th>Na$_2$HPO$_4$ %m/v</th>
<th>% Particle Yield</th>
<th>Encapsulation Efficiency</th>
<th>Supercoiled Content %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3%</td>
<td>48.20 ± 1.37</td>
<td>20.40 ± 1.29</td>
<td>54.79 ± 4.49</td>
</tr>
<tr>
<td>1%</td>
<td>76.78 ± 5.03</td>
<td>62.51 ± 2.15</td>
<td>51.62 ± 0.37</td>
</tr>
<tr>
<td>0.02%</td>
<td>51.85 ± 3.12</td>
<td>34.57 ± 4.09</td>
<td>26.85 ± 0.51</td>
</tr>
</tbody>
</table>

Table 4.7. Size distributions of particles made with different buffer concentrations. Two batches of particles were made and sizes are mean ± s.d of 3 readings from each batch.

<table>
<thead>
<tr>
<th>Na$_2$HPO$_4$ % m/v</th>
<th>MVD (µm)</th>
<th>D$_{[v,0.1]}$ (µm)</th>
<th>D$_{[v,0.5]}$ (µm)</th>
<th>D$_{[v,0.9]}$ (µm)</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>3%</td>
<td>3.65 ± 0.75</td>
<td>0.97 ± 0.05</td>
<td>2.47 ± 0.02</td>
<td>7.62 ± 2.24</td>
<td>2.70 ± 0.95</td>
</tr>
<tr>
<td>1%</td>
<td>2.67 ± 0.15</td>
<td>1.10 ± 0.02</td>
<td>2.41 ± 0.11</td>
<td>4.60 ± 0.40</td>
<td>1.45 ± 0.11</td>
</tr>
<tr>
<td>0.02%</td>
<td>4.05 ± 0.91</td>
<td>0.72 ± 0.02</td>
<td>1.74 ± 0.15</td>
<td>9.16 ± 3.14</td>
<td>4.73 ± 1.39</td>
</tr>
</tbody>
</table>
The SEM images also demonstrated that 1% \(\text{Na}_2\text{HPO}_4\) microparticles had less aggregation and that the large range of diameters (\(D[v,10]\) and \(D[v,90]\)) observed for 3% and 0.02% \(\text{Na}_2\text{HPO}_4\) is probably due to aggregation of the microparticles (Figure 4.12).

![SEM images of microparticles](image)

**Figure 4.12.** SEM images of microparticles made with different concentrations of \(\text{Na}_2\text{HPO}_4\) in the aqueous phases. (a) 3% m/v; (b) 1% m/v; (c) 0.02% m/v.

### 4.5. Discussion

For the studies that involved the effect of homogenization on the plasmid DNA conformation, it was shown that plasmid DNA was not affected by shear stress or presence of organic solvent. It was not until the addition of polymer that there was a change, and this is in agreement with previous studies (Walter et al., 1999). Although, in this case, homogenization did not degrade DNA that was in its original solution (i.e. without \(\text{NaHCO}_3\) or \(\text{Na}_2\text{HPO}_4\)), which is in contrast to Walter et al.'s findings. This may be due to Walter et al., using sonication, which has a higher energy output than homogenization. Degradation of the DNA by sonication was prevented in the presence of \(\text{NaHCO}_3\), but it did not prevent degradation during spray-drying (Walter et al., 1999). In a later study, the authors are unclear whether degradation was caused directly by the spray drying process, or whether the DNA degraded during sonication in the presence of polymer (Jilek et al., 2004).

A similar study was carried out with 0.9% \(\text{NaCl}\); 0.1M \(\text{NaHCO}_3\) (pH 8.0) and PBS (pH 8.0) as buffers to protect DNA during primary emulsification (Li et al., 2003). It also confirmed the protective effect of salt on the conformation of the plasmid DNA, with 0.1M \(\text{NaHCO}_3\), having the greatest protective effect. The
presence of polymer during primary emulsification also led to more 'degradation' of the plasmid DNA (Li et al., 2003).

It is uncertain why the addition of polymer into the emulsification step could cause the DNA to change conformation, but it could be through a number of ways. An increase in the viscosity of the solution could increase the shear stress on the DNA and therefore cause strand breakage. Or it could be due to the complex interactions that are occurring between the organic and aqueous phases, and entanglement effects between the polymer, PVA and DNA chains. What was conclusive, however, was the protective effect of buffers on the conformation of the DNA.

There are many other factors involved that affect DNA condensation, and it is beyond the scope of this section to discuss each of them in detail. It is still a highly active research area, but factors involved include, electrostatic forces, hydrodynamic interactions, base composition and length of the DNA and the ionic strength of the solution in which the DNA is present (Schlick et al., 1994; Bloomfield, 1996; Lyubchenko and Shlyakhtenko, 1997).

In general, results have shown that the structure of supercoiled DNA changed easily depending on the ionic strength of the solution it was in (Lyubchenko and Shlyakhtenko, 1997). Computational models on the effect of different NaCl concentrations demonstrated that Na+ ions shield the phosphates on the DNA backbone, which can lead to coils moving into closer proximity and therefore, to a reduction in diameter. If there are not enough ions present, then the coils are more open in structure (Schlick et al., 1994). It is highly likely that is how NaHCO₃ and Na₂HPO₄ buffers are exerting their protective effect - by reducing the diameter of the plasmid DNA, it is more able to avoid being damaged during homogenization, and this is the explanation given by other authors (Walter et al., 1999; Li et al., 2003).

In this study, the addition of buffers did improve encapsulation efficiency and conformation of plasmid DNA and is in agreement with previous studies (Walter et al., 1999; Li et al., 2003), but it did seem that Na₂HPO₄ offered more
advantageous characteristics with a narrower diameter distribution and higher encapsulation efficiency.

Not many researchers have used salt in the $w_1$ and $w_2$ phases, which makes it more difficult to interpret and compare results. In previous studies that have used 0.1M $\text{NaHCO}_3$ (Walter et al., 2001), the morphology of the particles shown in SEM images were not porous, although this is probably due to the different methodology used to make the particles (a single w/o emulsion, which was then spray-dried); and the different polymers used (a range of PLGA Resomer from Boehringer, Ingelheim, Germany). In another study where an o/w emulsion was used to entrap quinidine sulphate, the addition of various inorganic salts (NaCl, KSCN, $\text{Na}_2\text{SO}_4$, NaBr, NaSCN and NaClO$_4$) lead to different effects on the loading efficiency, with some salts increasing loading efficiency, whereas others reduced it. This was explained by the salts acting in two ways, either by depressing the drug aqueous solubility or by increasing the organic solvent solubility in the aqueous phase (Al-Maaieh and Flanagan, 2001). The salts used for this present study may also act in a similar way and could also explain the morphologies obtained – if the presence of salt increased the solubility of DCM, then it might mean that it would evaporate off faster, thus leading to the pores being left behind.

The porous nature of the particles made with buffer could mean a decrease in density (Yang et al., 2000), which could be advantageous in terms of delivery to the lung. It has been shown that a decrease in density of the particle can lead to a greater \textit{in vivo} deposition of the powder in the lung (Bosquillon et al., 2001).

The results obtained here for pH are in agreement with other studies; a range of 6-9 demonstrated an increase in plasmid DNA stability with pH 9 offering the best protection (Evans et al., 2000). The buffer in which the plasmid DNA is stored in might also have an added protective effect, with the presence of EDTA as a free-radical scavenger/chelator helping to reduce damage to the DNA through oxidation (Evans et al., 2000).
Entrapment of somatostatin acetate into PLA microspheres was investigated, when the pH was changed from 2.2 – 5.0 (using citric acid/HCl buffer system) and effect of addition of salts into the internal and/or external phase (NaCl and CaCl₂) on entrapment, morphologies and release rate. Microparticles that had buffer in both phases had a porous structure – salt in internal phase only had a more porous structure; salt in the external phase had a denser structure (Herrmann and Bodmeier, 1995). They also attributed the differences in morphology and encapsulation efficiencies on the changes in the solvent-water exchange. Although they explained the morphologies obtained when adding salt either to the internal or external phases (addition in the internal phase, promoted influx of water therefore more porous; addition into the external phase, led to more dense and homogeneous structure), they did not explain how the morphologies formed when salt was added to both phases.

A very important outcome was noticed with these results. There was a vast difference in the encapsulation efficiency obtained in these studies, compared to those made in the DofE. The DofE microparticles had encapsulation efficiencies of approximately 69% and a MVD of approximately 4 μm; but for these microparticles, the same formulation gave encapsulation efficiencies of 33% and MVD of 2 μm. The only item that was altered between the DofE and these microparticles was the type of DNA used. For the DofE, pCVM-luciferase was used (~5130 bp); for these particles, gWiz-luciferase was used (6732 bp). It may be that the larger plasmid was less easy to entrap. Also, impurities in the buffers may have also contributed to the difference in results – Evans et al compared different batches of PBS from different companies and noticed a wide range in stability of the plasmid DNA (Evans et al., 2000).

There seems to be a balance between the amount of “salt” in the particles and the amount of DNA loaded into the particles. Also, a relationship between the amounts of supercoiled DNA. This could be pH-related more than due to the salt content, although it has been mentioned that the higher the salt concentration, the more compacted the DNA structure.
4.6. Conclusions and Future Work

This study highlighted the lack of correlation between the results obtained from the DofE experiment, and the experiments performed here. There was a vast difference in the encapsulation efficiency and diameters of the microparticles produced, and demonstrated that there were still difficulties in reproducibility. What was clear however, were the protective effects of adding salt to the aqueous phases of the formulations. Addition of 1% m/v Na₂HPO₄ conferred more desirable characteristics in terms of amount of DNA entrapped and diameters of the microparticles; the additional porosity of the microparticles may aid in the delivery of these microparticles into the lung owing to their purported lower density. This is the first time that the buffer Na₂HPO₄ has been used in this way to entrap DNA into PLGA microparticles.

Detailed studies also demonstrated that DNA was damaged in the presence of polymer and in the primary emulsion step, indicating that the viscosity of the solutions were very important. Also the balance of salt and pH were also critical, with a clear demarcation between what was considered advantageous and what was not.

The following chapters outline the experiments conducted to assess the performance of the microparticles with regards to stability (e.g. is the polymer Tg still the same?) and also assessing the residual levels of water, solvent (DCM) and PVA levels. The viability of the plasmid DNA entrapped in the microparticles, as well as their efficiency for delivering plasmid DNA were studied in cell-culture models. Their potential to be used in a dry powder inhaler was also assessed.
Chapter 5

Analytical Experiments on Optimized Formulations
5.1. Introduction

As well as efficacy in a biological sense, the microparticle formulations must also demonstrate stability in terms of shelf-life and acceptable levels of residual solvent and/or other potential toxic materials. This section is dedicated to this aspect. There are many analytical techniques that can be used to characterise the polymeric formulations. The results of these tests can be used to give an indication of the formulations will behave and their stability under a range of conditions during in vivo use.

The parameters that were considered to be most important were:

1) Levels of residual water – to ascertain that the formulations were ‘dry’ after freeze-drying. It is believed that a low water content will be important for increased product stability.

2) Stability of the polymer – the polymer is an integral part of the formulation and it is therefore important that the polymer is stable under certain conditions. Parameters such as the glass transition temperature (Tg) are a good indication of the stability of the polymer matrix, and this is likely to have a significant effect of the stability of the formulation and its in vivo bioavailability.

3) Surface area of the microparticles – to understand the potential effect on release profiles and also how the particles might behave in a dry powder inhaler.

4) Residual DCM – to ensure that residual levels of the solvent were below those recommended in the regulatory guidelines.

5) Residual PVA – to ensure that residual levels of the stabilizer were below those recommended in the regulatory guidelines.

6) Release study – to ensure that the DNA was released at appropriate rates.

A detailed introduction to the theory and instrumentation of each analytical method would be beyond the scope of this thesis, but a brief outline of how they work will be given.

5.1.1. Thermogravimetric Analysis (TGA)

TGA belongs to a family of techniques termed ‘thermal analysis’. These involve measurement of a material’s specific physicochemical property under specific temperature-controlled conditions (Cheng et al., 2000). TGA is a measurement of weight changes, which normally is seen as a mass loss of a volatile component (Haines, 2002). Samples are weighed into an inert crucible and put onto a very sensitive
microbalance, which is then placed in a furnace. Most modern systems are attached to a computer system that control the temperature program, records the weight profile and process the results (Haines, 2002).

TGA measures mass loss (or gain) from a sample at a range of temperatures. The temperature of a thermal event is important as if a mass loss occurred at around 100°C, then it might be assumed that it is due to a release of water. The information from TGA is often easier to interpret if the sample has been well characterised by other analytical techniques, i.e. there is a knowledge of solvate and polymorph configuration. If not, then the evolved gas can be fed into a specific detector such as a mass spectrometer (MS) (Willard et al., 1988).

TGA is most useful when it complements differential thermal analysis studies (e.g. DSC), as not all energy-change processes are accompanied by changes in weight. This difference in the two techniques enables a clear distinction to be made between physical and chemical changes when the samples are subjected to both DSC and TG tests (Willard et al., 1988).

5.1.2. Differential Scanning Calorimetry (DSC)

Out of all the thermal analysis techniques, DSC is the most widely used, especially for the reporting of transitional behaviours such as melting point, crystallization temperature and glass transition temperature of polymers (Cheng et al., 2000; Haines, 2002).

A differential scanning calorimeter consists of two sensitive thermocouples, each positioned in a highly controlled thermal environment (furnace). The sample is placed in a small pan (or cup and lid arrangement) and placed in the first thermocouple. The second thermocouple is used as a reference, and normally has an empty pan. There are several methods of monitoring the thermal properties of the sample, but typically the electrical current is measured to each furnace when the sample and reference pans are taken through a certain thermal programme. A differential amplifier determines the relative current for the two pans, and when a thermal transition occurs in the sample, there is a differential current corresponding to whether an exothermic or endothermic reaction has occurred in the sample pan (Willard et al., 1988; Haines, 2002). The
changes in the signal obtained by the instrument (heat flow) are plotted against time or temperature (Haines, 2002).

Peaks that are obtained from a DSC curve can be due to endothermic or exothermic reactions occurring in the sample as the temperature changes. The shape of the peak can indicate reactions such as glass transitions; re-arrangements of the material’s structure; melting points and dehydration (Willard et al., 1988). Endothermic peaks generally represent physical changes, whereas exothermic peaks indicate chemical reactions (Willard et al., 1988). Determining the significance of DSC curves is not always a straightforward task. A reference library of curves of specific interest to a particular laboratory is vital (Willard et al., 1988).

5.1.3. Surface Area Analysis via Gas Adsorption

Although electron microscopy can give information about the external properties of powders, and in particular the shape and size of the particles, Gas adsorption is used to analyse the surface area properties and the internal pore structure of the powders (Lowell and Shields, 1984).

Dry samples are cooled down to a temperature at which inert gases, e.g. nitrogen, will physically adsorb onto the surface of the sample. An adsorption isotherm is obtained and the surface area is calculated by using the BET (Brunauer, Emmett and Teller) equation. A typical instrument for performing this test is manufactured by Beckman Coulter (Beckman Coulter).

5.1.4. Nuclear Magnetic Resonance (NMR)

Traditionally, NMR is used to identify atomic configurations in molecules, based on the characteristic absorption spectra of certain spinning nuclei under a strong magnetic field (Willard et al., 1988). The electron shift of certain atoms, which have a magnetic nucleus, such as protons (\(^1\)H), carbon-13 (\(^{13}\)C) or phosphorous (\(^{31}\)P), have a characteristic resonance and relaxation frequency, which can be used to determine the electronic equivalent of the atom. NMR can also be used for quantitative analysis as the area under the absorption band is proportional to the number of nuclei responsible for
the absorption (Willard et al., 1988). If a known amount of a reference compound (std) is included in the sample, the amount of the unknown present can be calculated by:

\[ W_{\text{unk}} = W_{\text{std}} \times \frac{N_{\text{std}} \times M_{\text{unk}} \times A_{\text{unk}}}{N_{\text{unk}} \times M_{\text{std}} \times A_{\text{std}}} \]

where \( N \) is the number of protons in the groups giving rise to the absorption peaks, \( A \) is the area under the curve and \( M \) are the molecular weights of the compounds (Willard et al., 1988).

5.1.5. Iodine reaction with PVA

PVA forms a blue complex with triiodide in the presence of boric acid, and this can be quantified using a spectrophotometer (Joshi et al., 1979). It is not entirely clear how the complex is formed, but it is suggested that the iodine molecules are adsorbed end to end at the microcrystalline sites of the PVA polymer in a highly specific manner (Rosenblatt, 1991). Boric acid is used to accelerate the complexation rate and increase sensitivity, i.e. less iodine solution is needed to form a blue colour, and it also further stabilizes the PVA/iodine complex (Rosenblatt, 1991). The high sensitivity of iodine for PVA and the stability of the complex formed make it an ideal method for quantifying residual PVA in microparticles.

5.1.6. Release Study

Release studies are commonly performed to predict or confirm the results obtained in cell culture experiments and in vivo experiments. It is usually carried out by suspending a known mass of microparticles in a physiological buffer (usually PBS) and incubating at 37°C under agitation. The supernatant is then taken out and analysed at specific time points to obtain a release profile of the DNA (Walter et al., 1999; Wang et al., 1999; Tinsley-Bown et al., 2000; Diez and Tros de Ilarduya, 2006).

5.2. Aims

To investigate the following parameters of the microparticulate formulations made in Chapter 4: 1) residual water; 2) \( \text{Tg} \); 3) residual DCM; 4) surface area; 5) residual PVA and 6) release profile.
5.3. Materials and Methods

Sodium formate (formic acid, sodium salt) and sodium azide were purchased from Sigma (Poole, UK). Acetic acid-d$_4$ (Cambridge Isotopes Lab Inc) and 5 mm NMR tubes (Wilmad-Labglass) were purchased from Goss Scientific (Essex, UK). Alum pans and alumina ceramic crucibles were purchased from PerkinElmer (UK).

All analytical experiments were performed using the microparticle formulations made in Chapter 4.

5.3.1. Thermogravimetric Analysis (TGA)

Determination of water content is normally carried out using Karl Fisher titration experiments by dissolving the sample in a suitable organic solution and titrating the sample with a titrant that is specific to water. The standard Karl Fisher approach is not suitable for PEG A samples, because the PLGA is not soluble in the normal solvents, and therefore water content would not be representative of the whole sample. For this reason TGA was used, as this will see the total water content of the sample.

TGA measurements were made using a Pyris 6 TGA (PerkinElmer Instruments, UK) and analysed using Pyris Software version 7.0.0.0110 (PerkinElmer LAS Ltd, UK). Empty alumina ceramic crucibles were tared before samples were weighed (1-5 mg) into them. Samples were heated from 30°C - 250°C at a rate of 10°C/min in an atmosphere of nitrogen gas, which was set at a flow rate of 20 ml/min. The machine was calibrated on a regular basis to ensure that the microbalance, heating rate and furnace were all within the ranges stated by the manufacturer. Three samples from a batch of microparticles were analysed and the mass loss was measured from the start at which the baseline deviated until it reached a constant mass - typically between the temperatures 40 -150°C.

5.3.2. Differential Scanning Calorimetry (DSC)

DSC is normally performed at a slow heating rate between 5-15°C/min. As there were a large number of samples to be analysed and the small masses involved, a fast scan rate was used, i.e. 200°C/min. This experimental configuration also resulted in an increased sensitivity of detection for the transitional states of the polymer (Mathot et al., 2003;
Saunders et al., 2004). As PLGA is an amorphous material, the glass transition (Tg) temperature was recorded. And although traditionally the Tg is recorded in the cooling cycle of the scan, it was not deemed appropriate in this case, due to the multiple components that are present in the formulations (Craig et al., 1999).

DSC measurements were made using a Pyris I DSC (PerkinElmer Instruments, UK) and analysed using Pyris v.3.80 (PerkinElmer LAS Ltd, UK). Samples were weighed (1-5 mg) accurately into alum pans and were hermetically sealed before being measured at a scan rate of 200°C/min, starting from -20°C up to 150°C. The reference was a sealed empty alum pan. All scans were carried out in an atmosphere of nitrogen gas, which was set at a flow rate of 10 ml/min. The machine was calibrated prior to experimental runs with Indium (Tg of 156.5°C, scanned at 200°C/min, starting at 20°C up to 240°C). Three samples from a batch of microparticles were analysed. The temperature of the Tg was recorded at the onset of the peak.

5.3.3. Nuclear Magnetic Resonance (NMR) Analysis of DCM

The method used to analyse residual levels of DCM was adapted from a standard method used at the London School of Pharmacy.

30 mg of microparticles and 20 mg of sodium formate were accurately weighed out into a 1.5 ml centrifuge tube. 1 ml of acetic acid-d₄ was added and sonicated until the samples were completely dissolved. For the microparticles, complete dissolution was not possible, so they were sonicated for at least 30 min before being centrifuged at 14,000 rpm for 1 min to pellet the undissolved material. 700 µl of the solution was then transferred into a 5 mm NMR tube. For the standards, sodium formate alone was dissolved in the d₄-acetic acid and known volumes of DCM were added before being transferred to an NMR tube.

The samples and standards were measured using a Bruker Advance 500 MHz NMR spectrometer (Bruker AXS Ltd, Coventry, UK) equipped with broadband and triple resonance (1H, 13C, 15N) inverse probes. The temperature was set to 300K and records of high field proton NMR (>400MHz) spectra were obtained using a 45 degree pulse and a 60 second pulse repetition time. The spectra were analysed using SpinWorks v2.5.3 (Kirk Marat, University of Manitoba, Canada).
The spectrum was referenced by setting the chemical shift of the methyl signals due to residual acetic acid to 2.03 ppm. The absorption values for sodium formate are 8.2 ppm and for DCM 5.4 ppm. Accurate integrals for DCM were obtained for the standards, and the concentrations of the samples were calculated from the standard curve obtained. All experiments were performed in duplicate.

5.3.4. Surface Area Analysis via Gas Adsorption

Surface area was measured by the Brunauer, Emmet, Teller (BET) technique using a Beckman Coulter SA3100 Surface Area Analyzer (Beckman Coulter Ltd, High Wycombe). The empty sample holder, stopper and filling rod were first weighed. Then the samples (between 0.5 mg and 1.0 mg) were added carefully into the holder and the whole assembly was weighed again. The samples were outgassed under helium at 30°C for 300 min (to remove any adsorbed gases from the surface of the particles) and then re-weighed. This was to ascertain an accurate mass for the microparticles being analysed.

After the samples were outgassed, they were immersed in liquid nitrogen to maintain a low temperature, which allows the gas to form a monolayer on the surface of the microparticles. The gas used for adsorption was nitrogen.

The surface area was calculated using the SA-VIEW™ Software (Beckman Coulter Ltd, High Wycombe). The same sample was measured.

5.3.5. Determination of Residual PVA

Determination of PVA content in the microparticles is modified from the method by Takeuchi (Takeuchi et al., 1998).

5 mg of microparticles was digested with 1 ml 0.5 M NaOH for 15 minutes at 60°C. 0.05 ml of the digested solution was mixed with 3 ml boric acid (4.0% w/v) and 0.6 ml I\(_2\)/KI test solution (0.05M I\(_2\)/0.15M KI) solution. The sample was made up to 10 ml by adding 6.35 ml water. This was mixed thoroughly before being filtered through a
0.45μm membrane. 200μl aliquots were dispensed into a clear 96-well plate and measured spectrophotometrically at a wavelength of 630 nm.

The PVA standard was based on the theory that the maximum PVA present would be 5 mg PVA/ml. This was then serially diluted to create a standard curve. The unknown samples were then calculated as the amount of PVA compared to 100%. PVA standards, which were subjected to the same NaOH digest as the samples.

5.3.6. Release Study

10 mg of particles were accurately weighed out into 1.5 ml centrifuge tubes. 1 ml of PBS containing 0.02% m/v sodium azide was added into each tube. These were placed horizontally into an orbital shaking incubator set at 150 rpm and a temperature of 37°C. At each time point the samples were centrifuged at 14,000 rpm for 2 min. 1 ml of the supernatant was taken out for analysis. Fresh PBS/sodium azide solution was added to the pellet and the pellet was fully re-suspended before placing back into the incubator. Supernatants were stored at -20°C if not analysed straight away. For each formulation, there were 3 samples for each time point. Samples of microparticles without encapsulated DNA were also incubated under the same conditions to serve as a reference value during analysis.

Supernatants were analysed using Picogreen® (Chapter 2, Section 2.4.4) and agarose gel electrophoresis (Chapter 2, Section 2.4.2). At the end of the study, the remaining pellets were subjected to a chloroform/TE buffer extraction process (Chapter 2, Section 2.4.1) to analyse the amount of DNA remaining in the pellets.

5.4. Results and Discussion

5.4.1. Thermogravimetric Analysis (TGA)

Two experiments were carried out with TGA. One was to determine the amount of residual water in formulations after freeze-drying and the second was to see if TGA could detect residual DCM in formulations.

For residual water, formulations made in Chapter 4 were analysed after freeze-drying. For detection of DCM, spiked formulations were used to determine the limit of
detection for TGA. Formulations were made as normal, but were freeze-dried immediately after the secondary emulsion to entrap the DCM into the microparticles. These were then analysed using TGA.

Freeze-drying (or lyophilization) is primarily used by pharmaceutical and bioindustries to enhance chemical stability of formulations, especially for peptides, proteins and DNA (Franks, 1998; Poxon and Hughes, 2000). Although exact levels of residual water vary for each entity, it is suggested that an amount of less than 1% is optimal for stability (Tang and Pikal, 2004).

Table 5.1 summarises the results obtained for microparticles made in Chapter 4. For microparticles that were prepared as normal (i.e. with a DCM evaporation step), it was demonstrated that freeze-drying is very effective at removing water from the formulations, with particles made with buffer having less than 1% m/m of water. This is in contrast to another study, where residual water was $3.50 \pm 0.02 \% \text{ m/m}$ (Passerini and Craig, 2001). The differences could be due to the different programmes used for freeze-drying. Passernini and Craig show that a longer drying time was required to reduce the levels of residual water in the samples.

An unusual result was obtained for particles made without buffer. For these samples there was a slight mass gain rather than the normal mass loss (Table 5.1). Figure 5.1 shows the trace obtained for particles made without buffer compared to one for particles made with 0.1M NaHCO₃. This may be an example of the 'buoyancy effect' sometimes seen with TGA analyses. If the mass of the sample is not enough, the gas surrounding the crucible and sample will decrease in density by a large amount as the temperature rises, and this can be detected by the system as a mass gain (Haines, 2002). A technique to avoid this is to increase the mass of sample to be analysed to cancel out the buoyancy effect. For this study the mass of particles was increased (from 2 mg to 6 mg), but the effect was still obtained, therefore it may be that some other reaction was taking place, which, without further experiments, cannot be fully explained.
Table 5.1. Summary of the mass loss of formulations made in Chapter 4. Mass loss took place between 40°C to 150°C and it is assumed that it is water (and DCM).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass loss (% m/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
</tr>
<tr>
<td>No buffer + DNA</td>
<td>1.291(+)</td>
</tr>
<tr>
<td>0.1M NaHCO3 + DNA</td>
<td>0.710</td>
</tr>
<tr>
<td>1% m/v Na3PO4 + DNA</td>
<td>0.804</td>
</tr>
</tbody>
</table>

Figure 5.1. Examples of TGA curves obtained for microparticles made in Chapter 4. a) particles made without buffer and b) particles made with 0.1M NaHCO3 buffer.

As a comparison, formulations were made where the DCM was not evaporated, to see if TGA could be used as a technique to determine levels of residual DCM. Unfortunately, due to the samples being in the open air before being analysed, it can be seen that on storage the DCM evaporated off at room temperature, such that the mass loss analysed was reduced over time for each sample (Table 5.2).

Table 5.2. Summary of mass loss obtained for formulations made in Chapter 4, but without DCM evaporation prior to freeze-drying.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass loss (% m/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
</tr>
<tr>
<td>No buffer + DNA</td>
<td>10.235</td>
</tr>
<tr>
<td>0.1M NaHCO3 + DNA</td>
<td>4.657</td>
</tr>
<tr>
<td>1% m/v Na3PO4 + DNA</td>
<td>0.679</td>
</tr>
</tbody>
</table>

If this experiment was to be repeated again, it would be important to load each sample into the furnace in a more controlled, randomised procedure to ensure that these effects are minimised. For this experiment, samples were placed on an automatic loader, and as the crucibles were open, DCM could easily have evaporated off (due to its volatile
nature) before the sample was selected to be run. Another alternative would be to use sealed pans and then pierce the lid (which can be performed automatically on some TGA machines) immediately before a scan.

However, accurate analyses of amount of the level of DCM can only be achieved if the TGA machine was attached to a mass spectrometer or FID, so that each component of material lost can be identified and proportions can be attributed accordingly. It can be concluded that TGA is not a suitable method to analyse residual levels of DCM in microparticles.

5.4.2. Differential Scanning Calorimetry (DSC)

DSC measurements were carried out on PLGA and PVA alone, as well as formulations made in Chapter 4 to compare Tgs. Table 5.3 summarises the Tg of the samples measured. All samples gave a distinct Tg peak in the temperature range 45°C to 70°C (Figure 5.2). The formulation that had 1% Na$_2$HPO$_4$ as the buffer (58.79 ± 2.0°C), most closely matched the Tg of the polymer alone (56.15 ± 1.0°C). The repeatability of the test for each sample was good; however, as the analyses were performed on a restricted set of samples, it is difficult to know how much of the variation in Tg is due to the individual sample preparation, and what was the influence of the additives for each microparticulate sample. Residual PVA, DCM or water in the microparticles will tend to reduce the Tg of a sample, and this will also affect the results. In terms of expected stability, the formulation that had no buffer would be expected to have the best stability, with the highest Tg of 66.63 ± 2.2°C, which is an increase of 10°C on the Tg of the polymer alone.

Table 5.3. Tg of samples measured. Samples were scanned at a rate of 200°C/min, starting from -20°C up to 150°C. Three different samples from one batch of microparticles (or material) were measured.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tg (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA 5050DL 4A*</td>
<td>Run 1</td>
</tr>
<tr>
<td></td>
<td>55.750</td>
</tr>
<tr>
<td>PVA 13-23kDa, 87-89% hydrolysed^</td>
<td>46.947</td>
</tr>
<tr>
<td>No buffer + DNA</td>
<td>68.628</td>
</tr>
<tr>
<td>0.1M NaHCO$_3$ + DNA</td>
<td>66.140</td>
</tr>
<tr>
<td>1% m/v Na$_2$PO$_4$ + DNA</td>
<td>57.237</td>
</tr>
</tbody>
</table>

*Tg according to manufacturer was 43-48°C. Program: Heating: 25°C – 100°C at 20°C/min. Cooling to 25°C at 20°C/min, then to -20°C at 10°C/min. Heating to 250°C at 10°C/min (Tg measured). ^Tg according to manufacturer – not specified. But according to DC Chemical Co., Ltd is 58°C.
The Tgs obtained here for the polymer and PVA alone were different to those reported in the literature, but this is probably due to the different scan rates and equipment used.

The temperature of glass transitions is not fixed and is dependent on the rate of heating (Craig et al., 1999; Haines, 2002).

In terms of storage and stability, the aim of the project was to produce a formulation that would not need a cold-chain for distribution. For amorphous materials, such as PLGA (the dominant material in the formulations), recommended temperatures for storage is dependent on a number of factors. Amorphous materials are metastable, and over time, will revert to the more stable crystalline form (Craig et al., 1999). This process can be slowed down if the material is stored below its Tg (to reduce molecular mobility) and in as dry a state as possible, to avoid plasticizing effects of solvents (Craig et al., 1999). From the results obtained in these experiments, it can be surmised that storage at room temperature under vacuum would be sufficient. However, it has been suggested by Hancock et al., that the storage temperature for amorphous materials should be 50°C below the Tg, where the molecular mobility of the material would be negligible over the lifetime of a typical pharmaceutical product (Hancock et al., 1995). For these formulations, that would be a recommended storage temperature of between 9 to 17°C. And so, it is expected that these formulations would not fulfil the aim of avoiding the cold-chain for storage and transportation.
Despite this, it is clear that amorphous materials are more difficult to characterise and quality control than crystalline materials, and the data available concerning the relationship between Tg and pharmaceutical product performance is not clear (Craig et al., 1999). It is therefore difficult to predict whether the formulations made in this study will be suitable as a pharmaceutical product without further studies.

5.4.4. NMR Method for Quantifying Residual Dichloromethane in PLGA Microparticles

The unique chemical and physical properties of DCM have led to its widespread use in industry (ECSA, 1995), and it is often used as the solvent of choice to dissolve PLGA in W_1/o/W_2 double emulsions (Denis-Mize et al., 2000; Stern et al., 2003; De and Robinson, 2004). Although the exact effects of DCM exposure on human health is unclear (Andersen et al., 1987; ECSA, 1995), residual DCM is also important in terms of product stability. For example residual DCM may act as a plasticizer and reduce the polymer Tg (Witschi and Doelker, 1997) or it may cause nano-sized particles to aggregate in an irreversible manner (De and Robinson, 2004).

It was therefore important to ensure that DCM was evaporated off as much as possible during the manufacturing process and that if there was any residual DCM in the microparticles, that it was below the regulatory limits of 500 ppm for the US and European Pharmacopoeia’s and 600 ppm (or permitted daily exposure of 6.0 mg/day) for the International Conference on Harmonization (ICH) (Witschi and Doelker, 1997).

Although sealed cap, head space analysis using gas chromatography-mass spectrometry (GC-MS) is recommended as the method to be used to quantify levels of DCM (Witschi and Doelker, 1997), it has been suggested that this would be difficult for PLGA particles owing to the small sample sizes available, and difficulties in extracting DCM from within the particles (De and Robinson, 2004). NMR has been used previously and therefore, is not a completely unknown methodology (Passerini and Craig, 2001), its suitability has also been demonstrated in the studies here.
5.4.4.1. Limit of detection for DCM levels using NMR

As NMR was a novel method in the department for analysing the levels of DCM in microparticles, a limit of detection for the instrument was first established. Figure 5.3. is an example of a trace where a peak for DCM is clearly visible.

Figure 5.3. An NMR spectrum of sodium formate dissolved in d₄-acetic acid and spiked with DCM at 500 ppm concentration. Sodium formate has a resonance peak at 8.2 ppm and DCM at 5.4 ppm. The peaks of different DCM concentrations were integrated using the software SpinWorks v2.5.3 (Kirk Marat, University of Manitoba, Canada) to produce a standard curve for DCM.

Using known quantities of DCM, it was established that the detection of DCM over a narrow and wide range of concentrations was linear in relationship (Figures 5.4 and 5.5.) and that the limit of detection was approximately 5 ppm (Figure 5.6). Once this was determined, levels of DCM for unknown samples could be calculated with confidence.
Figure 5.4. Standard curve of DCM over a concentration range of 5 ppm – 500 ppm. Bars represent the range from two readings.

Figure 5.5. Standard curve of DCM over a concentration range of 5 ppm – 100 ppm. Bars represent the range from two readings.

Figure 5.6. Standard curve DCM, 1.25 ppm, 2.5 ppm and 5 ppm, showing the loss of linearity. It was concluded that the detection limit of DCM using NMR is about 5 ppm. Bars represent the range from two readings.
5.4.4.2. Levels of Residual DCM in formulations

There were difficulties in having enough sample mass for the NMR analysis, so experiments were carried out once only. As with the TGA experiments, two batches of microparticles were made; one which had undergone an evaporation step, and the second which were freeze-dried soon after the secondary emulsion.

The results demonstrate clearly that if there is not a DCM evaporation step, there are high levels of DCM trapped within the microparticles (Table 5.4). Despite this, the amount is still below the regulatory concentration of 500 ppm. A trend can be seen, with microparticles made with buffer having much higher levels of residual DCM than those made without buffer. This may be due to the higher surface area of these particles, which can mean a higher entrapment of DCM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Residual DCM ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In 30 mg particles</td>
</tr>
<tr>
<td>No buffer</td>
<td>77.5</td>
</tr>
<tr>
<td>0.1M NaHCO3</td>
<td>120.0</td>
</tr>
<tr>
<td>1% m/v Na2HPO4</td>
<td>152.5</td>
</tr>
<tr>
<td>No buffer</td>
<td>0.0</td>
</tr>
<tr>
<td>0.1M NaHCO3 + DCM evap</td>
<td>-2.5</td>
</tr>
<tr>
<td>1% m/v Na2HPO4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

These results highlight the importance of an evaporation step in the manufacturing process. If the particles are left to stir on a magnetic stirrer plate at room temperature for 4 hours, the DCM evaporates off so that levels are below the detection limit for NMR (5 ppm). Although the level of residual DCM for microparticles prepared with phosphate buffer had a measured level of 2.5 ppm, this is close to the limit of detection for the instrument and therefore is considered not to be accurate. If more accurate measurements are required, it would be important to repeat these experiments on more samples.
5.4.5. Determination of the Surface Area of Microparticles using Gas Adsorption

BET analysis of the surface area of the formulations entrapping DNA, given in Table 5.5, showed that microparticles made with buffer had twice the surface area than particles made without buffer. This is in agreement with the SEM pictures, which showed the microparticles prepared with buffer to have a porous nature and hence a larger surface area.

Table 5.5. Surface area analysis of microparticles encapsulating plasmid DNA made in Chapter 4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass (g)*</th>
<th>BET Surface Area m²/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reading 1</td>
<td>Reading 2</td>
</tr>
<tr>
<td>No buffer</td>
<td>0.81844</td>
<td>6.705</td>
</tr>
<tr>
<td>0.1M NaHCO₃</td>
<td>0.72456</td>
<td>12.814</td>
</tr>
<tr>
<td>1% m/v Na₂HPO₄</td>
<td>0.75351</td>
<td>12.976</td>
</tr>
</tbody>
</table>

*Mass of microparticles after outgassing

5.4.6. Analysis of Residual PVA

Polyvinyl alcohols (PVA) are synthetic polymers used in a wide range of industrial, commercial, medical and food applications (DeMerlis and Schoneker, 2003). PVA is a GRAS material (FDA, 2004) and studies have shown that oral toxicity of PVA is very low (with LD₅₀s in the range of 15-20 g/kg); orally administered PVA is very poorly absorbed from the gastrointestinal tract; does not accumulate in the body when administered orally and is not mutagenic or clastogenic (DeMerlis and Schoneker, 2003).

However, because of the effects on the lung are not known and because of potential drawbacks of residual PVA on the particle surface including reduction in cellular uptake (Sahoo et al., 2002); lower gene transfection (Prabha and Labhasetwar, 2004) and changes in hydrophobicity and digestibility of the particles (Lee et al., 1999), it was of importance to ensure that the levels of PVA on the formulations were as low as possible.

For the formulations made in Chapter 4, it was shown that levels of residual PVA
ranged from 4.85 ± 1.12 % m/m for blank particles made without buffer, up to 22.45 ± 1.65 % m/m for blank particles made with 1% m/m Na$_2$HPO$_4$ (Figure 5.7). The general trend was that particles made with buffer had higher levels of residual PVA, and this is probably due to the higher surface areas of these particles (see previous section).

![Figure 5.7. Percentage of residual PVA in different microparticle formulations.](image)

Particles with buffer in their formulation have higher percentage of residual PVA than those without buffer. Mean ± s.d; n = 3.

Additional experiments demonstrated that most of the PVA is removed by the second wash, after which, the PVA remained the same regardless of further washings (Figure 5.8). This is in agreement with other studies that have shown that PVA binds to the surface of particles in an irreversible manner (Boury et al., 1995; Lee et al., 1999; Sahoo et al., 2002). It is proposed that the hydrophobic vinyl acetate part of partially hydrolysed PVA serves as anchor polymer at the oil interface, and once it is entangled with the polymer, it is much more difficult to remove from the surface (Boury et al., 1995; Lee et al., 1999).
Figure 5.8. Percentage of residual PVA in different microparticle formulations after a number of washes. Most of the PVA is removed after the second wash, with minimal being removed in subsequent washes. Mean ± s.d; n = 3.

A further study was carried out to observe if the use of a higher molecular weight PVA would make a difference on the levels of residual PVA. In the present study, PVA of an average molecular weight of 13-23 kDa (87-89 % hydrolysed) was used. When PVA of 30-70 kDa (87-90 % hydrolysed) was used, the levels of residual PVA were very similar (results not shown). Indicating that in this case, molecular weight of PVA had no effect on the level of residual PVA on the microparticles.

Despite the disadvantages associated with residual PVA on microparticles (e.g. unknown long-term effects of PVA in the lung), it was shown in one study that PVA enhanced the aerosolisation performance of catalase and Isyozyme in a HFA MDI formulation (Liao et al., 2005), which could be interesting in terms of the aerosolisation properties of the formulations prepared in this study (see Chapter 7).

5.4.7. Release Study of DNA from Microparticles

Experiments were carried out to observe the release profile of DNA from the different formulations made in Chapter 4. A burst release of DNA was observed within 24 h of the study, which then proceeded at a steady rate (Figure 5.9). This is in agreement with
previous studies that have encapsulated model plasmid DNA into PLGA 50:50 micro/nanoparticles using the double emulsion evaporation method (Cohen et al., 2000; Stern et al., 2003; Diez and Tros de Ilarduya, 2006; Mohamed and van der Walle, 2006). Formulations made with 0.1M NaHCO₃ demonstrated the most rapid release rate, and also releasing the most DNA within the time studied. In contrast, particles made with 1% m/m Na₂HPO₄ had the lowest release rate (only 57.81 ± 7.70 % after 28 days), but with intermediate amount of DNA released, owing to its higher encapsulation efficiency.

![Graph](image)

**Figure 5.9. Cumulative percentage and total amount of DNA released from each formulation over a period of 28 days.** No buffer (♦) 0.1M NaHCO₃ (■) 1% Na₂HPO₄ (▲). Mean ± s.d; n = 3.

The actual mechanics of how substances are released and how PLGA degrades in a microparticulate form are still a matter of debate. There have many studies that have analysed or modelled release rates of proteins or other entities from microparticles (O'Hagan et al., 1994; Cleland et al., 1997; Capan et al., 1999; Yang and Alexandridis, 2000; Wang et al., 2002); and the degradation profiles of PLGA polymer (Reed and Gilding, 1981; O'Hagan et al., 1994; Gopferich, 1996; Shive and Anderson, 1997) and therefore, details will not be discussed here. It was also not the intention of this study to analyse how polymer degradation and release profiles were related, and so, this aspect will not be discussed further. However, it is generally thought that the burst release is related to the rapid release of DNA that is located at the surface of the microparticles and therefore be more readily available to diffuse out (O'Hagan et al., 1994; Cleland et
al., 1997; Capan et al., 1999); and the slower release is according to the gradual erosion of the polymeric microparticle, which will also depend on the degree of porosity and the size of the pores (Herrmann and Bodmeier, 1995; Ehtezazi and Washington, 2000; Lemaire et al., 2003; Klose et al., 2006). The results obtained here do not present a clear relationship between degree of porosity and release rate, especially for those made with 1% Na$_2$HPO$_4$, which had the lowest rate in contrast to its surface area. It is not possible to make a conclusion regarding the correlation between surface properties and dissolution rates without further experiments.

At the end of the study, any remaining DNA was extracted out of the pellets to observe the conformation of DNA and to ensure that the amount of DNA released into the medium was calculated correctly. It was observed that for particles made with 0.1M NaHCO$_3$ in particular, there was an overestimation of the DNA that was released into the medium and what remained in the pellet, with more than 100% calculated (Table 5.6). This might be the cause of error in the experimentation, which could affect the data presented in Figure 5.9.

Table 5.6. A comparison of percent of DNA released into the medium and percent of DNA remaining in the pellets after the study. Mean ± s.d; n =3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Released</th>
<th>% Remaining</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No buffer</td>
<td>76.88 ± 11.99</td>
<td>27.60 ± 1.67</td>
<td>104.09 ± 11.82</td>
</tr>
<tr>
<td>0.1M NaHCO$_3$</td>
<td>117.08 ± 5.90</td>
<td>28.38 ± 2.20</td>
<td>145.46 ± 5.63</td>
</tr>
<tr>
<td>1% m/v Na$_2$HPO$_4$</td>
<td>57.81 ± 7.70</td>
<td>22.33 ± 4.58</td>
<td>80.14 ± 10.56</td>
</tr>
</tbody>
</table>

It has been suggested that analysis of DNA using Picogreen®, which was the method used here, can overestimate concentrations because of the sensitivity of the dye to different conformations of the plasmid DNA (Barman et al., 2000). The authors recommended using UV spectroscopy instead, which is insensitive to plasmid DNA isoform. It is uncertain whether this is the case here, especially as it was not uniform occurrence for the results. Because of the discrepancies, it could be inferred that other contaminants might be skewing the results, e.g. presence of salt, PVA etc at different concentrations for each formulation (Barman et al., 2000; Invitrogen, 2005; Probes, 2005), although this would have been taken into account because of the ‘blank’ particles that were also incubated at the same time.
An agarose gel of samples taken over time showed no visible bands, probably because of the low concentration released. However, extraction of DNA from the pellets at the end of the study demonstrated that for particles made with buffer, the DNA had degraded mostly into the open-circular conformation, but also with linear and supercoiled DNA too (results not shown). For particles made without buffer, the conformation of DNA remained the same (linear and open-circular). The pH of the medium was similar throughout the study at approximately pH 8-7 (results not shown).

Various studies have highlighted the effect of using different polymers (e.g. different ratios of lactide to glycolic acid and molecular weights) to control the release rate of DNA (Wang et al., 1999; Tinsley-Bown et al., 2000; Diez and Tros de Ilarduya, 2006), however, it is unclear how or what release profile would be ideal for DNA vaccines. Others have argued that release profiles obtained in vitro do not take into account enough factors that would be useful to predict an immune response in vivo (Balenga, 2006) and so improved strategies for analyzing release profiles of drugs from microparticles are needed. A number of researchers have previously looked at this, including the use of a continuous sampling system (Wang et al., 2002), and the use of more suitable buffers (Blanco-Príetoa et al., 1999; Jiang et al., 2002) for a more accurate in vitro and in vivo correlation.

5.5. Conclusions and Future Work

These experiments demonstrated clearly the difficulties encountered when trying to formulate microparticles that fulfil all requirements in terms of biological efficacy and product stability. From these studies, microparticles made with 1% Na₂HPO₄ were optimal in terms of suitable diameter for pulmonary delivery, encapsulation efficiency of DNA and best retention of DNA configuration. However, the DSC experiments suggest that these microparticles may have the poorest stability on storage, and slow and incomplete release rates. They may also have the highest residual DCM and PVA levels.

It may be that 0.1M NaHCO₃ particles are more suitable and further experiments would be needed to confirm or refute these ideas, which could include a range of DoE experiments to optimize the formulation in terms of thermal stability and residual levels of PVA.
Experiments to analyse the release profile of drugs entrapped in microparticles are still debated, but they do offer an indication of the processes involved in polymer degradation and rapidity of drug release. More work is needed to evaluate the analytical techniques used in this study, especially due to the over-estimation of DNA released from particles made with 0.1M NaHCO₃. Further studies are also needed to correlate release rates obtained in vitro with responses seen in vivo.
Chapter 6

Cell Culture Studies on Microparticle Formulations
6.1. Introduction

As well as investigating the physical and chemical characteristics of the particles, it is essential to monitor the stability and transfectivity of DNA within the formulations and methods for assessing biological potency of encapsulated DNA include transfection of mammalian cells followed by an enzyme assay; bacterial transformation or cell free transcription/translation systems (Hao et al., 2000).

Traditionally, cell culture experiments are used to assess the viability of the plasmid DNA after the micro-encapsulation process and to evaluate the efficiency of microparticles in delivering DNA (Walter et al., 1999; Cohen et al., 2000; Sandor et al., 2002; Dunne et al., 2003; Li et al., 2003). Although, there are still disparities in extrapolating cell culture results to in vivo results, they remain the mainstay of pre-in vivo assessment of the potential of new formulations.

The general assumption is that the higher the expression of the protein which the DNA encodes for, the higher the immune response. This is logical, as it is important for the antigen to be expressed to induce immunity. This has been demonstrated by Andre et al., (1998) where the use of a modified and wild-type gp120 gene sequence from HIV-1 env, demonstrated that there was a correlation between levels of gene expression in different cell lines (293T, HeLa, NIH 3T3 and COS-7) and the level of immune response in mice. The modified gp120 sequence had much higher levels of expression compared to the wild-type and resulted in increased antibody titres and cytotoxic T-lymphocyte reactivity (Andre et al., 1998).

There are a number of cell culture systems that can be used to assess the transfection efficiency and uptake of the formulations. As the aim is to target the lung, pulmonary endothelium was preferred. There have been several reviews that suggest various in vitro and in vivo methods to assess pulmonary absorption (Mobley and Hochhaus, 2001; Forbes and Ehrhardt, 2005). Although primary cell lines allow more accurate modelling of transport and uptake mechanisms; immortalized cell lines offer more controlled, reproducible and sustainable methods of investigation (Mobley and Hochhaus, 2001). Airway epithelial cell lines are represented by 16HBE14o- and Calu-3; both having characteristics of cilia, mucus production and tight junctions. Calu-3 cells grown under
air-interfaced culture allowed a more relevant and quantitative characterization of therapeutic aerosol particles intended for delivery to the tracheobronchial region of the lung or to the nasal passages (Fiegel et al., 2003). The only alveolar epithelial cell line available for study is the A549 cell line (Type II). This cell line bears very little resemblance to native epithelia, and is therefore not used very often for drug uptake studies. But, despite the disadvantages of A549, it was still selected for studying the uptake and transfection efficiencies of the formulations produced in Chapter 4. The rationale being that the area of the lung that was to be targeted was the alveolar region.

A study comparing the uptake ability of A549 and Calu-3 cells was carried out by Foster et al (2001). They found that A549 cells had a higher phagocytic activity compared to Calu-3, and that the size and charge of the particles influenced the degree of uptake in these cells (Foster et al., 2001). This has also been confirmed indirectly in another study investigating the uptake of deslorelin (a leuteinizing hormone releasing hormone (LHRH) agonist) (Koushik and Kompella, 2004). So, for a primary investigation, it will be useful to observe the transfection efficiency of PLGA particles (of micron size) encapsulating DNA in A549 cells; a study of which there is little literature.

Also, as discussed in the introduction, macrophages and dendritic cells play an important role in regulating and inducing the immune response to antigens, therefore, transfection efficiency of the microparticles in macrophages was also investigated and methods of these studies have been well established (Thiele et al., 2001; Walter et al., 2001; Lutsiak et al., 2002; Makino et al., 2003).

6.2. Aims

The aims of the work for this chapter are:

1) To evaluate the viability of DNA after encapsulation into PLGA microparticles.

2) To evaluate the efficiency of PLGA microparticles as a vehicle to deliver DNA into cells.

3) To observe if the sustained release of DNA from the particles as demonstrated in Chapter 5, will lead to sustained expression of the gene in cells.
6.3. Materials and Methods

6.3.1. Microparticle Formulation

Formulations made in Chapter 4 were tested in cells. The plasmid DNA encoding the reporter gene DNA gWiz Luciferase was encapsulated into the microparticles. For all the experiments, microparticles with a higher theoretical loading were made to increase the actual loading of DNA into the particles. Previous formulations have had a theoretical loading of 0.66 µg DNA/mg polymer. For these experiments, particles with a theoretical loading of 5 µg DNA/mg of polymer were made. The method of making the microparticles was the same as outlined in Section 4.3.2 except that the amount of DNA in the internal aqueous phase was 0.5 % m/m of the polymer weight. So, for 100 mg of polymer, the amount of DNA added was 500 µg (500 µl of a 1 mg/ml solution). Characterization of the particles was carried out as detailed in Chapter 2.

6.3.2. Cell Culture of Immortalised Cell Lines

Standard cell culture and aseptic techniques were used throughout. GIBCO media and supplements were purchased from Invitrogen (Paisley, UK). Phosphate buffered saline (PBS) (Dulbecco A, pH 7.3 ± 0.2) was purchased from Oxoid Ltd (Basingstoke, UK). Cell culture flasks (TPP) were from Helena BioSciences Europe (Gateshead, UK). Dimethyl sulphoxide (DMSO), cell-culture tested grade, and 1.0 ml cryovials were purchased from VWR International (Lutterworth, UK). Novagen GeneJuice® was from Merck Biosciences (Beeston, UK). Luciferase Assay Kit was from Promega (Southampton, UK). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Sigma-Aldrich (Poole, UK). Pierce BCA™ Protein Assay Kit was from Perbio Science (Cramlington, UK). All solutions (e.g. water, PBS) and materials (e.g. pipette tips) were sterilised by autoclaving or bought in pre-sterilised.

A549 cells were a generous gift from Dr Len Seymour, Department of Clinical Pharmacology, University of Oxford. J774A.1 cells were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cells were maintained at 37°C, 5% CO2 and 100% humidity levels. For further information regarding cell culture techniques in general and for specific cell lines, please refer to the European Collection of Cell Cultures (http://www.ecacc.org.uk).
6.3.2.1. A549 (Human Caucasian lung carcinoma) Epithelial Cells

A549 cells were maintained in F-12K Ham's medium supplemented with 10% foetal calf serum (FCS), 100U penicillin and 100 μg streptomycin in a 500 ml bottle of medium. When cells were about 90% confluent, old medium was removed and cells were washed with a volume of PBS. Cells were detached from the surface of the tissue culture flask using trypsin/EDTA and re-suspended in an appropriate volume of growth medium. Cells were passaged at a ratio of 1:3 or 1:4 and put into a new culture flask and then transferred back into the incubator.

Long-term stocks were prepared by freezing cells at a density of 3 x 10^6 cells/ml. The freezing medium consisted of the usual growth medium of the cells supplemented with 10% v/v of DMSO. Cells were aliquoted into cryovials at 0.5 ml or 1.0 ml volumes and initially frozen at -80°C for 24 h, before being transferred to liquid nitrogen for long-term storage.

6.3.2.2. J774A.1 (Murine BALB/c Monocyte Macrophage) Cells

Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FCS, 2mM glutamine, 100U penicillin and 100 μg streptomycin in a 500 ml bottle of medium. When cells were about 90% confluent, the cell culture medium was removed and centrifuged (1500 rpm for 5 minutes) to harvest growing cells that were in suspension. The cell pellet was re-suspended in fresh growth medium. Cells attached to the surface of the tissue culture flask were removed using a cell scraper and re-suspended together with the cells that were harvested from the medium. Cells were passaged at a ratio of 1:3 or 1:4 and put into a new culture flask and then transferred back into the incubator.

Long-term stocks were prepared by freezing cells at a density of 3 x 10^6 cells/ml. The freezing medium consisted of the usual growth medium of the cells supplemented with 10% v/v of DMSO. Cells were aliquoted into cryovials at 0.5 ml or 1.0 ml volumes and were initially frozen at -80°C for 24 hours, before being transferred to liquid nitrogen for long-term storage.
6.3.3. Cell Transfection Assays

6.3.3.1. Seeding of Cell Culture Plates

24 h before transfection, cells were seeded into a 48-well cell culture plate at a density of 40,000 cells/well (500 μl medium/well). Cells that were growing in tissue culture flasks were removed (either by trypsinization or scraping depending on cell-line) and re-suspended in a volume of cell culture medium. A small aliquot of cells were counted on a haemacytometer to determine the number of cells/ml. This suspension was then diluted down to give a cell suspension of 80,000 cells/ml (to give 40,000 cells/500 μl).

On the day of transfection, the cells were checked for the degree of confluency. A confluence of between 50-80% was used for transfection assays.

6.3.3.2. Viability of DNA Extracted from Microparticles

The method for extracting DNA from microparticles is outlined in Chapter 2, Section 2.4.1 To reduce variability of transfection efficiency due to different DNA concentrations, DNA extracted from microparticles and DNA that was not processed were all diluted so that they were of the same concentration (e.g. 25 μg/ml). Purification of extracted DNA was carried out using the sodium acetate, ethanol method as outlined in Chapter 2, Section 2.5.4.

For transfection into cells, DNA was complexed with GeneJuice® (Novagen, USA) and added to the cells according to the manufacturer's protocol (Novagen, 2003) with appropriate controls. For a 48-well plate, cells were transfected with 0.15 μg DNA/well. For each experiment, 6 wells were transfected; 3 wells were analysed for expression of the gene and for total protein quantifications and the remaining 3 were for the cell viability assay. Therefore, each experiment was performed in triplicate. The cells were assayed after 2-3 days (within a set of experiments, the time of assay was kept constant to ensure reproducibility).

6.3.3.3. Transfection of Cells with Microparticles

The amount of microparticles added to cells was such that 0.15 μg of DNA was added per well, so that a direct comparison of transfection efficiency to GeneJuice® could be made. For example, if an encapsulation efficiency of 50% was obtained for a
microparticle formulation (with theoretical loading of 5 µg DNA/mg polymer) then the mass of microparticles to add to one well would be 0.06 mg. The same mass of microparticles without DNA encapsulated was also added to cells as a control. For each formulation, 6 wells were transfected; 3 wells were analysed for expression of the gene and for total protein quantifications and the remaining 3 were for the cell viability assay. Therefore, each experiment was performed in triplicate.

Microparticles were re-suspended into cell culture medium (500 µl/well) and added onto cells and left to incubate for 2 h. After 2 h, the microparticles were removed and the cells washed with PBS before fresh cell culture medium was added. The cells were then returned to the incubator and assayed after 2-3 days (within a set of experiments, the time of assay was kept constant to ensure reproducibility).

6.3.4. Quantification of Luciferase Expression using Promega Luciferase Assay Kit

A luciferase assay kit was used to quantify the amount of luciferase expressed from the cells. The assay is based on the enzymatic reaction of luciferase and luciferin, which produces light which can be quantified using a luminometer (Figure 6.1).

![Figure 6.1. Schematic diagram of the reaction between luciferin and luciferase. Taken from Promega Luciferase Assay Technical Bulletin (Promega, 2005).](image)

The protocol was carried out according to the manufacturer's instructions (Promega, 2005), except that 50 µl of the luciferase assay buffer was used instead of the recommended 100 µl (to conserve reagent). 50 µl of the lysis buffer was added per well and 20 µl was used for the luciferase assay and the remainder was used for the BCA assay (Section 6.3.6). Assays were carried out in black 96-well plates and read on a Wallac Victor2™ 1420 Multilabel Counter (PerkinElmer Wallac, UK), using the luminescence filter. The software Wallac 1420 Workstation v.2.00.0.10 was used to collect the data. Results were expressed as relative light units (RLU) and normalized against the total protein content in the well (see section 6.3.6).
6.3.5. Cell Viability Assay using Tetrazolium Dye (MTT)

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals, which are largely impermeable to cell membranes (Mosmann, 1983). This results in an accumulation of the crystals inside healthy cells. Addition of a detergent is used to lyse the cells and dissolve the crystals. The amount of formazan produced by the cells is proportional to the cell viability. The colour is quantified using a spectrophotometer. The method used to analyse a 48-well plate is presented below.

5 mg/ml of MTT (Sigma, USA) was dissolved in PBS and filter-sterilised. 3 hours before the end of incubation, 50 µl of the MTT solution was added to each well containing cells. The plate was incubated in a CO₂ incubator for 3 hours at 37°C. After 3 hours, the medium was aspirated from the wells gently and 500 µl of DMSO was added to each well and pipetted up and down to dissolve crystals. 200 µl of each sample was dispensed into a clear 96-well plate and the plate was read at the wavelength of 570 nm on a Wallac Victor2™ 1420 Multilabel Counter. Cell viability was expressed as a percentage of untreated cells, whereby viability of untreated cells is 100%. For other sized cell culture plates, the volumes were adjusted accordingly (e.g. for 96-well plate, 20 µl of MTT solution was added per well and 200 µl of DMSO added. The plate was read directly). For all experiments, untreated cells were used as a positive control for cell viability.

6.3.6. Quantification of Total Protein by Pierce BCA™ Protein Assay Kit

Protein quantification was carried out using a kit from Pierce Biotechnology Inc., (USA) according to the manufacturer’s instructions (Pierce, 2003). Note that the cell lysis buffer used in the luciferase assay (Section 6.3.4) has been shown not to interfere with the BCA assay (Betz, 2003). The BSA standards were made up in the same lysis buffer that was used in the luciferase assay. Assays were carried out on clear 96-well plates and read on a Wallac Victor2™ 1420 Multilabel Counter, at a wavelength of 570 nm. The result from the luciferase assay (Section 6.3.4) was normalized against the total protein assayed and the results were re-calculated to be expressed as RLU/ mg of protein as demonstrated in another study (Li et al., 2003).
6.4. Results

6.4.1. Microparticle Formulations with Higher Theoretical DNA Loading

Microparticles made with a higher theoretical loading of DNA (0.5% m/m) demonstrated similar characteristics to those made with a lower theoretical amount of DNA (0.06% m/m) (Tables 6.1 and 6.2). However, looking at the microparticles under the scanning electron microscope, there were differences in the morphology (Figure 6.2). For all formulations, the microparticles demonstrate intense aggregation, and there are string-like structures that are covering particles made with buffer. It is unclear what the "strings" might be; but they seem to be water soluble, as the particle diameters measured are consistent with previous results.

Table 6.1. Encapsulation efficiency and the percentage of supercoiled content of the DNA extracted out from the microparticles made with a higher theoretical loading. Mean ± s.d. of 3 readings.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>% Encapsulation Efficiency</th>
<th>% Supercoiled Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>No buffer</td>
<td>23.39 ± 1.00</td>
<td>0 ± 0.00</td>
</tr>
<tr>
<td>0.1M NaHCO₃</td>
<td>61.05 ± 14.42</td>
<td>69.67 ± 1.08</td>
</tr>
<tr>
<td>1% m/v Na₂HPO₄</td>
<td>28.57 ± 7.22</td>
<td>55.42 ± 3.21</td>
</tr>
</tbody>
</table>

Input DNA % supercoiled = range between 70.12 – 75.16 %

Table 6.2. The diameter distributions of the microparticles made with a higher theoretical loading. Mean ± s.d. of 3 readings.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MVD µm</th>
<th>D[ν10] µm</th>
<th>D[ν50] µm</th>
<th>D[ν90] µm</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>No buffer</td>
<td>1.57 ± 0.06</td>
<td>0.66 ± 0.00</td>
<td>1.18 ± 0.01</td>
<td>2.94 ± 0.17</td>
<td>1.93 ± 0.13</td>
</tr>
<tr>
<td>0.1M NaHCO₃</td>
<td>5.35 ± 0.44</td>
<td>1.90 ± 0.18</td>
<td>4.86 ± 0.56</td>
<td>9.80 ± 0.47</td>
<td>1.66 ± 0.13</td>
</tr>
<tr>
<td>1% m/v Na₂HPO₄</td>
<td>2.23 ± 0.04</td>
<td>0.72 ± 0.01</td>
<td>1.56 ± 0.01</td>
<td>4.77 ± 0.17</td>
<td>2.59 ± 0.13</td>
</tr>
</tbody>
</table>

Figure 6.2. SEM images of particles made with and without buffer with a higher theoretical loading of DNA. Note 'strings' across the particles made with buffer. a) No buffer; b) with 0.1M NaHCO₃; c) with 1% m/v Na₂HPO₄.
6.4.2. Viability of DNA Extracted from Microparticles

DNA extracted from the different microparticle formulations showed varying degrees of supercoiled content (Figure 6.3) and this was reflected in the transfection efficiencies of the extracted DNA in A549 cells. It was clear that if there was no supercoiled content, there was very little gene expression ($p > 0.05$ compared to cells alone) as demonstrated by the DNA extracted from particles made without buffer (Figure 6.4). At the other extreme, unprocessed DNA had the highest level of gene expression (Figure 6.4). For DNA that was extracted from particles made with buffer, there was significant transfection efficiency ($p < 0.05$ compared with cells alone) and there was no significant difference between the two types of buffer ($p > 0.05$). Cell viability between all extracts was similar ($p > 0.05$), indicating that there was no other material that may have been extracted out with the DNA that could be detrimental to the cells (Figure 6.5).

![Agarose gel image of DNA extracted from the particles and degree of supercoiled content for each sample.](image)

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Supercoiled (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No buffer</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.1M NaHCO₃</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>1% m/v Na₂HPO₄</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td>Unprocessed</td>
<td>73</td>
</tr>
</tbody>
</table>

La = λ /Hind III Ladder

**Figure 6.3.** Agarose gel image of DNA extracted from the particles and degree of supercoiled content for each sample. The above image is representative of the profile of conformations of DNA extracted from multiple batches of microparticles; as are the percentages of degree of supercoiled content for each.
Figure 6.4. Transfection efficiency of DNA extracted from microparticles on A549 cells. Cells were transfected with DNA extracted from particles which were then complexed with GeneJuice®. Viability of the DNA was compared with un-processed DNA. Experiments were carried out in triplicate on each cell culture plate and 3 plates were transfected. Results are mean ± s.d; n = 9. Statistical test was ANOVA with Tukey post-hoc test.

<table>
<thead>
<tr>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unprocessed DNA + GeneJuice®</td>
</tr>
<tr>
<td>2 No buffer DNA + GeneJuice®</td>
</tr>
<tr>
<td>3 0.1M NaHCO₃ DNA + GeneJuice®</td>
</tr>
<tr>
<td>4 1% m/v Na₂HPO₄ DNA + GeneJuice®</td>
</tr>
<tr>
<td>5 Unprocessed DNA Alone</td>
</tr>
<tr>
<td>6 GeneJuice® Alone</td>
</tr>
<tr>
<td>7 No treatment (Cells Alone)</td>
</tr>
</tbody>
</table>

Figure 6.5. Cell Viability of DNA extracted from microparticles and complexed with GeneJuice® on A549 cells. Cells were transfected with DNA extracted from particles which were complexed with GeneJuice®. Viability of the cells was compared with untreated cells. Experiments were carried out in triplicate on each cell culture plate and 3 plates were transfected. Results are mean ± s.d; n = 9. Statistical test was ANOVA with Tukey post-hoc test. See table in Figure 6.4 for key to x-axis.
6.4.3. Transfection of A549 Cells with Purified or Non-Purified DNA Extracted from Microparticles

From the above results, the transfection efficiencies for DNA that was extracted from particles made with buffer were far from equal to that of unprocessed DNA. After searching through literature, it was puzzling how some authors manage to observe similar levels of gene expression between untreated DNA and DNA extracted from particles. After looking closer at their methodology, it was observed that the DNA extracts were put through a purification process (Hao et al., 2000; Atuah et al., 2003). This may have affected the observed gene expression, and so experiments were then carried out to test this hypothesis.

A large batch of DNA was extracted from particles and half the amount was purified. Therefore, the DNA is from the same source. Cell viability was not investigated; therefore no results are shown for this aspect.

Although there was no difference observed on an agarose gel in the conformation of the extracted DNA between purified and non-purified (Figure 6.6), there is a difference in the transfection efficiencies (Figure 6.7). With DNA extracted from particles made with carbonate buffer, there is no difference ($p > 0.05$), but a very large difference was observed with particles made with phosphate buffer ($p < 0.05$). It is unclear why this is so. It may be that contaminants extracted out with the DNA affect complexation efficiency between the DNA and the transfection reagent, and this is more marked in DNA extracted from particles made with phosphate buffer. Particles made with phosphate buffer retained higher levels of PVA compared with particles made with carbonate buffer (see Chapter 5, Section 5.4.6), so this may contribute towards a reduction in transfection efficiency into the cells.
<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Supercoiled (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No buffer</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.1M NaHCO₃</td>
<td>68.2</td>
</tr>
<tr>
<td>3</td>
<td>1% m/v Na₂HPO₄</td>
<td>38.3</td>
</tr>
<tr>
<td>4</td>
<td>No buffer - P</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.1M NaHCO₃ - P</td>
<td>66.2</td>
</tr>
<tr>
<td>6</td>
<td>1% m/v Na₂HPO₄ - P</td>
<td>38.0</td>
</tr>
</tbody>
</table>

Figure 6.6. Agarose electrophoresis gel of DNA extracted from particles. Purified (-P) or non-purified. It can be seen that there is no difference in the conformation or percentage supercoiled content of the DNA before or after purification with sodium acetate and ethanol.

Figure 6.7. Transfection efficiency of purified or non-purified DNA extracted from microparticles complexed with GeneJuice® on A549 cells. Experiments were carried out in triplicate on each cell culture plate and 3 plates were transfected. Results are mean ± s.d; n = 9. Statistical test was Student's Independent T-test (2-tailed).
6.4.5. Transfection of Cells with Microparticles

The next set of experiments involved transfection of A549 cells with the microparticles themselves. From the release studies (Chapter 5, Section 5.4.7), it was observed that there was a difference in the amount of DNA available over time from each formulation, and so a study was carried out over a period of 7 days. The amount of DNA added into each well was calculated to be equivalent to the amount used for the positive control – which is unprocessed DNA complexed with GeneJuice®. Because of the poor performance of DNA extracted out from particles made without buffer; these experiments were carried out with particles made with 0.1M NaHCO$_3$ and 1% m/v Na$_2$HPO$_4$ only.

Both microparticle formulations demonstrated very little transfection efficiency in A549 cells in the time period tested (Figure 6.8). Levels were not above background (p > 0.05), and gene expression was only observed with DNA that was extracted out from the microparticles and then complexed with GeneJuice®. But, if statistical analysis is performed using data for the transfection levels of the microparticles alone, it can be seen that the particles made with carbonate buffer are higher than that of the empty particles (Figure 6.9). Particles made with phosphate buffer still have insignificant transfection efficiency (p > 0.05).

The microparticles (blank and DNA-loaded) also demonstrated significant toxicity to the cells (p < 0.001), which was only diminished at day 7 of the study (Figure 6.10). This effect may be due to the washing step of the transfection protocol. After the microparticles are left on for 2 h, the excess is washed off with PBS, and this action may also have dislodged cells. This is enforced by the fact that cells that had no microparticles added, which did not go through the washing step, had higher initial viability levels than those that had microparticles added. The cells recovered well over time; therefore, it is unclear whether cell loss is attributed to the toxic nature of the microparticles or the reduction in the number of cells in the wells. The washing step may also have contributed to the low levels of gene expression observed with the microparticle formulations. Washing off the particles could have led to a lower ‘dose’ of DNA available in each well.
<table>
<thead>
<tr>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Unprocessed DNA + GeneJuice®</td>
</tr>
<tr>
<td>2 0.1M NaHCO₃ + DNA Particles</td>
</tr>
<tr>
<td>3 1% m/v Na₂HPO₄ + DNA Particles</td>
</tr>
<tr>
<td>4 0.1M NaHCO₃ Blank Particles</td>
</tr>
<tr>
<td>5 1% m/v Na₂HPO₄ Blank Particles</td>
</tr>
<tr>
<td>6 0.1M NaHCO₃ DNA Extract + GeneJuice®</td>
</tr>
<tr>
<td>7 1% m/v Na₂HPO₄ DNA Extract + GeneJuice®</td>
</tr>
<tr>
<td>8 No Treatment (Cells Alone)</td>
</tr>
</tbody>
</table>

Figure 6.8. Comparison of transfection efficiencies of different microparticle formulations in A549 cells over time. Cells were transfected with formulations made in Chapter 4. Their efficiency was compared with DNA extracted out of the particles and un-processed DNA complexed with GeneJuice® (as in Section 6.4.2). Experiments were carried out in triplicate on each cell culture plate and 3 plates were transfected. Results are mean ± s.d; n = 9. Statistical test was MANOVA with Tukey post-hoc test.

Figure 6.9. Comparison of transfection efficiencies of different microparticle formulations in A549 cells over time (particles alone shown). Experiments were carried out in triplicate on each cell culture plate and 3 plates were transfected. Results are mean ± s.d; n = 9. Statistical test was Student’s Independent T-test (2 tailed). See table in Figure 6.8. for key to x-axis.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unprocessed DNA + GeneJuice®</td>
</tr>
<tr>
<td>2</td>
<td>0.1M NaHCO₃ + DNA Particles</td>
</tr>
<tr>
<td>3</td>
<td>1% m/v Na₂HPO₄ + DNA Particles</td>
</tr>
<tr>
<td>4</td>
<td>0.1M NaHCO₃ Blank Particles</td>
</tr>
<tr>
<td>5</td>
<td>1% m/v Na₂HPO₄ Blank Particles</td>
</tr>
<tr>
<td>6</td>
<td>0.1M NaHCO₃ DNA Extract + GeneJuice®</td>
</tr>
<tr>
<td>7</td>
<td>1% m/v Na₂HPO₄ DNA Extract + GeneJuice®</td>
</tr>
</tbody>
</table>

Figure 6.10. Comparison of the cell viability of A549 cells over time when transfected with different microparticle formulations. Cells were transfected with formulations made in Chapter 4. Their cell viability was compared with cells that had no treatment. Experiments were carried out in triplicate on each cell culture plate and 3 plates were transfected. Results are mean ± s.d; n = 9. Statistical test was MANOVA with Tukey post-hoc test.
6.4.6. Comparison of Transfection Efficiency Between Different Cell Lines – A549 and J774A.1

As the ultimate target of formulations is the lung for vaccine delivery, it would also be prudent to investigate the transfection efficiency of particles with a white blood cell line. Macrophages are numerous in the lung and also play a pivotal role in regulating immune responses (Nicod et al., 2000).

With the macrophages, efficiency was compared over a 24 hour incubation period with A549 cells.

For J774A.1 cells there was no significant expression of luciferase (p > 0.05 for all compared to background), with the exception of unprocessed DNA with GeneJuice®, which showed some transfection efficiency compared to background (p < 0.01) (Figure 6.11). The particles showed significant toxicity to the J774A.1 cells (p < 0.01) (Figure 6.12).

When comparing the transfection efficiencies of the formulations between the two cell lines, it is clear that macrophages are extremely difficult to transfect. Levels of luciferase expression were significantly higher for all treatments in A549 cells (p < 0.01) compared to J774A.1 cells (Figure 6.11). The macrophages also seemed to be more sensitive against microparticle exposure compared to the epithelial cells (Figure 6.12).
Figure 6.11. Comparison of transfection abilities of different cell lines. For the transfection efficiencies, there is a significant difference between all treatments ($p < 0.01$) when comparing efficiency of A549 with J774A.1 (Inset is of particles alone). Experiments were carried out in triplicate on each cell culture plate and 3 plates were transfected. Results are mean ± s.d; $n = 9$. Statistic test was Student’s Independent T-test.

Figure 6.12. Comparison of cell viabilities of different cell lines. For cell viability $p < 0.005$ for treatment numbers 2, 3, 4, 5 and maybe 7 ($p = 0.043$) when comparing efficiency of A549 with J774A.1. Experiments were carried out in triplicate on each cell culture plate and 3 plates were transfected. Results are mean ± s.d; $n = 9$. Statistic test was Student’s Independent T-test. See Figure 6.11 for key to x-axis.
6.5. Discussion

The objective of this study was to evaluate the viability of the DNA after micro-encapsulation into PLGA and to evaluate the efficiency of the microparticles as a delivery system for DNA into cells. Firstly, it was established that the extracted DNA from the microparticles transfected the cells with varying degrees of efficiency. This has been attributed mainly to the degree of supercoiled content remaining in the conformation and correlates with other results where the linear conformation of the plasmid DNA had a much reduced level of transfectibility in cells (Adami et al., 1998; Hao et al., 2000). Despite the addition of the purification step onto extracted DNA, the levels were still not equivalent to that of unprocessed DNA, which has not been observed in other papers (Hao et al., 2000; Atuah et al., 2003). This may be due to the different cell line that was used in this project compared to the others. Hao et al, used COS-7 (African green monkey kidney fibroblast) cells; Atuah et al, used HEK293 (human embryo kidney fibroblast) cells. The COS and HEK cell lines, together with the Chinese hamster ovary (CHO) cell line, are commonly used for transfection because they are ‘easy’ cell-lines to transfect (Florea et al., 2002; Gamper et al., 2005; Thomas and Smart, 2005). This is mainly because they take up DNA and express recombinant proteins very efficiently (Parham et al., 1998).

A549 is not the easiest of cell lines to transfect as demonstrated by Dokka et al., (2000). They compared the transfection efficiencies of liver HepG2 cells, kidney embryonic 293 cells, A549 cells and RAW 264.7 and NR 383 macrophage cells with a liposomal system to deliver the DNA and the efficiencies they obtained were as follows: Hep G2 > 293 > A549 > RAW 264.7 > NR 383 (Dokka et al., 2000). This highlights the differences between cell lines for transfection efficiency, and also acts as a caution for interpreting studies that have demonstrated high transfection efficiencies.

Another result observed was the differences seen with the extracted DNA from the microparticles that underwent a purification process before complexation. For the DNA that was extracted from particles made with phosphate buffer, there was a marked increase in the level of gene expression, not seen with the other extracts. However, this could not be attributed to an increase in the supercoiled content of the DNA as agarose gel electrophoresis and densitometry revealed that the content and conformation of the
DNA was equivalent to that of the unpurified DNA. It may be that other factors are involved that cannot be detected by agarose gel electrophoresis; Poxon and Hughes (2000) investigated the efficiency of DNA that was lyophilized with or without carbohydrate. After lyophilization, the viability of the DNA was tested by transfection into COS-1 cells. There was a decrease of up to 75% in transfection efficiency. No changes were observed on agarose gel, with percentage of supercoiled being the same. After further investigation they showed that the DNA had changed – plasmid hyperchromic effect – there was change at 260 nm and a change in the Tm of the DNA. This was prevented with the addition of carbohydrates such as glucose, lactose and sucrose (Poxon and Hughes, 2000). That may be the case here, or it may be due to other factors. Further investigations would be necessary to elucidate the mechanism for reduction.

For the efficiency of the particles themselves, again there was a very marked difference between the positive control and the microparticles. Microparticles demonstrated extremely low efficiency over the time period investigated for both cell lines tested. For A549 cells it may be due to the fact they are a ‘difficult’ cell-line to transfect as alluded to in the previous paragraph. It is unlikely that the cells wouldn’t be able to take the particles up, it has been demonstrated that A549 cells readily internalise polystyrene particles (size up to 3 μm) of both negative and positive charge within 15 minutes of addition onto the cells (Foster et al., 2001).

The most likely explanation is probably the reduction in DNA available to the cells from the particles. Either because of the washing step involved in the transfection procedure or the differences in the actual number of particles added per well or both. Especially as the controls (positive and negative) did not undergo the additional washing step. The ‘dose’ of particles was added according to the encapsulation efficiency of DNA in the formulations. Particles made with phosphate buffer had higher encapsulation efficiency, and therefore, fewer particles were needed to be added to each well compared with particles made with carbonate buffer. The rate of DNA released from the formulations could also explain the difference in gene expression levels. Particles made with carbonate buffer released up to 85 ± 5% of the DNA encapsulated within 7 days (Chapter 5, Section 5.4.7), whereas, particles made with phosphate buffer only released 34 ± 4%. The reduction in number of particles added, coupled with the
extremely low release rate of the DNA probably led to the low levels of gene expression in cells transfected with particles made with phosphate buffer. This highlights again, the nuances of formulation development.

For the J774A.1 cell line, levels of gene expression were extremely low, even for the un-processed DNA complexed with GeneJuice®. Macrophages in general are extremely difficult to transfect with regards to gene expression and this has been demonstrated before (Walter et al., 2001; Walter and Merkle, 2002; Jilek et al., 2004) In these experiments, macrophages demonstrated less than 1% gene expression compared with A549 cells (using non-treated DNA). With particles therefore, there was an even more marked difference, although cell viability was comparable over a 24 hour period. It may be that the DNA is rapidly degraded inside the macrophage and therefore is unavailable for transcription. (Yoshinaga et al., 2002)

Overall, the particles performed poorly in cell culture experiments compared with the commercial transfection reagent and this has been experienced by others (Diez and Tros de Ilarduya, 2006). Despite these results though, it is not clear how these particles will perform in an *in vivo* situation. Studies that have had “good” results in cell culture experiments do not go on to have the same results *in vivo* (Foged et al., 2004) or vice versa (Roques et al., 2006). This highlights the problems that are inherent in trying to extrapolate *in vitro* results to an *in vivo* setting.

**6.6. Conclusions and Future Work**

In answer to the aims that were given at the beginning of the chapter:

1) It was demonstrated that DNA that had been microencapsulated could transfect A549 and J774A.1 cells with varying degrees of success. There was a clear relationship between the amount of DNA that was in the supercoiled conformation and the level of gene expression.

2) Microparticles were shown to be poor in efficacy for delivering DNA into cells.

3) The results obtained from the release study carried out in Chapter 5 were reflected in the results obtained for the transfection efficiencies of the microparticles. Particles made with carbonate buffer, where the DNA was
released rapidly, also had higher levels of gene expression compared with particles made with phosphate buffer.

For future work, a number of approaches could be used:

Leaving the microparticles on the cells for a longer period of time could be used to determine if transfection is increased, but from experience after 24 hours, there was significant cell loss (results not shown). Jilek *et al.*, (2004) only observed detectable levels of transfection in a murine dendritic cell line on day 7 (experiments lasted 27 days) in contrast to cationic transfection reagents (Jilek *et al.*, 2004). Despite this, the levels were also very low with only 2-18 cells out of 80,000 cells being transfected. Increasing the number of particles added onto the cells could also be investigated.

Confocal microscopy could be used to elucidate uptake mechanisms and movement of DNA in the cells. This would involve labelling of the microparticles and DNA separately; as well as specific cellular compartments, such as the nucleus and the endosome.

A more accurate prediction for *in vivo* success could be to look at mRNA expression levels rather than detection of the reporter gene itself. It may be that the DNA is transcribed, but not translated; or that the levels of expression were below the detection limit. Denis-Mize *et al.*, (2000) reported that attempts to detect reporter gene activity in bone marrow derived dendritic cells (BMDC) were unsuccessful, although they did detect intracellular fluorescence with rhodamine-labelled PLG-CTAB-DNA and readily detected HIV gag peptide presentation in PLG-CTAB transfected dendritic cells (Denis-Mize *et al.*, 2000).

Since it is widespread knowledge that there is a poor correlation between transfection efficiency in cell culture and *in vivo* (Pouton *et al.*, 1998), it would be interesting to try these microparticles in vivo to see if they were able to generate immune responses against the DNA-encoded protein. However, the additional development of better *in vitro*-in *vivo* extrapolation models would be important.
Chapter 7

Dry Powder Dispersion Experiments
7.1. Introduction

The ultimate aim of the project is the development of formulations suitable for delivery to the lung. This chapter looks at the performance of various microparticle formulations as a dry powder - as a dry powder inhaler (DPI) would probably be the device of choice for further development. There are currently 3 main types of inhalers on the market, the nebuliser, the pressurised metered dose inhaler (pMDI) and the DPI. Each has their advantages and disadvantages according to the drug that is to be delivered; a summary of these is outlined in Table 7.1.

Table 7.1. Advantages and disadvantages of different inhalation devices.
Adapted from (Labiris and Dolovich, 2003b)

<table>
<thead>
<tr>
<th>Device</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizers (Jet, Ultrasonic)</td>
<td>Specific inhalation technique or co-ordination not required.</td>
<td>Time consuming</td>
</tr>
<tr>
<td></td>
<td>Aerosolises most drug solutions</td>
<td>Bulk, not portable</td>
</tr>
<tr>
<td></td>
<td>Delivers large doses</td>
<td>Contents easily contaminated</td>
</tr>
<tr>
<td></td>
<td>Suitable for infants and or those physically unable to use other devices</td>
<td>Relatively expensive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poor delivery efficiency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Drug wastage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wide performance variations between different models</td>
</tr>
<tr>
<td>Pressured Metered Dose Inhalers (pMDI)</td>
<td>Compact, portable</td>
<td>Inhalation technique and patient co-ordination required</td>
</tr>
<tr>
<td></td>
<td>Multidose (~200 doses)</td>
<td>High oral deposition</td>
</tr>
<tr>
<td></td>
<td>Inexpensive</td>
<td>Maximum dose of 5 mg</td>
</tr>
<tr>
<td></td>
<td>Sealed environment (no degradation of drug)</td>
<td>Limited range of drugs available</td>
</tr>
<tr>
<td>Dry Powder Inhalers (DPI)</td>
<td>Compact, portable</td>
<td>Respirable dose dependent on inspiratory flow rate</td>
</tr>
<tr>
<td></td>
<td>Breath actuated</td>
<td>Humidity may cause powders to aggregate</td>
</tr>
<tr>
<td></td>
<td>Easy to use</td>
<td>Dose lost if patient inadvertently exhales into DPI</td>
</tr>
<tr>
<td></td>
<td>No hand-mouth co-ordination required</td>
<td></td>
</tr>
</tbody>
</table>

It is not the intention for this project to discuss the details of the different devices as other articles deal with this excellently (Ashurst et al., 2000; Labiris and Dolovich, 2003b; Anderson, 2005; Leach, 2005), but it is worth noting that there is an increasing interest in developing formulations for the DPI. The main reason for this is because of difficulties encountered by companies when chlorofluorocarbons (CFCs) were phased out as a propellant in pMDIs (Ashurst et al., 2000; Frijlink and De Boer, 2004). Hydrofluoroalkanes (HFAs) are the main replacement propellant, but these have led to a whole new realm of formulation development for existing drugs as well as new drugs due to the different properties of HFAs compared to CFCs (Ashurst et al., 2000). DPIs,
therefore, have become a more attractive delivery system due to their perceived simplicity in drug and delivery development.

The investigation of aerosols is an exact science, be they in a liquid or dry form, and involves complex experiments for predicting their performance in vivo. Several reviews are available regarding this matter (Newman et al., 2000; Mobley and Hochhaus, 2001; Frijlink and De Boer, 2004; Mitchell, 2004). Cascade impactor analysis and liquid impingers are the two main methods recommended by the US and European Pharmacopeia to analyse medical aerosols (Mitchell, 2004). But these methods are very time consuming and laborious to perform. At present, there is a focus towards the use of the laser diffraction technique as an alternative method to analyse aerosols (de Boer et al., 2002; Martin et al., 2006). If this is successful, then a marked reduction in time for analyses can be achieved i.e. a day for cascade impactor analyses, compared to a few minutes with laser diffraction – although it has to be noted that laser diffraction cannot determine which is active and which is excipient; in these cases, cascade impactors are more suitable for analysis of the particle size distribution of the drug.

For this section, a preliminary study was carried out to observe the diameters of the microparticle formulations when dispersed under pressure as a dry powder using laser diffraction. The diameters of the formulations are considered to be suitable for lung delivery when measured in a wet state (Chapter 4), but will they show the same diameters when measured in a dry state?

Successful aerosolisation of the microparticles as a dry powder will depend on the ease in which the forces that occur between the particles (cohesive), and those between the particles and the device (adhesive), are broken. Interparticulate (cohesive) forces are dominated by Van der Waals, electrostatic and capillary forces (Hickey et al., 1994). Adhesive forces are dependent on the composition of the surface, duration and velocity of contact as well as the humidity of the atmosphere (Hickey et al., 1994). As different DPIs work in different ways and for this project, the choice of a particular DPI to be used had not been made, the
microparticle formulations were analysed alone, thereby reducing the amount of forces that need to be taken into consideration for dispersion.

In addition, to increase the dispersibility of the formulations it was theorised that the addition of surfactants could aid in the dispersibility of the particles in a dry powder form. Dipalmitoylphosphatidylcholine (DPPC) and lecithin were chosen as the two surfactants for investigation as these are both endogenous in the lung and as excipients, also have GRAS status (Evora et al., 1998; Vanbever et al., 1999; Kibbe, 2000; Cryan, 2005).

Not many reports are available that use DPPC or lecithin in PLGA microparticles, particularly not in the context of using it in DNA-loaded particles. DPPC has been incorporated into PLGA microparticles to evade macrophage uptake in the lung (Evora et al., 1998); increase entrapment of paclitaxel (Mu and Feng, 2001) and to increase porosity, thereby reducing density, of microparticles for delivery to the lung (Vanbever et al., 1999). Lecithin has been used as an absorption enhancer for delivery of salmon calcitonin to the lung (Kobayashi et al., 1996) and also as a stabiliser for the entrapment of the enzyme carbonic anhydrase into PLGA particles (Sandor et al., 2002).

It would be interesting to observe the interactions of these two surfactants with DNA and their properties for enhancing or reducing the ability of the microparticles to be aerosolised.

7.2. Aims

The ultimate aim of the formulations developed in this thesis was that they were to be put into an inhaler device for aerosol delivery of the vaccine. In contrast to formulations for pMDIs, dry powder inhalation is simple as no other surfactants or excipients would need to be considered that might affect the formulation. The aim of this work therefore is to investigate the dispersibility of different microparticle formulations as a dry powder using laser diffraction.
7.3. Materials and Methods

The materials that were used in this Chapter were the same as for formulations made in Chapter 4.

For those with added surfactants, dipalmitoyl-phosphatidylcholine (DPPC) was purchased from Genzyme Pharmaceuticals, USA, and soy lecithin from Lipoid GmbH, Germany.

7.3.1. Microparticle Formulations

Microparticles used for dry dispersion experiments were made according to materials and methods in Chapter 4. The difference in dispersibility between formulations made with 1) no buffer; 2) with 0.1M NaHCO₃ and 3) 1% m/v Na₂HPO₄ were investigated.

For microparticles with added surfactant (Table 7.2), 5% m/m polymer mass of DPPC or soy lecithin was added into the organic phase of the formulation together with the polymer and then homogenized as usual. To reduce the number of formulations to be made, particles were made with (+) or without (−) 1% m/v Na₂HPO₄ only (i.e. no microparticles with 0.1M NaHCO₃ buffer were made). All formulations were then characterised according to methods outlined in Chapter 2.

Table 7.2. Formulation parameters for microparticles with added surfactant. Percentage mass refers to mass of polymer. Buffer = 1% m/v Na₂HPO₄.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Buffer</th>
<th>Surfactant 5% m/m</th>
<th>DNA % m/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>DPPC</td>
<td>0.067</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>DPPC</td>
<td>0.067</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>Lecithin</td>
<td>0.067</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>Lecithin</td>
<td>0.067</td>
</tr>
</tbody>
</table>
7.3.2. Dry Powder Dispersion Experiments

Microparticle formulations were measured after being freeze-dried and were carried out by Dr Jag Shur. The dry powders were dispensed with compressed air at 1 or 2 bar through a RODOS dry powder feeder (Sympatec GmbH, Germany) before sizing with a Helium Neon Laser Optical System (HELOS) laser diffractometer (Sympatec GmbH, Germany). The particle size analysis was performed using the WINDOX 4.0 software (Sympatec GmbH, Germany), and as these were preliminary experiments, only the $X_{50}$ value was recorded so that a comparison could be made to the MVD diameters obtained when analysing the microparticles using laser diffraction (Chapter 2, Section 2.3.1). 1 and 2 bar pressures were selected for initial studies, to avoid attrition of the formulations, which might occur at higher pressures, and lead to inaccurate results.

Measurements were only carried out once, this was because of the large amounts of sample needed and also because these were initial studies to assess the performance of the machine and the formulations.

7.4. Results

7.4.1. Microparticle Characterisation

The microparticles made with additional surfactant differed from those made without surfactant. There was a reduced yield of the formulation and the encapsulation efficiency and percentage supercoiled content of the plasmid DNA was also lower compared to those made without surfactant (Table 7.3, Table 4.3).

Table 7.3. Characteristics of particles made with the addition of surfactants.
Two batches of particles made for each formulation. For the particle yield, mean ± s.d. of two batches of particles. For the encapsulation efficiency and % supercoiled content, each batch was analysed twice; mean ± s.d. 4 readings.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Yield %</th>
<th>Encapsulation Efficiency %</th>
<th>% Supercoiled</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60.34 ± 3.91</td>
<td>8.29 ± 1.65</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>37.06 ± 12.63</td>
<td>33.71 ± 6.65</td>
<td>18.76 ± 3.73</td>
</tr>
<tr>
<td>3</td>
<td>52.68 ± 1.17</td>
<td>3.89 ± 1.46</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>48.35 ± 11.05</td>
<td>30.05 ± 5.59</td>
<td>26.53 ± 1.43</td>
</tr>
</tbody>
</table>
For particles made without buffer, the DNA extracted out from the particles was in the linear and open-circular form (results not shown). The surfactants did not protect from degradation. This was partially prevented by the addition of the phosphate buffer, although complete protection was not achieved – the percentage supercoiled content of unprocessed DNA being 65.05 ± 1.87 compared to 18.76 ± 3.73 for DPPC and 26.53 ± 1.43 lecithin.

There was also a difference in the diameters measured in the wet state using laser diffraction (Table 7.4, Table 4.4); for the particles made without buffer and with DPPC or lecithin, there was a marked difference in the measurements. The addition of DPPC led to the particles being very difficult to disperse in water and this is reflected in the large MVD and the range of Dv10, v50 and v90 diameters. The complete opposite is achieved with the addition of lecithin – the distribution becomes much narrower. For the formulations where 1% Na₂HPO₄ was used; the addition of DPPC or lecithin had little effect on the diameters, although there seemed to be a slight increase compared to formulations without surfactant. Scanning electron micrographs of the formulations (Figure 7.1) confirmed the results obtained using laser diffraction.

Table 7.4. Size distributions of formulations made with surfactant. Each batch of particles was measured three times in a wet state using a Mastersizer (Malvern, UK). Mean ± s.d. of 6 readings.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MVD μm</th>
<th>D[v,10] μm</th>
<th>D[v,50] μm</th>
<th>D[v,90] μm</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.87 ± 0.33</td>
<td>0.79 ± 0.04</td>
<td>2.59 ± 0.42</td>
<td>39.06 ± 17.01</td>
<td>16.13 ± 2.71</td>
</tr>
<tr>
<td>2</td>
<td>3.17 ± 0.59</td>
<td>1.32 ± 0.06</td>
<td>2.78 ± 0.32</td>
<td>5.31 ± 0.63</td>
<td>1.44 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>1.16 ± 0.00</td>
<td>0.64 ± 0.01</td>
<td>1.01 ± 0.00</td>
<td>1.88 ± 0.01</td>
<td>1.24 ± 0.10</td>
</tr>
<tr>
<td>4</td>
<td>3.34 ± 0.17</td>
<td>1.24 ± 0.13</td>
<td>2.82 ± 0.14</td>
<td>6.21 ± 0.27</td>
<td>1.77 ± 0.08</td>
</tr>
</tbody>
</table>
7.4.2. Dry Powder Experiments

7.4.2.1. Microparticles without Surfactant

The microparticles made without buffer dispersed poorly at both 1 and 2 bar, with an $X_{50}$ of 95 and 78 μm, respectively (Figure 7.2). The large $X_{50}$ suggested the presence of large agglomerates, which meant that the cohesive forces are particularly strong and therefore, a higher air pressure would be needed to disperse them to a finer powder.
Figure 7.2. Particle size distribution (cumulative distribution and density distribution) of microparticles produced without buffer dispersed at 1 bar (■) and 2 bar (●). The X$_{50}$ at 1 bar was 95 μm and at 2 bar it was 78 μm, demonstrating that an increase in pressure was needed to increase the dispersibility of the dry powder. (Note that the “Incorrect lens range – data for information only” is because the size measurements are larger than what the lens can record, not because of experimental error).

Microparticles produced with 0.1M NaHCO$_3$ buffer were also difficult to disperse at both 1 and 2 bar with an X$_{50}$ of 88 and 82 μm, respectively (Figure 7.2). However, Figure 7.3 also shows the presence of a secondary peak suggesting liberation of finer particles in the size range of 2–6 μm. This suggests that despite poor dispersion, the interparticulate forces are not as strong as particles produced without buffer (Figure 7.2). Results for microparticles made with 1% m/v Na$_2$HPO$_4$ (X$_{50} = 70$ μm) also liberated finer material compared to the X$_{50}$ (result not shown).
Figure 7.3. Particle size distribution (cumulative distribution and density distribution) of microparticles produced with 0.1M NaHCO$_3$ dispersed at 1 bar (■) and 2 bar (●). The $X_{50}$ at 1 bar was 88 μm and at 2 bar it was 82 μm, showing little difference between the 2 pressures compared to microparticles made without buffer. (Note that the “Incorrect lens range – data for information only” is because the size measurements are larger than what the lens can record, not because of experimental error).

For comparison, Figure 7.4 shows the size distributions of the same formulations measured in the wet state. The D[$v$,50] for the formulations according to analysis with a Malvern Mastersizer were a) without buffer - 1.80 μm; b) 0.1M NaHCO$_3$ - 4.14 μm; c) 1% Na$_2$HPO$_4$ – 2.69 μm. There was an agreement with the diameters obtained for the microparticles made with buffer, 1% m/v Na$_2$HPO$_4$ particles had a reduced diameter compared with 0.1M NaHCO$_3$ particles when measured either in the dry or wet state; even though the dry readings were much larger than the wet readings. This was not true, however, for the microparticles made without buffer – there was a very large disparity between the wet and dry readings.
Figure 7.4. Comparison of sizes achieved with laser diffraction between formulations (wet reading). Histograms are representative of 9 readings (for more details see Chapter 4, Section 4.4.2). The $D_{v,50}$ for the formulations according to analysis with a Malvern Mastersizer were a) without buffer - 1.80 $\mu$m; b) 0.1M NaHCO$_3$ - 4.14 $\mu$m; c) 1% Na$_2$HPO$_4$ – 2.69 $\mu$m.

7.4.2.2. Microparticles with Surfactant

There was difficulty in dispersing microparticles made with 1% m/v Na$_2$HPO$_4$ buffer and DPPC or lecithin, therefore data was not possible to collect. However, Figure 7.5 shows the size distributions of the microparticles made without buffer and with DPPC added; the results demonstrated that the addition of DPPC helped aerosolise microparticles to give an $X_{50}$ of 69 $\mu$m (compared to without DPPC when an $X_{50}$ 95 $\mu$m was obtained). Lecithin particles showed a similar distribution, with an $X_{50}$ of 73 $\mu$m (results not shown), the large reduction in the $X_{50}$ of these microparticles suggest that surfactant may prevent excessive particle interaction by acting as force controlling agents.
7.5. Discussion

Agglomeration (cohesive) and adhesion to other surfaces, mainly via Van der Waals forces, are a major problem in dry powder inhalation development and this has clearly been demonstrated in this work. Unfortunately, the ideal size of particles for deep lung delivery (1-5 μm) is also the size when particles have strong cohesive and adhesive forces (Frijlink and De Boer, 2004).

The pressures used to disperse the powders using the Sympatec HELOS system were chosen to be 1 and 2 bar, to avoid attrition of the formulations. Previous studies have used the Sympatec system to size their formulations as a dry powder, but have used pressures of 3 bar (Sethuraman and Hickey, 2002) and 4 bar (LiCalsi et al., 2001). For both studies, there was also a lack of agreement between the diameters of the particles analysed using the Sympatec system, and other measurements, such as SEM and laser diffraction using the Malvern Mastersizer (wet measurement). For this work, it may be useful to increase the
pressure at which the powders are dispersed (up to 5 bar), but there is the risk of attrition of the formulations, which could damage the plasmid DNA.

Surfactants were therefore used as an excipient in the formulations to try and reduce the inter-particulate forces. Leithin and DPPC were chosen because of their GRAS status and suitability to be used for pulmonary delivery. The addition of these into the microparticle formulations had varied results. When added to microparticles that had phosphate buffer included, there was extreme difficulty in dispersing them through the Sympatec system. It is unclear why this is so, but it may be that the salt and the surfactant interacted in such a way that the forces between them were too strong. An indication of this can be seen in the SEM pictures of these formulations where there is intense aggregation, such that, no discrete particles can be discerned. This is not reflected however, in the diameters obtained when measured in a wet state.

For the other formulations (no buffer + surfactant), the addition of lecithin or DPPC had the opposite effect, whereby the diameters measured using the Sympatec machine were much reduced compared to the formulations without surfactant. Other groups that have used surfactants in PLGA particles have shown that DPPC aids in dispersion of different spray dried microparticle formulations, with the inclusion of 60% DPPC having the best performance in terms of the respirable fraction obtained. They suggested that DPPC worked by helping droplet formation in the atomisation step of spray-drying as well as decreasing the particle surface energy and thereby powder cohesiveness (Bosquillon et al., 2001). Another aspect in which surfactants can aid in aerosolisation of microparticles, are their hydrophobic properties. Studies have shown that if DPPC is incorporated into a formulation, it dominates the surface of the particle (Evora et al., 1998; Bosquillon et al., 2004) and it has been suggested that hydrophobic materials are easier to de-agglomerate, due to smaller interactions, compared to hydrophilic materials (Hickey et al., 1994). Without further experiments to elucidate the surface properties of the different formulations, it would be difficult to explain with certainty, why some formulations disperse better than others.
An interesting outcome of note was for the formulation of no buffer + lecithin. The diameters measured using the Malvern Mastersizer were extremely unimodal and small compared to other formulations made so far. The reduction in particle diameter has been experienced before, and has been attributed to the increased stability of the primary emulsion provided by the lipophilic properties of lecithin (Alonso et al., 1993).

As mentioned above, Van der Waals forces are the dominant forces that cause particle cohesion, but others also contribute, such as electrostatic and capillary forces (liquid bridges between particles caused by increase in moisture). Methods used to reduce these effects include, increasing the particle size, increasing the roughness of the particle surface and drying of the powder (Hickey et al., 1994). From the TGA results obtained in Chapter 5, it is unlikely that excessive moisture content in the powder would be the main reason why there was difficulty in dispersing the particles. For the particles made with buffer especially, moisture content was less than 1%, but for particles made without buffer, the TGA results reported a net ‘gain’ in mass, although it is unclear what this means, it may be that these particles are too dry and hence there is a reduction in powder flow due to increase in electrostatic forces (Hickey et al., 1994). An additional favourable characteristic of particles made with buffer was the increase in surface roughness compared to those made without buffer; this too may have helped in the dispersibility of the particles. But the addition of surfactants complicates the picture, and it is difficult to say which forces dominate without extra experiments.

7.6. Conclusions and Future Work

In conclusion, all of the formulations presented in this chapter aerosolised poorly as a dry powder. The presence of residual water content may have led to increased inter-particulate interactions, which prevented efficient dispersion of the particles, but this is probably unlikely due to the efficient drying of the particles after manufacture. Particles made with carbonate and phosphate buffers had improved dispersion, which may be attributed to the surface roughness of the particles, which could reduce particle-particle contact. The addition of
surfactants led to mixed results; it improved the dispersibility of particles made without buffer, but it had the opposite effect for particles made with buffer.

For a clearer idea of the mechanisms in action with regards to the ease at which the particles disperse, future experiments could include:

Increasing the pressure at which the particles are dispersed at for measurement on the Sympatec system.

As the small diameters of the particles are inherent to large cohesive forces, addition of a bulking agent, such as lactose, may help to disperse the smaller particles from each other as well as from an inhaler and the filling the capsules. (LiCalsi et al., 1999).

Although laser diffraction is emerging as a new method to predict performance of aerosols, it would be useful to test the powders in a cascade impactor, be it an Anderson or Next Generation impactor.

An investigation of the surface energies/properties of the particles would be important to decipher what mechanisms are contributing to the flowability of the particles. Methods include X-ray photoelectron spectroscopy (XPS) for analysis of the chemical nature of the microparticle surface (Evora et al., 1998) and inverse phase gas chromatography (IGC) for surface energy measurements of the powders (Sethuraman and Hickey, 2002).

At the end of the day, because of the large disparity between the diameters obtained in the wet and dry measurement, it may be that these formulations are unsuitable for a DPI and another inhaler system, such as a nebuliser, would be more suitable, where the presence of a liquid would provide the conditions suitable to produce the appropriate sized range particles for pulmonary delivery.
Chapter 8

Use of Alternative Stabilisers in $w_1/o/w_2$

Emulsion Oligolactic Acids (OLA)
8.1. Introduction

As mentioned in the main introduction, to stabilize emulsions made using the w<sub>1</sub>/o/w<sub>2</sub> double emulsion solvent evaporation method, addition of an emulsifier is added into the aqueous phases. Various ionic and non-ionic surfactants are available that can be used, however non-ionic surfactants are preferred because of their lower toxicity and lower interaction with ionic compounds (Florence and Whitehill, 1982).

PVA is widely used as the emulsifier of choice for many (Alpar and Almeida, 1994; Coombes et al., 1998; Yang et al., 2001; Jeong et al., 2003), and although PVA is generally regarded as safe (Chapter 5, Section 5.4.6), alternative emulsifiers for the production of microparticles were sought, because of potential drawbacks of residual PVA on the particle surface including reduction in cellular uptake (Sahoo et al., 2002) and lower gene transfection (Prabha and Labhasetwar, 2004).

Oligolactic acids (OLA) are short-chain (3-15 repeat units) versions of the polylactide polymer and have been used in industry as an excipient in MDIs to aid the suspension of drugs in the propellant HFA (Stefely, 2002). Their biocompatibility and non-toxicity to the lung in various animal studies have been established (Leach et al., 2000), therefore there was an opportunity here to develop a new formulation which would utilise an alternative stabiliser to PVA, and to also enhance the dispersibility of the microparticles as a dry powder. The first step though, was to see if the replacement of PVA with OLA would produce microparticles.

8.2. Aims

Observe the effect of using OLA as an alternative to PVA as a primary emulsifier in w<sub>1</sub>/o/w<sub>2</sub> double emulsion.
8.3. Materials and Methods

Two types of OLAs were generously provided by 3M, Loughborough. No physicochemical characteristics are given due to confidentiality restrictions. The two different OLAs are therefore designated as OLA-1 and OLA-2.

As OLAs are not soluble in water (Stefely, 2002), OLA-1 or OLA-2 was added into the organic phase of the \( w_1/o/w_2 \) double emulsion. Two investigations were carried out;

1) addition of OLA into the formulation as an excipient – with the intention of observing the effects of adding it on dispersibility of the microparticles as a dry powder.

2) replacement of PVA in the internal aqueous phase altogether, with the additional parameter of reducing the concentration of PVA in the external water phase.

Details of the formulation parameters are outlined in Tables 8.1 and 8.2. As there was a limited amount of OLA available, only two batches of particles were made for each formulation.

Table 8.1. Formulation parameters of microparticles made with OLA as an additional excipient in the microparticles. OLA was added at 5% m/m polymer mass, so that a comparison could be made to those made in Chapter 7, where DPPC or lecithin were also added at 5% m/m.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>OLA 5% m/m</th>
<th>Buffer</th>
<th>DNA % m/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OLA-1</td>
<td>None</td>
<td>0.67</td>
</tr>
<tr>
<td>2</td>
<td>OLA-1</td>
<td>1% m/v Na(_2)HPO(_4)</td>
<td>0.67</td>
</tr>
<tr>
<td>3</td>
<td>OLA-2</td>
<td>None</td>
<td>0.67</td>
</tr>
<tr>
<td>4</td>
<td>OLA-2</td>
<td>1% m/v Na(_2)HPO(_4)</td>
<td>0.67</td>
</tr>
</tbody>
</table>
Table 8.2. Formulation parameters of microparticles made with OLA as a replacement for PVA in the internal aqueous phase (\(w_i\)), with a concomitant reduction in the concentration of PVA in the external aqueous phase (\(w_z\)). OLA was added at 10% m/m of polymer mass, so that a comparison could be made to those made in Chapter 4.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Buffer</th>
<th>OLA % m/m</th>
<th>External PVA % m/v</th>
<th>DNA % m/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>10</td>
<td>2.5</td>
<td>0.67</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>10</td>
<td>0.5</td>
<td>0.67</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>10</td>
<td>0.25</td>
<td>0.67</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>10</td>
<td>0</td>
<td>0.67</td>
</tr>
<tr>
<td>5</td>
<td>1% m/v Na(_2)HPO(_4)</td>
<td>10</td>
<td>2.5</td>
<td>0.67</td>
</tr>
<tr>
<td>6</td>
<td>1% m/v Na(_2)HPO(_4)</td>
<td>10</td>
<td>0.5</td>
<td>0.67</td>
</tr>
<tr>
<td>7</td>
<td>1% m/v Na(_2)HPO(_4)</td>
<td>10</td>
<td>0.25</td>
<td>0.67</td>
</tr>
<tr>
<td>8</td>
<td>1% m/v Na(_2)HPO(_4)</td>
<td>10</td>
<td>0</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Microparticles were then prepared and characterised as outlined in Chapter 2.

8.4. Results

8.4.1. OLA as an Excipient

All formulations formed microparticles with similar characteristics to those made with PVA in the internal phase (Figure 8.1) with addition of buffer still leading to more porous particles compared to those without buffer. The encapsulation efficiencies (Table 8.3) are similar to those obtained previously (no buffer - 32.77 ± 12.96%; 1% Na\(_2\)HPO\(_4\) - 45.65 ± 5.27%, Chapter 4). Addition of OLA did not help in preserving the supercoiled conformation for particles made without buffer (Table 8.3), but unfortunately the amount of supercoiled DNA preserved is not very good for particles made with buffer with a marked reduction to 23% compared to 46% achieved previously.
Figure 8.1. SEM images of formulations with added 5% m/m OLA as an excipient. Image A is of particles made without buffer, B is of particles made with 1% Na$_2$HPO$_4$. Addition of OLA-1 or OLA-2 showed no difference in morphologies.

Table 8.3. Characteristics of particles made with OLA as an additional excipient. For the particle yield, this is the mean of two batches made, with the range of these two shown. For the encapsulation efficiencies and amount of supercoiled content in the DNA, mean ± s.d total of 4 readings – two from each batch of particles made.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Yield %</th>
<th>Encapsulation Efficiency %</th>
<th>% Supercoiled</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63.04 ± 3.08</td>
<td>10.96 ± 2.19</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>66.04 ± 8.45</td>
<td>46.02 ± 7.31</td>
<td>23.06 ± 4.13</td>
</tr>
<tr>
<td>3</td>
<td>74.42 ± 1.40</td>
<td>12.07 ± 2.02</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>74.38 ± 2.33</td>
<td>40.74 ± 6.75</td>
<td>23.44 ± 4.56</td>
</tr>
</tbody>
</table>

The diameters of the microparticles also demonstrated similar characteristics to previous results, with the mean volume diameter being in the range of 1.96 μm – 2.74 μm. Although, it seemed that microparticles that had OLA-2 added, were slightly smaller than those that had OLA-1 added (Table 8.4).

Table 8.4. Size distributions of formulations made with OLA as an additional excipient. Each batch of particles was measured 3 times; mean ± s.d. total of 6 readings.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MVD μm</th>
<th>D[v,10] μm</th>
<th>D[v,50] μm</th>
<th>D[v,90] μm</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.02 ± 0.10</td>
<td>0.79 ± 0.02</td>
<td>1.70 ± 0.06</td>
<td>3.69 ± 0.22</td>
<td>1.71 ± 0.10</td>
</tr>
<tr>
<td>2</td>
<td>2.74 ± 0.20</td>
<td>0.89 ± 0.01</td>
<td>2.24 ± 0.16</td>
<td>5.28 ± 0.44</td>
<td>1.96 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>1.96 ± 0.12</td>
<td>0.74 ± 0.04</td>
<td>1.56 ± 0.15</td>
<td>3.66 ± 0.21</td>
<td>1.88 ± 0.10</td>
</tr>
<tr>
<td>4</td>
<td>2.67 ± 0.09</td>
<td>0.93 ± 0.03</td>
<td>2.25 ± 0.02</td>
<td>5.00 ± 0.35</td>
<td>1.82 ± 0.20</td>
</tr>
</tbody>
</table>
### 8.4.2. OLA-2 Replacing PVA in $W_1$ and Reduction of PVA in $W_2$

OLA-2 was used in order to assess the suitability of it as a replacement for PVA. All formulations produced particles, with the exception of those that had no PVA in the external phase, where the polymer precipitated out into an amorphous mass.

The diameters of the formulations were measured using PCS because from observing the SEM images (Figure 8.2), most formulations looked to be less than 1 μm in diameter and therefore, for a meaningful comparison, it was deemed appropriate to measure the diameters using PCS. It can be seen that the SEM images and the diameters measured by PCS do not correlate (Table 8.5). It is probable that for the especially small particles, they were not dispersed enough prior to reading because of intense aggregation, but it seemed that there was a general trend of the diameters of the microparticles getting smaller as the amount of PVA in the external phase was reduced according to the SEM images.

![Figure 8.2. SEM images of formulations made with OLA as a replacement of PVA. PVA in the $w_1$ phase was replaced with OLA-2 at 10% m/m of polymer and the amount of PVA in the $w_2$ phase was gradually decreased from 2.5% m/v to 0.25% m/v. Top 3 images are microparticles made without buffer (A); bottom 3 images are microparticles made with 1% m/v Na$_2$HPO$_4$ (B).](image-url)
Table 8.5. Characteristics of particles made with OLA as a replacement of PVA in internal aqueous phase. For the particle yield, this is the mean of two batches made. For the Z-Average, size was measured once only (polydispersity in parenthesis). For the encapsulation efficiencies and amount of supercoiled content in the DNA, mean ± s.d total of 4 readings – two from each batch of particles made.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Yield %</th>
<th>Z-Average (nm)</th>
<th>Encapsulation Efficiency %</th>
<th>Supercoiled</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69.69 ± 0.83</td>
<td>785.10 (0.30)</td>
<td>15.96 ± 5.16</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>65.43 ± 1.06</td>
<td>1571.20 (0.509)</td>
<td>11.37 ± 3.97</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>64.43 ± 1.47</td>
<td>1932.55 (0.387)</td>
<td>18.17 ± 6.65</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>No particles formed</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>81.19 ± 0.52</td>
<td>1017.15 (0.368)</td>
<td>24.24 ± 3.12</td>
<td>36.14 ± 18.83</td>
</tr>
<tr>
<td>6</td>
<td>67.09 ± 2.73</td>
<td>787.45 (0.480)</td>
<td>40.03 ± 3.09</td>
<td>8.37 ± 3.50</td>
</tr>
<tr>
<td>7</td>
<td>77.70 ± 1.26</td>
<td>2564.50 (0.644)</td>
<td>46.35 ± 4.23</td>
<td>5.90 ± 0.60</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>No particles formed</td>
<td></td>
</tr>
</tbody>
</table>

For particles made without buffer, replacement of PVA with OLA-2 did not offer any additional protection against DNA change in conformation or encapsulation efficiency; the efficiencies were similar regardless of how much PVA was in the external phase. For particles made with buffer, a different picture was seen. OLA-2 did not improve amount of supercoiled content, and in fact, seemed to decrease the presence of supercoiled form for all formulations. There was an interesting dynamic occurring, in that as the amount of PVA in the external phase decreased, the amount of supercoiled decreased, but with an increase in the encapsulation efficiency. This is similar to the result obtained when the pH of the buffer was altered from pH 5-12 (Chapter 4, Section 4.4.3).

8.5. Discussion

OLAs have not been used as an alternative surfactant in microparticle manufacture before. It seemed that the addition of 5% m/m of OLA into the formulation reduced the stability of the emulsions, because from the SEM images, it can be seen that for particles without buffer in particular, there are many collapsed and irregular-shaped particles. This is in contrast to particles made with 10% m/m OLA, where the particles demonstrated smooth surfaces, similar to that of particles that have 10% m/v PVA in the internal aqueous phase,
indicating that OLA could be a viable substitute for PVA as an alternative surfactant.

These results have also shown the importance of the level of PVA in the external aqueous phase. Diameters seemed smaller as the concentration of PVA in external phase was reduced, according to the SEM images. This is opposite to a majority of observations where an increase in PVA concentration led to a decrease in particle diameter (Jeffery et al., 1993; Kempen et al., 2004; Maia et al., 2004). Few have had the same result as presented here (Vandervoort and Ludwig, 2001). In most cases it seemed that the concentration of PVA in the external phase was a balancing act – too little and there was not enough to stabilise the emulsions leading to a coalescence of droplets; too much and the solution was too viscous and therefore harder to emulsify (Yang et al., 2001; Maia et al., 2004).

In this study, it seemed that the concentration of 0.5% m/v PVA in the external phase was the limit of stability for particles made without buffer. Anything below that, then particles grew larger or more unstable, and this was seen in the SEM images of particles which had 0.25% m/v PVA in the external phase, where very large particles were mixed in together with particles of less than 1 μm in diameter. This was not the case for particles made with phosphate buffer, where the distribution of particles was still quite homogeneous. The presence of salt may have offered additional stability to the emulsion droplets in this case (for more information see Chapter 4, Section 4.5). The presence of stabiliser in the external phase is important, as without it, the polymer precipitated out into an amorphous mass. This is similar to a previous study where a critical concentration of 1.4 mM or 2.5% m/v PVA was needed before PVA acquired its surfactant characteristics. For concentrations below 2.5% m/v, the PVA solution contains single molecules of PVA and this can lead to unstable emulsions (Zambaux et al., 1998).

As well as size, DNA encapsulation efficiency was also an important parameter to consider. Unfortunately, the use of OLA did not improve encapsulation efficiency or the amount of supercoiled content for both sets of formulations. An
interesting result was obtained for the formulations where the amount of PVA in the \( w_2 \) phase was reduced from 2.5% m/v to 0.25% m/v. The trend of increasing encapsulation efficiency, but decrease in supercoiled content as the amount of PVA was reduced, was similar to the trend seen where the \( \text{pH} \) of the buffer solution was varied from \( \text{pH} \) 5-12 (see Chapter 4, Section 4.4.3). It is unclear at this stage what the mechanisms are, it may be due to the change in viscosity, \( \text{pH} \) or the dynamics at the oil/water interface, without further experiments, it would be difficult to say with certainty why this trend occurs.

8.6. Conclusions and Future Work

OLAs have potential to replace PVA as an alternative stabiliser in \( w_1/o/w_2 \) double emulsions. Unfortunately, their role in the reduction in supercoiled DNA conformation may preclude them for further work, unless another DoE is used to optimize the formulation. However, their potential for increasing stability of formulations in MDIs has been proven (Stefely et al., 2000), and so it is an interesting step forward in development for the production of a DNA vaccine for pulmonary delivery.

Extensive further work would be required to fully examine the properties and potential of using OLA as a replacement surfactant in double emulsions. In particular, it would also be interesting to see whether these microparticles disperse well in a dry powder state.
Chapter 9

Addition of Spermine as a DNA Condenser
9.1. Introduction

The addition of a cation moiety to enhance loading of DNA on or into microparticles is not a new phenomenon. Cations work by neutralising the negative charge of the phosphate backbone of the DNA molecule, thereby condensing the DNA into a more compact form, which in turn has aided in increasing the encapsulation efficiency. Cations used so far include lipids such as cetyltrimethylammonium bromide (CTAB) (Denis-Mize et al., 2000) and 1,2-dioleoyxy-3-trimethylammonium propane (DOTAP) (Seville et al., 2002) and polymers such as polyethylenimine (PEI) (Oster et al., 2005) and poly-L-lysine (PLL) (Gebrekidan et al., 2000).

Unfortunately, their success has been limited because of their cytotoxicity (Filion and Phillips, 1998; Lv et al., 2006). Alternative cationic molecules were therefore sought to overcome this issue. A positive charged molecule was needed to help increase the entrapment of DNA into PLGA particles, because at the current loading, formulations are implausible to use for in vivo studies. The mass of particles needed for a dose was too high for the volume of liquid used for pulmonary delivery.

Spermine is a linear aliphatic polyamine that is widely distributed in the body and one of its natural functions is to condense DNA in the nuclei (Tabor et al., 1961; Blagbrough et al., 2003). As it is an endogenous source in the body it was considered that it would be more biocompatible than other positively charged molecules such as PEI. Spermine has 4 protanable amine groups and these are all protonated at physiological pH, which should make its condensing capacity for DNA quite high (Morgan, 1998).

9.2. Aims

To increase encapsulation efficiency of plasmid DNA into PLGA microparticles by prior complexation with spermine.
9.3. Materials and Methods

Spermine tetrahydrochloride and poly(aspartic acid) were purchased from Sigma (Poole, UK). For all other materials, please see Chapter 2.

9.3.1. Calculation of Nitrogen:Phosphate ratio (N:P)

Nitrogen (N) refers to the amine group of spermine. Phosphate (P) refers to the phosphate groups on the DNA plasmid backbone. The molecular weight of one DNA base is 330 Daltons and has 1 negatively charged phosphate (Stephenson, 2003). The molecular weight of spermine is 202.35 Daltons and has 4 positively charged nitrogen’s (Sigma, 2006).

N:P ratio of 1:1 would therefore be calculated as \( \frac{202.35 - 4}{330} = 0.153 \)
N:P 1:1 = \( \frac{50.6}{330} = 0.153 \)
N:P 5:1 = \( \frac{50.6 \times 5}{330} = 0.766 \)

If the amount of DNA = 10µg, then amount of spermine to be added would be \( 10 \times 0.76 = 7.6 \) µg.

9.3.2. Condensation Ability of Spermine

Initial experiments were carried out to observe the ability of spermine to condense plasmid DNA in various buffer solutions. DNA was kept constant at 5 µg and the volume of water or buffer (0.1M NaHCO\(_3\) or 1% m/v Na\(_2\)HPO\(_4\)) was kept constant at 12.5 µl (this was the amount of DNA/buffer normally used in a formulation, but scaled down, i.e. in a formulation, 200 µg DNA is used with a volume of 500 µl for the internal aqueous phase). A stock spermine solution was made at 100 mg/ml in sterile water and the volume of spermine added was calculated according to the following N:P ratios: 0, 50, 100, 200, 500 and 1000:1

Samples were briefly centrifuged to collect the solutions into the bottom of the tube and incubated at room temperature for 4 hours before being analysed using agarose gel electrophoresis (Chapter 2, Section 2.4.2) and the Picogreen® assay (Chapter 2, Section 2.4.4).
9.3.3. Formulation parameters

N:P ratio 100:1 was decided upon for complexation of plasmid DNA with spermine into PLGA particles. The formulation parameters were the same as those in Chapter 4, Section 4.3.2.1, but with the additional spermine amounts outlined in Table 9.1.

Table 9.1. Formulation parameters for microparticles made with additional spermine.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>PLGA (mg)</th>
<th>DNA* (µg)</th>
<th>Spermine^ (µg)</th>
<th>Internal Phase</th>
<th>External Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300</td>
<td>None</td>
<td>3066</td>
<td>dWater</td>
<td>dWater</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>None</td>
<td>3066</td>
<td>0.1M NaHCO₃</td>
<td>0.1M NaHCO₃</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>None</td>
<td>3066</td>
<td>1% m/v Na₂HPO₄</td>
<td>1% m/v Na₂HPO₄</td>
</tr>
<tr>
<td>4</td>
<td>300</td>
<td>200</td>
<td>3066</td>
<td>dWater</td>
<td>dWater</td>
</tr>
<tr>
<td>5</td>
<td>300</td>
<td>200</td>
<td>3066</td>
<td>0.1M NaHCO₃</td>
<td>0.1M NaHCO₃</td>
</tr>
<tr>
<td>6</td>
<td>300</td>
<td>200</td>
<td>3066</td>
<td>1% m/v Na₂HPO₄</td>
<td>1% m/v Na₂HPO₄</td>
</tr>
</tbody>
</table>

* 200 µl DNA of stock 1 mg/ml gWiz Luciferase DNA in TE buffer pH 8.0.
^ 30.66 µl spermine of stock 100 mg/ml solution made up in sterile distilled water.

DNA and spermine were incubated in the 10% PVA solution overnight at 4°C. All solutions were homogenized on ice as outlined in Chapter 2 (Section 2.2). “Blank” particles consisted of spermine, but no DNA, where the volume of DNA was replaced with TE buffer pH 8.0.

9.3.4. Size and Zeta Potential

Measurement of diameters and charge of the microparticles were carried out using the same methods as in Chapter 2, Section 2.3.1 and 2.3.3 respectively.

9.3.5. Extraction of DNA from microparticles

Difficulties were encountered when trying to extract out the DNA entrapped into the PLGA particles. For DNA that has been complexed with positively charged moieties, DNA is released by addition of competing anions – that is these anions will preferentially bind to the cationic polymer and release the DNA, which can then be quantified. Various anions have been used, including heparin sulphate (Tsai et al., 2002), sodium dodecyl sulphate (SDS) (Dunne et al., 2003) and poly(aspartic acid) (Howard et al., 2004). The usual process is that the aqueous
extract is treated with a solution of the competing anion. When this was carried out in this study, no DNA could be detected. Through a process of trial and error, it was discovered that DNA can only be extracted if the competing anion was also present during the extraction process, that is, DNA could not be detected when the aqueous extract was treated after the extraction process. An outline of the process is below.

10 mg of particles were weighed out into a 1.5 ml centrifuge tube. 200 µl of TE buffer was added together with 20 µl of poly(aspartic acid) solution (5 mg/ml) and the mixture was vortexed briefly to re-suspend the particles. 500 µl chloroform was added and again the sample was vortexed. The suspension was shaken horizontally in a 37°C incubator for 90 min. After 90 min, the mixture was centrifuged at 14,000 rpm for 10 min. The aqueous layer was taken off and put into a fresh tube. The aqueous layer was then analysed via agarose gel electrophoresis (Chapter 2, Section 2.4.2) and the Picogreen® (Chapter 2, Section 2.4.4).

9.3.6. Adsorption Assay

5 mg of spermine microparticles with or without encapsulated DNA was weighed out into a centrifuge tube. 100 µg of DNA was added to the particles (100 µl of 1 mg/ml solution) and the volume of solution was brought up to a total of 500 µl with TE buffer (pH 8.0). A control where only 500 µl of TE buffer was added to the particles was also carried out in parallel. Adsorption was also performed with 0.9% m/v NaCl (physiological concentration) replacing TE buffer to observe if the use of different salts can enhance adsorption of DNA onto the particles.

The microparticles were re-suspended by flicking the tubes gently and they were then placed on a platform rocker at room temperature for 4 hours. The particles were then centrifuged at 7,500 rpm for 5 min and the supernatant was removed for analysis. Adsorption was calculated indirectly by the amount of DNA remaining in the supernatant.
9.3.7. Release Study

Release study was carried out as per Chapter 5, Section 5.3.6. An initial study demonstrated that PBS was sufficient to release the DNA from the spermine, therefore no additional poly (aspartic acid) was needed in the release medium.

9.3.8. Cell Culture Studies

Transfection studies were carried out with A549 cells according to the same methods as outlined in Chapter 6, Section 6.3.3.

9.4. Results

9.4.1. Condensation Ability of Spermine

It can be seen that addition of spermine does retard the migration of DNA slightly, but it does not completely exclude the ethidium bromide dye (Figure 9.1). This is in contrast to other cationic polymers such as PEI, where there is no visible migration of DNA when complexed with PEI (results not shown).

![Figure 9.1. Agarose gel electrophoresis image of the migration of plasmid DNA after incubation with various amounts of spermine.](image)

The same N:P ratios were used in the Picogreen® assay to determine if addition of spermine excludes the dye. Samples were diluted so that they were within the detection range of the assay. Figure 9.2 demonstrated that spermine completely quenches the Picogreen® dye, indicating the differences in which ethidium bromide and Picogreen® bind to DNA. The presence of spermine does not
preclude the binding of ethidium bromide, which is why DNA is still visible on an agarose gel; but this is not the case for Picogreen® dye, where the presence of spermine excludes the dye, and hence, no fluorescence is detected.

![Figure 9.2. Effect of spermine on the interaction between DNA and Picogreen® dye. The addition of the spermine prevents the Picogreen® dye from binding to the DNA and therefore, no fluorescence is emitted. No buffer (♦) 0.1M NaHCO3 (■) 1% Na2HPO4 (▲).](image)

### 9.4.2. Microparticle Characterisation

Addition of spermine did not affect the yield of particles, nor did it affect the zeta potential of the particles, all of them being negative in charge (Table 9.2). Microparticles made without spermine were also measured at the same time and these too showed similar zeta potentials to those made with spermine (results not shown).

### Table 9.2. Characteristics of particles made with spermine.

For the particle yield, this is the mean of two batches made. For the encapsulation efficiencies mean ± s.d total of 4 readings – two from each batch of particles made. For the zeta potential mean ± s.d. of 3 readings.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle Yield % m/m</th>
<th>Encapsulation Efficiency %</th>
<th>Zeta Potential mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82.52</td>
<td>n/a</td>
<td>-35.8 ± 0.33</td>
</tr>
<tr>
<td>2</td>
<td>87.22</td>
<td>n/a</td>
<td>-34.7 ± 0.38</td>
</tr>
<tr>
<td>3</td>
<td>83.71</td>
<td>n/a</td>
<td>-38.2 ± 0.87</td>
</tr>
<tr>
<td>4</td>
<td>88.07</td>
<td>58.99 ± 15.23</td>
<td>-36.1 ± 0.33</td>
</tr>
<tr>
<td>5</td>
<td>87.73</td>
<td>36.36 ± 5.52</td>
<td>-35.1 ± 0.33</td>
</tr>
<tr>
<td>6</td>
<td>87.78</td>
<td>41.80 ± 3.44</td>
<td>-37.7 ± 0.39</td>
</tr>
</tbody>
</table>
The loading efficiencies were slightly lower for particles made with buffer compared to efficiencies obtained for particles made without spermine (Chapter 4, Section 4.4.2); but for particles made without buffer, there was a marked increase in the encapsulation efficiency (Table 9.2). After extraction from the microparticles and decomplexation with poly (aspartic acid), the DNA for all formulations was mostly in the open-circular or supercoiled form, with different degrees of both (Figure 9.3). For microparticles made without buffer, the addition of spermine helped preserve the supercoiled conformation slightly. There was also presence of an extra band above the open-circular DNA, which may be a dimer of plasmid DNA or some other fragments of the DNA. Further investigations would be needed for a definitive answer.

![Agarose image of DNA extracted from particles](image)

**Table 9.2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Supercoiled</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Input</td>
<td>88.16 ± 6.42</td>
</tr>
<tr>
<td>B No Buffer</td>
<td>7.47 ± 1.40</td>
</tr>
<tr>
<td>C NaHCO₃</td>
<td>58.48 ± 1.90</td>
</tr>
<tr>
<td>D Na₂HPO₄</td>
<td>44.64 ± 1.34</td>
</tr>
</tbody>
</table>

**Figure 9.3. Agarose image of DNA extracted from particles.** For the amount of supercoiled content in the DNA, mean ± s.d total of 4 readings – two from each batch of particles made.

All formulations had a larger diameter than microparticles made without spermine (Table 9.3), but it is unclear as to why the particles made with carbonate buffer exhibit such large size distributions. SEM images (Figure 9.4) indicate that the sizes measured by laser diffraction do not correlate with the images and it may be due to aggregation. Aggregation does not occur with particles made without buffer or phosphate buffer; or alternatively, particles made with distilled water or with phosphate buffer disperse more efficiently in water than particles made in carbonate buffer. All particles exhibited porous morphology (Figure 9.4).
Table 9.3. Summary of Particle characteristics. Mean ± s.d; n=3

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MVD μm</th>
<th>D[v10] μm</th>
<th>D[v50] μm</th>
<th>D[v90]</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.92 ± 0.04</td>
<td>1.39 ± 0.03</td>
<td>2.71 ± 0.04</td>
<td>4.73 ± 0.05</td>
<td>1.24 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>11.16 ± 2.00</td>
<td>2.42 ± 0.09</td>
<td>5.85 ± 0.72</td>
<td>27.25 ± 5.42</td>
<td>4.21 ± 0.98</td>
</tr>
<tr>
<td>3</td>
<td>4.10 ± 0.07</td>
<td>2.08 ± 0.08</td>
<td>3.84 ± 0.03</td>
<td>6.45 ± 0.11</td>
<td>1.14 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>3.55 ± 0.02</td>
<td>1.61 ± 0.04</td>
<td>3.26 ± 0.02</td>
<td>5.87 ± 0.01</td>
<td>1.30 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>10.95 ± 2.36</td>
<td>2.37 ± 0.09</td>
<td>5.41 ± 0.25</td>
<td>21.31 ± 3.56</td>
<td>3.46 ± 0.84</td>
</tr>
<tr>
<td>6</td>
<td>4.69 ± 0.03</td>
<td>2.58 ± 0.03</td>
<td>4.41 ± 0.03</td>
<td>7.22 ± 0.05</td>
<td>1.05 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 9.4. SEM images of PLGA particles with encapsulated DNA complexed with spermine. A) no buffer; B) 0.1M NaHCO₃; C) 1% m/v Na₂HPO₄. Note the porous morphology of all the formulations. Bar = 10 μm.

9.4.3. Adsorption Assay

Unfortunately, there was no DNA adsorption observed with these particles, in either TE buffer or 0.9% m/v NaCl solution. It may be that the spermine molecule was too small to exert an effect to adsorb DNA and this was corroborated with the negative zeta potentials observed.

9.4.4. Release Study

For the release of DNA from the microparticles, there were very different curves compared to those without spermine (Chapter 5, Section 5.4.7). For those without spermine, there is a burst release, followed by a steady release of small amounts of DNA over the 28 days of the study. For those with added spermine, the DNA is rapidly released within 72 h, but without any additional release of DNA thereafter (Figure 9.5). Rapid release is probably due to the highly porous nature of the microparticles, although, like previous results, particles made with phosphate buffer did not completely release the entire DNA within 14 days and the DNA remained in the pellet. For particles made without buffer, the small
amount of supercoiled DNA was lost during the release; for particles made with buffer, the supercoiled conformation was maintained. This was true for even the DNA that was left in the pellet (results not shown).

![Figure 9.5](image)

**Figure 9.5.** Cumulative percentage and total amount of DNA released from each formulation over a period of 14 days for microparticles with added spermine. No buffer (♦) 0.1M NaHCO₃ (■) 1% Na₂HPO₄ (▲). Mean ± s.d; n = 3.

### 9.4.5. Transfection of A549 Cells

Disappointingly, the addition of spermine did not enhance gene transfection of microparticles in A549 cells. For all formulations, the level of expression was barely above background over the 7 days (Figure 9.6). The large change on day 3 of RLU/mg protein is probably due to a change in the reagent used compared to the other days, but it can be seen that the trend is the same for the different treatments on the same day. On a positive note, it seemed that the particles still maintained their non-toxic characteristic, with the cells recovering well over the time period of the experiment (Figure 9.7).
Figure 9.6. Transfection efficiency of microparticles made with spermine in A549 cells. (1) No buffer + DNA (2) 0.1M NaHCO₃ + DNA (3) 1% m/v Na₂HPO₄ + DNA (4) No buffer w/o DNA (5) 0.1M NaHCO₃ w/o DNA (6) 1% m/v Na₂HPO₄ w/o DNA (7) No Treatment.

To ensure that the DNA was still viable, the DNA was extracted out of the particles and complexed with a commercial transfection reagent (Novagen GeneJuice®). It can be seen that the DNA was still viable (Figure 9.8) but again with varying degrees of transfection efficiency.
9.5. Discussion

The initial complexation studies yielded interesting results regarding the binding modes of spermine, ethidium bromide and Picogreen® to DNA. It is clear that different mechanisms are involved. The mode of binding of Picogreen® reagent to DNA is unclear, although it is suggested that it may bind via intercalation (Singer et al., 1997). Ethidium bromide binds DNA in two distinct modes; 1) insertion of its phenanthridinium ring between the bases of the DNA (intercalation) and 2) binding externally to the DNA via the phosphate backbone (Bugs and Cornelio, 2002). There have been many studies for elucidating the binding mode of spermine with DNA, and to date, there has been no conclusive model that has been accepted. It is clear that the form of DNA used for the investigations plays an important role in the different results obtained and this complicates interpretation (Burton et al., 1981; Deng et al., 2000; Keniry, 2003).

As well as the binding specifics of spermine to DNA, there have also been many studies regarding the condensation of DNA in the presence of multivalent and monovalent counterions, such as spermine and NaCl (Burton et al., 1981; Raspaud et al., 1998; Raspaud et al., 1999; Burak et al., 2003). In general, it seems that spermine condenses DNA through electrostatic interactions with the phosphates of the DNA backbone via the “Ion-bridging” model (Raspaud et al., 1998; Raspaud et al., 1999; Burak et al., 2003).
and there is a fine balance between condensation and precipitation of the DNA molecule out in solution, with a complex interplay between the concentrations of DNA, polyamine and monovalent cations.

In agreement with a previous study, spermine is unable to retard DNA mobility through an agarose gel (Kim et al., 2005), indicating that even with 4 nitrogen’s available, spermine is not a strong condenser of DNA. This was reflected in the negative zeta potentials obtained for microparticles with spermine added in. For most cationic polymers, the cross-over from negative to positive zeta potential occurs at an N:P ratio of 1:1, in contrast, small, multivalent cations like spermine, do not exhibit strongly positive zeta potentials, even at high excess of cation (Tang and Szoka, 1997). Spermine associates with DNA in a reversible manner, which is why positive zeta potentials are never achieved, and aggregation generally results under physiological buffer conditions (Tang and Szoka, 1997).

The morphologies of the particles were different to previous formulations, especially for particles made without buffer. It may be because spermine was in its salt form, so the presence of salt may have led to the formation of pores (see Chapter 4, Section 4.5. for a more detailed explanation). The extra pores could have led to the more rapid escape of DNA from the microparticles (with the exception of particles made with phosphate buffer, where complete release of the DNA was not achieved).

The encapsulation efficiency of DNA was lower in particles made with buffer; it may be that the presence of salt in the aqueous phase competed with spermine for binding to the DNA and spermine therefore was not in high enough concentrations to exert any extra effect; in contrast, it may have even precipitated the DNA out of solution.

Because essentially there was no change in the encapsulation efficiency or amount of supercoiled form of DNA in these microparticles, it was difficult to explain why there was no transfection seen with the A549 cells. It may be that the DNA was unavailable for the cell to transcribe, but it is unlikely. The intense
burst release of DNA from the microparticles may be the reason why there is no transfection seen. It could be that the DNA was released out into the cell culture medium before the microparticles were taken up into the cells. A similar result was seen when a complex of DNA with spermine as an emulsion was unable to enhance gene expression in COS-1 cells (Kim et al., 2005).

9.6. Conclusions and Future Work

The use of spermine to condense plasmid DNA and therefore increase encapsulation efficiency has had varied success. It increased the loading in particles made without buffer, and also increased the amount of supercoiled conformation to a small degree. Unfortunately, the microparticles still perform poorly in an in vitro assay, with low gene transfection efficiencies in the cell line studied for all formulations. However, the non-toxic nature of PLGA particles was maintained.

It may be that a larger cationic molecule would be needed to significantly increase the loading and therefore enhance transfection efficiency in cells, such as protamine sulphate and poly-l-lysine, which, in previous studies, performed better in comparison to spermine (Kim et al., 2005).
Chapter 10

*Overall Conclusions and Further Studies*
A summary of the conclusions obtained for each chapter is given below. At the end, a discussion of the future work needed to develop microparticulate DNA vaccines for pulmonary delivery is also presented.

10.1. Fractional Factorial Design

The use of experimental design allowed a rapid and economical process for optimizing a microparticulate formulation with suitable diameters for delivery to the lung (1-5 μm), and with reasonable encapsulation efficiencies of plasmid DNA (66.93 ± 1.56 % m/m of original DNA loading). It also allowed assessment of the reproducibility of the method used to make the particles, which was confirmed to be repeatable between the batches made. Certain relationships between the factors and responses were elucidated and the results of the experimental design also correlated well with results from previous publications where one factor at a time was changed and results then observed. For example, the inner aqueous phase had a large effect on the diameters of the particles, with a larger inner aqueous volume leading to larger diameters. The results also highlighted the importance of interpreting ‘statistically significant’ findings according to the knowledge of the techniques used to make the microparticles as well as the techniques used to analyse the responses. For example, the program interpreted statistical significance for particles that had DNA incorporated into them with a higher zeta potential value. This was considered to be an artefact of the equipment used to measure the zeta potential, rather than a significant result.

The software used for experimental design, also allowed the prediction of a formulation that was optimal for the parameters required. In this case, it turned out to be the centre point of the design, and this formulation was therefore chosen to be used as a platform for further experiments.
10.2. Use of Buffers to Protect Plasmid DNA Conformation

DofE was more concerned with obtaining high encapsulation efficiencies of DNA and suitable diameters of the microparticles. The conformation of the entrapped DNA was not looked at. This issue was addressed in this thesis as it was important to maintain the supercoiled conformation of the plasmid DNA as much as possible. This was for biological efficiency, as supercoiled DNA was considered the most efficient form for gene transfection into cells. The protective effects of adding salt (either 0.1M NaHCO₃ or 1% m/v Na₂HPO₄) to the aqueous phases of the formulations was evaluated. It is thought that this is the first time that buffers have been used in this way for formulation of microparticles entrapping DNA, especially for 1% m/v Na₂HPO₄. Addition of 1% m/v Na₂HPO₄ conferred more desirable characteristics in terms of the amount of DNA entrapped and the diameters of the microparticles compared with those without buffer and 0.1M NaHCO₃. The additional porosity of the microparticles may also aid in the delivery of these microparticles into the lung owing to their purported lesser density.

Detailed studies also demonstrated that DNA was damaged in the presence of polymer and in the primary emulsion step, indicating that the viscosity of the solutions were very important. Also the balance of salt and pH in the aqueous phases were also important, with a clear demarcation between what was advantageous and what was not.

10.3. Physicochemical Characterization Studies

Analytical experiments were carried out to determine the stability of the microparticles in terms of predicted storage conditions and shelf life, as well as ensuring that the levels of residual solvent and stabilizer were below those set by the regulatory authorities. These experiments demonstrated the difficulties encountered when trying to formulate microparticles that fulfilled all requirements for biological efficacy and stability. So far, microparticles made with 1% Na₂HPO₄ were optimal in terms of suitable diameter for pulmonary delivery and encapsulation efficiency of DNA. But, unfortunately, compared to
the other two formulations, they also had the lowest stability according to DSC measurements (low Tg), low and incomplete release rates and also had the highest residual DCM and PVA levels (although these were below regulatory levels). It could be in fact that 0.1M NaHCO₃ particles would be a more suitable formulation, but further experiments would be needed to confirm this hypothesis.

10.4. Cell Culture Studies

Cell culture studies were used to ensure that the plasmid DNA entrapped in microparticles was still able to transfect cells and to assess the efficiency of the microparticles to deliver DNA into cells.

It was demonstrated that DNA that had been microencapsulated could transfect A549 and J774A.1 cells, but with varying degrees of success. There was a positive correlation between the amount of DNA that was in the supercoiled conformation and the level of gene expression, i.e. when there was no supercoiled conformation, there was very little gene expression. Microparticles were shown to be poor in efficacy for delivering DNA into cells, with minimal gene expression seen in both A549 and J774A1 cells for all formulations tested. The results obtained from the release study were reflected in the results obtained for the transfection efficiencies of the microparticles. Particles made with 0.1M NaHCO₃, where the DNA was released rapidly, also had higher levels of gene expression compared with particles made with 1% m/v Na₂HPO₄. These results also highlight the fact that even though particles made with 1% m/v Na₂HPO₄ had more suitable diameters and encapsulation efficiencies, 0.1M NaHCO₃ particles performed better, a result that confirmed those obtained for the analytical experiments, in terms of the more rapid and complete DNA released from these particles.

10.5. Dry Powder Testing

All of the formulations tested aerosolised poorly as a dry powder, with diameters of greater than 65 µm measured. The presence of residual water content may have lead to increased inter-particulate interactions, which prevented efficient dispersion of the particles, but this is unlikely due to the efficient drying of the
particles after manufacture. Particles made with 0.1M NaHCO₃ and 1% m/v Na₂HPO₄ buffers had improved dispersion, which may be attributed to the surface roughness of the particles which could reduce particle-particle contact. The addition of surfactants (DPPC or lecithin) in the formulations led to mixed results, with improved dispersibility of particles made without buffer, but increasing agglomeration for particles made with buffer. As the diameters of the microparticles measured using laser diffraction were in the 1-5 μm range, it might be that the microparticles would perform better in a metered-dose inhaler (MDI), where they will be in a ‘wet’ environment. And again, further experiments would be needed to confirm this.

10.6. Use of OLAs as Alternative Stabilisers

OLAs were shown to have the potential to replace PVA as an alternative stabiliser in w₁/o/w₂ double emulsions. Unfortunately, their reduction in supercoiled DNA content (from 77.01% with 1% Na₂HPO₄ and PVA, to 23.44% with 1% Na₂HPO₄ and OLA-2) could preclude them for further work, unless another DoE is used to optimize the formulation. However, their potential for increasing stability of formulations in MDIs has been proven (Stefely et al., 2000), and so it is an interesting step forward in development for the production of a DNA vaccine for pulmonary delivery.

10.7. Use of Spermine to Increase DNA Entrapment

The use of spermine to condense plasmid DNA and therefore increase encapsulation efficiency had varied success. It increased the DNA-loading in particles made without buffer, and also increased the amount of supercoiled conformation to a small degree. For those made with buffer, there was no change in encapsulation efficiency or diameter of the particles. Unfortunately, the microparticles still performed poorly in an *in vitro* assay, with low gene transfection efficiencies in the cell line studied for all formulations. However, the non-toxic nature of PLGA particles was maintained.
10.8 Overall Conclusions and Future Work

As a final comment, the work presented in this thesis is at the earliest stages of formulation development. It demonstrated clearly the complexities involved in formulating microparticles entrapping DNA that are suitable for pulmonary delivery. Many factors need to be taken into account, and the main issues were trying to balance biological efficacy with stability of the formulation as a pharmaceutical product.

One aspect of the project that can be carried through to future studies is the use of experimental design. Once the desired parameters are decided (e.g. encapsulation efficiency, ideal Tg, residual DCM, particle diameter, etc) DofE could reduce the time it would take to obtain a suitable formulation that would fulfil requirements. The sophisticated methodology is a powerful tool to gain meaningful results in a rapid time frame.

And although this work showed little success, the future of delivery of a vaccine as a dry powder has hope, as a measles vaccine is currently being developed as a dry powder for pulmonary delivery (LiCalsi et al., 1999; LiCalsi et al., 2001; de Swart et al., 2006; de Swart et al., In Press 2006). Another aspect is that for many respiratory therapies, there is a target area in the lung for the drug to work, for example, the bronchioles are optimal for asthma, and for systemic therapeutics (e.g. insulin) the deep lung is the optimal site, as this area has the greatest absorption capacity for drug uptake. For vaccines though, there has been very little research for the optimum site of delivery. LiCalsi et al., (2001) mention that because there has been no assessment yet as to the optimal size distribution for the measles vaccine, it is difficult to assess whether to target the deep lung or to the tracheobronchial region. Therefore, it may be that the usual constraints of aerosol development for drug delivery, such as unimodal particle diameters and exact calculation of dose delivered, may not be so strictly observed for vaccine development. Macrophages and dendritic cells, are abundant in all areas of the lung (Massard et al., 1996; Suarez and Hickey, 2000), and it might be that there is no specific diameter of microparticle size needed either – with the exception
that 1 μm diameter particles are the size at which they are most avidly taken up (Ahsan et al., 2002; Carcaboso et al., 2003; Makino et al., 2003).

There has been a wealth of reviews regarding DNA vaccines lately, demonstrating that this area warrants continued research (Doria-Rose and Haigwood, 2003; Bivas-Benita et al., 2005; Donnelly et al., 2005; Jilek et al., 2005; Patil et al., 2005; Scheuch et al., 2006). The subject was given a significant boost when two DNA vaccines were licensed in 2005 – one against West Nile Virus (WNV) in horses (CDC, 2005), and one against Infectious Haematopoietic Necrosis Virus (IHNV) in salmon (Vical, 2005). Their success in humans, however, remains to be seen, and in the reviews mentioned, a number of aspects still need to be addressed a summary of which is given below:

1) A more focused approach is needed in developing a DNA vaccine. Basic questions need to be asked such as, what is the target? Is it the toxin or the actual microorganism? These are important because depending on the target, then the immune responses required will differ. For example, if it is a toxin, then a humoral (Th2) response is more suitable, and for a virus, a humoral and/or Th1 approach could be ideal depending on the epitope(s).

2) Adjuvant selection and improved immunomodulatory efficiency and presentation of the vaccine to the target cells are also needed.

3) Route and timing of administration is an aspect that needs careful consideration, as well as the dose amount.

4) A fuller understanding of exactly how the DNA is taken up and processed by cells will aid in a more targeted approach. And this also ties into the use of appropriate animal models for the evaluation of the vaccines.

These are just a few aspects that need to be considered in designing a DNA vaccine, and it illustrates the different scientific areas involved – molecular biology, immunology, analytical, formulation and so on. This is perhaps the
reason why this project was not as successful as predicted. Its hypothesis was simple in theory, but in practical terms, was much more difficult that expected.

However, it did provide a means to understand the variability of the processes and difficulties encountered in producing formulations for a new purpose. As well as success in vivo, the formulation would also have to demonstrate stability and compatibility with the device that will be used to deliver the formulation. It has been said that collaborations between formulation and drug delivery scientists is crucial, especially in the area of pulmonary delivery, if a successful product is to be developed (Atkinson and Klum, 2001; de Boer et al., 2003).

It is unfortunate that this project did not fulfil all its aims. However, much of the work presented here provides invaluable insights and enhances the understanding needed for the future development of microparticulate carriers for pulmonary delivery of DNA vaccines.
References and Bibliography


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Appendix 1 – List of Posters and Presentations

- M. T. Tse, C. Blatchford, H. O. Alpar
  *Investigation of PLGA Microparticles for DNA Vaccine Delivery*

- M. T. Tse, C. Blatchford, H. O. Alpar
  *Design and Development of Novel Micro-carriers for Non-Invasive Vaccine and Gene Delivery*
  SET for Britain. 8 May, House of Commons, London, UK (2006). Poster#L2-76

- M. T. Tse, C. Blatchford, H. O. Alpar
  *Protection of Plasmid DNA During Microparticle Manufacture Using Buffers.*

- M. T. Tse, F. Koppenhagen, C. Blatchford, H. O. Alpar
  *Use Of Experimental Design to Optimise a Microparticle Formulation for Encapsulation of Plasmid DNA.*