A STUDY OF DNA/DENDRON NANOPARTICLES FOR GENETIC IMMUNISATION AGAINST ANTHRAX

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This thesis describes research conducted in the School Of Pharmacy, University of London between October 2002 and October 2006 under the supervision of Professor Alexander T Florence. 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 text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature: [Signature]

Date: Oct 2006
"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

Albert Einstein
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ABSTRACT

A series of cationic polylysine dendrons of diverse lipophilicity were synthesised and characterised. These cationic dendrons were selected to have the properties required for complexation with DNA. The dendrons series used in this thesis all have branched asymmetrical polylysine dendritic head group attached to a central core containing none or maximum of three lipidic chains of varying chain lengths. Dendrons interact with and condense DNA producing a complex “dendriplex” with a small residual negative surface charge, and enhance cellular internalization of DNA, in part protecting the DNA from degradation.

The colloidal properties of the dendriplexes were measured. These included size, morphology and zeta potential as a function of charge ratio of dendron to DNA (the N/P ratio) accomplished by standard dynamic light scattering and transmission electron microscopy. The relative strengths of DNA/dendron interactions were determined by ethidium bromide efficiency dye displacement together with assessment of the release of the DNA when challenged with anionic compounds. A double emulsion method was employed to encapsulate the dendriplexes in poly-lactide-co-glycolide (PLGA) particles, characterised by the same biophysical methods as for the dendriplex formulations. The DNA was radiolabelled prior to formulation of PLGA-dendriplexes using a nick translation kit with $^{35}$S catalyzed DNA polymerase I, to introduce radioactive labelled nucleotides into DNA to allow the measurement of encapsulation efficiency, release and in vitro DNA uptake studies.

The preliminary studies of both dendriplexes and PLGA-dendriplexes demonstrate their potential application as a genetic vaccine. Both particulate systems encapsulated protective antigen (PA), one of the protein components required for specific immunity to anthrax. Two types of plasmid DNA were developed one encoding PA 83 cloned into the eukaryotic expression plasmid pSecTag 2B (7.3kbp) and a control plasmid without PA 83. In vitro assays were conducted analysing expression of PA in cells. The assays verified that complexation with dendron aids the stability and uptake of PA DNA. In vivo immunisation with naked PA DNA even with multiple dosing did not induce sufficient antibody response even after secondary boosting post primary i.m immunisation, whereas, both dendriplexes and PLGA particles produced strong anti-PA antibody responses. However, they did not provide protection against lethal toxin challenge in the toxin neutralisation studies.

To our knowledge this is the first in vivo study using dendriplexes and encapsulated dendriplexes as a vaccine against anthrax. Further work needs to be conducted to investigate what levels of antibodies are needed to protect humans against anthrax after vaccination.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>AVA</td>
<td>Anthrax vaccine adsorbed</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-Butyloxycarbonyl</td>
</tr>
<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIEA</td>
<td>N,N-diisopropylethyamine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>EF</td>
<td>Edema factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked ImmunoSorbent assay</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenlymethoxycarbonyl</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HBTU</td>
<td>O-benzotriazolyl-N,N,N',N'-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrogen fluoride</td>
</tr>
<tr>
<td>HOBt</td>
<td>1-hydroxybenztriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>LF</td>
<td>Lethal factor</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionisation time of flight mass spectrometry</td>
</tr>
<tr>
<td>MBHA</td>
<td>4-methyl benzhydralamine</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino-acids</td>
</tr>
<tr>
<td>O.D</td>
<td>Optical density</td>
</tr>
<tr>
<td>PA</td>
<td>Protective antigen</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly(amidoamine) dendrimers</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon correlation spectroscopy</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly vinyl alcohol</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>Te</td>
<td>T cytotoxic cells</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TNA</td>
<td>Toxin neutralisation assay</td>
</tr>
<tr>
<td>XPS</td>
<td>Xray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

1.1 MODERN VACCINE TECHNOLOGY

Vaccine technologies must keep evolving as the world’s transmissible infectious diseases grow. HIV, tuberculosis and influenza are just some of an ever expanding list of diseases. Vaccination programs are successful and have been implemented, which explains the reduced incidence of measles, polio, rubella, mumps, and diphtheria. Most of these vaccines were discovered by trial and error, whereas the next generation of vaccines have to be designed in a more rational manner (Wack and Rappuoli, 2005). The increased knowledge of immunology and molecular genetics has enabled novel strategies against diseases that thus far have evaded successful control. One of the newer vaccination strategies involve DNA vaccines. DNA vaccination is being actively explored as an alternative for treatment of various infectious diseases. However naked DNA vaccines elicit limited immunogenicity even when a different delivery strategy such as gene gun-mediated delivery was attempted (Ludwig-Portugall et al., 2004). The work in this thesis examines the use of dendrons as non-viral DNA carriers for prophylaxis against anthrax. A secondary formulation of poly(lactide-co-glycolide)-DNA/dendron nanoparticles were additionally evaluated both with DNA encoding protective antigen (PA), the protein inducing anti-PA antibodies for protection against anthrax.
1.2 DNA VACCINES

DNA vaccines were discovered serendipitously by biologists attempting to replace faulty or missing genes. Wolff and colleagues initially injected intramuscularly and were able to show transduction of local muscle cells for at least 19 months with a high dose of 100 μg of DNA (Wolff et al., 1990). They demonstrated that skeletal and cardiac muscles have the ability to take up and express naked DNA. Since then, a major problem with current DNA vaccines is their transfection efficiencies and the actual amount of protein synthesised as a consequence (Fynan et al., 1994). The design of the plasmid DNA backbone can increase its adjuvant effect by the addition of immunostimulatory sequences and induce a particular type of immune response (Zelenay et al., 2003).

1.2.1 DNA design

The DNA is in a form of a plasmid which is bacterially derived and contains the genetic sequence of a desired antigen. There are certain elements that plasmid DNA must contain in order to be used as a viable DNA vaccine, as shown in figure 1.1. These include:

(I) A bacterial backbone with origin of replication (normally from E.coli) which aids the amplification of large quantities of DNA for purification. Evidently, to reduce the possibility of integration into the host genome; this should not be active in mammalian cells

(II) An antibiotic resistance (prokaryotic marker) gene to facilitate the selection of organisms carrying the plasmid;

(III) A eukaryotic promoter (as the name indicates) promotes the expression of the antigenic gene, common ones used being cytomegavirus (CMV) or Simian virus 40 (SV40);

(IV) A transcription terminator to make certain that the mRNA is terminated during transcription;

(V) A DNA sequence encoding the antigenic properties of interest and a mammalian signal sequence to enhance protein secretion may be required.
Figure 1.1: A schematic illustration of a DNA plasmid. The vector requires a bacterial origin of replication, an antibiotic selection marker, a strong eukaryotic promoter, and a transcription terminator.

DNA vaccines have several advantages in comparison to recombinant protein. They are generally regarded as safe and have the potential to be produced on a large scale and they are highly stable, allowing for repeated dosing and facilitated combination vaccines (Johnston et al., 2002). A challenging problem is faced of efficiently transferring stably expressing transgenes in the appropriate tissue. Direct gene transfer can be accomplished in various tissues by different means such as particulate delivery systems which allow mass immunisation (Huygen, 2005). Whereas indirect gene transfer involves re-implantation of genetically modified cells this includes more risks, inadequate efficacy, and large expense. Direct gene transfer can be further considered, for discussion, in two vector categories: viral and non viral vectors.

1.2.2 Viral vectors

Viruses are attractive vectors due to their natural ability to incorporate DNA into the host genome (Lee et al., 2005). Modification of the viral carrier, eliminating pathogenic effects and insuring safe transgene expression makes them ideal for therapeutic purposes. The disadvantage of viral vectors is that they are detected by
the immune system and are considered as foreign pathogens thus initiating an immune response. Repeat dose administration which is essential for genetic immunisation is fraught with difficulty due to the adverse effects produced by viral vectors. This has not stopped the growing interest in viral DNA vaccine delivery research which has been mainly applied to adenoviruses and poxviruses (vaccinia virus). Studies by Moss and coworkers (2001), have focused on the development of vaccinia virus (VV) as a vaccine vector for protection against a range of pathogens.

A recombinant vaccinia virus-vectored anthrax vaccine was constructed by cloning protective antigen (PA) gene from B.anthracis into two strains of vaccinia virus, elicited high antibody titers and protection against anthrax but the level of protection was dependent on the strain used (Lee et al., 2005).

Combination vaccines expressing two or more proteins have been used for the pox virus as it has two infectious forms. The insertion of these antigenic proteins into the vector has attracted research as a viable approach. This controlled mixing of antigens may reduce the impact of immunodominance, which has been seen in single antigen DNA vectors (Hutchings et al., 2001).

There are still lingering concerns about the association of viral vectors with human disease and immune responses, making repeated administration of the genetic material difficult. This is why attention has turned to non-viral vectors as they are stable and easier to scale up and produce.

1.2.3 Non viral vectors

Non-viral vectors have fewer disadvantages than their viral counterpart but they are less capable of inducing high transgene expression levels. These carriers can be encapsulated, complexed and absorbed normally onto polymeric system, to protect the DNA from nuclease degradation and to allow release of the DNA in a controlled manner, reducing the need for vaccine booster doses. This area will be discussed further in section 1.6.
1.3 IMMUNE SYSTEM RESPONSES TO DNA VACCINATION

The mechanism of immune stimulation by genetic immunisation has been something of a mystery, the outcome (increase or decrease in antibody production) was known but the processes involved were not clearly defined. Over the past decade mechanisms have been clarified with the growing knowledge of immunological responses of hosts to infection. Recent discoveries by several groups (e.g. Ahuja et al., 1999) have shown that the role played by APC (antigen presenting cells) or resident dendritic cells, play in the induction of the immune response was evoked by vaccination with a plasmid DNA specific epitope. These cells are critical costimulatory molecules that help T cells prepare for action.

1.3.1 Innate immune system

The innate or natural immune system compromises of cells and proteins involved in inflammatory and acute phase responses. They are called innate as they exist prior to infection by a pathogen, although the amount of each component may increase as a response to infection (Janeway C.A., Traver P, Walport M, Shlochik M. (2004) Immunobiology, 6ed. Churchill Livingstone.)

The body has chemical and physical means of removing any pathogens trying to gain entry into the body as shown in Table 1.1. The physical and chemical barriers are very effective at preventing entry and exclude more than 99.9% of infectious organisms (Janeway C.A., Traver P, Walport M, Shlochik M. (2004) Immunobiology, 6ed. Churchill Livingstone.). However, organisms infecting the body start a cascade of events involving other components of the innate system. These include the macrophages as they are the first line of defence against pathogens. They act by engulfing microbes and dead cells using phagocytosis. Macrophages are one type of phagocyte, the other being neutrophils or polymorphonuclear leucocytes. These are mobile cells that can travel through the blood stream to the site of infection. The phagocytes together with proteins assist in the removal of the pathogen. This process is known as the inflammatory response.
Table 1.1: A comparison of the physical and chemical barriers that are part of the immune system.

<table>
<thead>
<tr>
<th>Chemical barriers</th>
<th>Physical barriers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lysozyme in sweat and tears.</strong></td>
<td><strong>Skin and mucosa.</strong></td>
</tr>
<tr>
<td>Kills bacteria by breaking down peptidoglycan in bacterial cell walls.</td>
<td>Intact skin and mucosa provide a physical barrier preventing entry of organisms.</td>
</tr>
<tr>
<td><strong>HCl in the stomach.</strong></td>
<td><strong>Cilia.</strong></td>
</tr>
<tr>
<td>This is lethal to most bacteria and does not only occur in the stomach. The lactic and propionic acid produced in the vagina produces low pH inhibiting the division of most bacteria.</td>
<td>The respiratory tract is lined with hair like structures that beat and propel particles towards the throat where they will be expelled by coughing or swallowing and excretion.</td>
</tr>
<tr>
<td><strong>Fatty acids.</strong></td>
<td><strong>Mucus.</strong></td>
</tr>
<tr>
<td>The sebaceous glands produce fatty acids which have antimicrobial properties.</td>
<td>This is secreted by epithelial cells of gut, respiratory tract, and genitourinary tract. The adhesive properties traps and eliminates microbes.</td>
</tr>
</tbody>
</table>

Adapted from (Goldsby et al. (1997) Kuby, Immunology 3 ed. W.H Freeman)

1.3.2 Cytokines

Cytokines are small proteins (~ 20 kDA) and resemble hormones, enabling cells to communicate with each other. They are produced in a paracrine or autocrine manner facilitating communication of cells in a microenvironment. There are many different types of cytokines, divided into different families with common functions as seen in Table 1.2. Cytokines control many aspects of cell behaviour, including proliferation, differentiation, and cell function. Cells are never exposed to one single cytokine, but to multiple cytokines which can promote a response or act antagonistically inhibiting a reaction.

There is an overlap between the innate and systemic systems shown in the cases of delayed type hypersensitivity (DTH) reactions, differentiation of CD4 T cells into Th cells (see section 1.3.3) and other responses. DTH reactions involve the activation and recruitment of monocytes and resident tissue macrophages. These specialised DTH Th cells have evolved enabling them to migrate through tissue. If they encounter antigen, they can stimulate the production of cytokines.
Table 1.2: List of members of the cytokine families.

<table>
<thead>
<tr>
<th>Family</th>
<th>Member</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin (IL)</td>
<td>IL-1 to IL-22</td>
<td>Different ILs has a variety of functions and is secreted by a number of cells.</td>
</tr>
<tr>
<td>Interferon (IFN)</td>
<td>IFNα</td>
<td>Leucocyte IFN. Inhibits viral replication.</td>
</tr>
<tr>
<td></td>
<td>IFNβ</td>
<td>Fibroblast IFN. Inhibits viral replication.</td>
</tr>
<tr>
<td></td>
<td>IFNγ</td>
<td>Secreted by T cells and natural killer (NK) cells.</td>
</tr>
<tr>
<td>Tumour necrosis factor (TNF)</td>
<td>TNFα</td>
<td>Factor activates macrophages and endothelium.</td>
</tr>
<tr>
<td></td>
<td>TNFβ</td>
<td>Secreted by T cells and has similar activity as TNFα.</td>
</tr>
<tr>
<td>Colony-stimulating factor (CSF)</td>
<td>G-CSF, M-CSF, GM-CSF and others</td>
<td>It has been identified by the ability of make bone marrow cells and aid differentiation. It has an effect on mature cells of the same lineage e.g. monocytes and macrophages.</td>
</tr>
<tr>
<td>Chemokine</td>
<td>MCP, Eotaxin</td>
<td>Controls the migration of cells between tissues.</td>
</tr>
</tbody>
</table>


The main cytokine produced is TNFα, making the endothelium more adhesive for monocytes and resulting in more of them extravasating and entering infected tissue. The Th cells also secrete IFNγ and IL-2, which, together with TNF activate the tissue macrophages and recruited monocytes to become more efficient at killing intracellular pathogens. This shows that they work in conjunction with each other, especially if the innate immune system cannot deal with the infection alone.

1.3.3 Specific immune system

*The relationship between T cells and B cells in the mechanism of DNA vaccines*

T (thymus-derived) lymphocyte precursors, like those of B lymphocytes originate in the bone marrow. Whereas B cells complete their maturation in the bone marrow, T lymphocytes migrate to the thymus where they develop into mature T lymphocytes. T cells use a receptor, the T cell receptor (TcR), for recognising antigen. The TcR consists of two glycoprotein chains, α and β, both showing a typical Ig-like domain structure. Similar to Ig (immunoglobulin), each chain of the TcR has a constant
domain near the cell membrane surface. The variable domains contain hyper-variable regions and adopt a globular structure (figure 1.2)

![Figure 1.2: Interaction of TcR with antigen/class I MHC.](image)

The antigen binding site of TcR is made up of Vα and Vβ chains the hyper variable region and Cα and Cβ the constant region highlighted in the left hand side box. The binding site interacts with the antigenic peptide bound in the groove of the MHC molecule (MHC I or MHC II illustrated in right hand side box), only when this occurs does the T cell recognise the foreign antigen. Adapted from (Goldsby et al. (1997) Kuby, Immunology 3 ed. W.H Freeman)

T cells follow the same rules as B cells in that they both are specific for antigen (small peptide) recognition. However T cell recognition of antigen differs in one fundamental aspect compared to the way B cells use antibodies on their surface for antigen recognition. They recognise antigen associated with the major histocompatibility complex (MHC) molecules on the surface of cells. The term major histocompatibility complex refers to a region of DNA approximately 4 Mbp and containing over 100 genes. Each species has a specific MHC; for humans it is HLA (human leucocyte antigen) of which there are two different classes: class I and class II are involved in T cell recognition.

Broadly, T cells can be subdivided into T helper cells (Th) and T cytotoxic cells (Tc). These cells can be distinguished from one another based on the presence of different glycoproteins on their surfaces; CD4 on Th cells and CD8 on Tc cells. Simplistically, Th cells dictate the type of immune response that is produced,
whereas Tc cells are involved in actual destruction of infected cells (Esser et al., 2003). As mentioned previously, Th cells have specific antigen binding receptors on their surface. On encountering these receptors Th cells become activated and differentiate into Th\(_1\) or Th\(_2\) cells. The dominance of one of these cell types will influence the type of immune response that develops. The exact mechanism of differentiation is unknown, but is believed to be linked to the presence of interleukin (IL)-12 for Th\(_1\) cells and IL-4 for Th\(_2\) cells (O’Garra and Arai, 2000).

**Figure 1.3:** Schematic representation of the mechanism for the induction of T cell responses. The DNA is transfected in cells and processed by two routes: (1) MHC class I-restricted presentation of antigen inducing CTL responses; or (2) Professional antigen presenting cells take up exogenous antigens via MHC Class II using the endo-lysosomal pathway. Specific T helper cells recognise class II MHC molecules, inducing cytokine production.

Generally with DNA vaccination the direct intramuscular injection of the plasmid DNA, containing encoded polypeptide sequences that allows eukaryotic transcription and translation, results in the *in vivo* synthesis of protein (antigen).
This endogenous processing can take place in a similar way to processes that are seen following an infection with intracellular pathogens. DNA vaccines stimulate both the exogenous (MHC class II) and the endogenous (MHC class I) antigen presentation pathway illustrated by figure 1.3 (Sharma and Khuller, 2001). In particular it is this class I restricted presentation that results in a strong CD8 mediated immune response that is a hallmark of viral vaccines. Recombinant proteins do not exhibit this even in the presence of a strong ‘Th1’ promoting adjuvant. As well as directly transfected plasmid DNA dendritic cells (bone marrow derived cells), found in most tissue, can also take up antigen from transfected myocytes and present the relevant peptides to CD4 and CD8 T cells. DNA vaccines based on protein antigens or killed pathogens are preferentially processed through the exogenous presentation pathway that generates MHC class II restricted CD4 responses.

Generation of humoral responses required direct stimulation of B cells with antigen via MHC II molecules presented at their surfaces (Ahuja et al., 1999). The antigen may then be recognised by Th2 lymphocytes and subsequently stimulate proliferation and antibody class switching, finally forming plasma cells (figure 1.4).

Muscle tissue contains immature dendritic cells that exist in a high phagocytic state thus expressing low levels of MHC class I and II molecules, rendering them poor initiators of immune response. These dendritic cells are activated by proinflammatory cytokines which migrate via the afferent lymph to the lymph nodes where they undergo maturation and increase in number (Ridge and Matzinger, 1998). The mode of administration of the vaccine developed in this thesis was via the intramuscular route. It is the most frequently used route and normally forms the basis for comparison to other existing vaccines. It still has less dendritic cells when compared to the mucosal route. Ear skin models conducted by Akbari et al. (1999) have shown that the dendritic cell is the key for immunity after administration of a DNA vaccine.

To measure the success of the vaccine, antibody response must be stimulated (humoral) and this can only happen in conjunction with CD8 T cells and CD4 T cells (cell-mediated) (figure 1.5).
**Figure 1.4: Interaction between B cell and Th cell to stimulate antibody production.** B cell binds antigen via their membrane Ig, associated with class II MHC on the surface of T cell also containing CD40 and CD154 molecules. This interaction between CD40 and CD154 are essential for class switch and without it only IgM is produced. Finally, differentiation into plasma cells is influenced by cytokine production (IL-4 and IL-5) and antibody secretion by IL-6.
B cells differentiate into plasma cells which are specialised cells, as they are designed to produce enormous amounts of antibody. The other fate of B cells is to become memory B cells, and retain specific Ig on their surface. After administration of the vaccine with these stored memory cells a secondary exposure to the antigen, the immune response is ready to respond and has the specific components to remove the antigen.

**Figure 1.5:** Summary of the main aspects of the immune system connecting the innate and systemic systems with T cell (cellular) and B cell responses (humoral).
1.4 ROUTES OF ADMINISTRATION

Traditionally most vaccines have been administered intramuscularly (I.M) which allows repeat boosting with a larger dosing capacity. In our animal studies the intramuscular route was used to investigate antibody expression. Wolff et al (1992) were the first group to show that I.M administration of naked DNA resulted in expression in skeletal muscles tissue that was evident for at least 19 months. This makes it an “attractive target tissue for induction of plasmid DNA for the purpose of gene therapy”. Others such as Jiao et al. (1992) administered naked DNA to monkeys via the intraarterial route rather than by intramuscular injection. They put this down to the decrease in efficiency in DNA uptake or decrease in delivery to the myofibres. Studies evaluating the amount of anti-DNA antibodies after rodent and primate administration were difficult to elicit even after repetitive inoculation (Le et al., 2000).

Gene gun delivery is another alternative method of delivering DNA to the epidermis, where skin associated lymphoid tissue such as Langerhans cells present antigen to the immune system. Williams et al. (1991) used a gene gun to deliver DNA-coated gold beads, and found that foreign genes were expressed in liver and skin cells of live mice by using a new apparatus to accelerate DNA-coated microprojectiles into tissues. Huygen, (2005) used plasmid encoding tuberculosis proteins in saline administered via the intramuscular route to mice, inducing strong T helper type immune responses; the same plasmid coated in gold particles was delivered using gene gun but did not successfully induce T cells. The combination of two administration routes such as intramuscular with mucosal (intranasal delivery) stimulates a greater immunological response than a single route (Manocha et al., 2005).

The cause of disease and where it manifests is essential in deciding the route of administration. As the majority of infections commence from the mucosal surface it seems logical to select this local route for protective immunisation. Most proteins have poor transport characteristics across biological barriers in contrast to the parental route and this is one of the major problems that is faced with the mucosal route. For these proteins to be delivered, new design strategies have developed (particularly for particulate systems), to enable such barriers to be overcome (Eriksson and Holmgren., 2002).
A number of alternative routes have been investigated: these include the oral, intranasal, ocular, rectal and intravaginal routes. Several existing oral vaccines, are based on live-attenuated organisms including polio virus, *Vibrio cholerae* and *Salmonella typhi*. One of the most attractive routes is the oral route, due to ease of administration without the complication of needles. However, the acidity of the stomach and digestive enzymes in the intestines need to be considered, as well as the thickness of the mucus forming an additional barrier to penetration. Novel systems using adjuvants may enhance immune responses. In mice, the oral administration of PLG microparticles containing antigen has shown these to be potent inducers of systemic and mucosal immunity (O’Hagan *et al.*, 1991). For oral vaccines to be successful they have to overcome very harsh environments, this is why much emphasis has been placed on designing a potential delivery system (Green and Baker, 2002), involving biodegradable microparticulate carriers.

Studies have investigated nasal delivery, utilising the nasal-associated lymphoid tissue as it contains dendritic cells and M cells that aid the processing of the antigen. Various intranasal immunisations have taken place with relevant antigen using encapsulated polymeric systems and liposomes (Cerchiara *et al.*, 2005). In this case the particulate surface and size properties play a vital role in uptake and mucosal and systemic responses.

DNA vaccines have typically been delivered by intramuscular injections as previously mentioned and the public associates the parenteral route with vaccine delivery, which is still the main method of immunisation. Hence it is used as a starting point when designing a new vaccine delivery system.

1.5 ADJUVANTS

The purpose of an adjuvant is clarified by its Latin root “adjuvare” meaning “to help” (Hunter, 2002) i.e to enhance by whatever means, immunological responses. Adjuvant immunology has been a neglected area of research, whereas discovering new protective antigens for various infectious diseases became a priority. However, the injection of naked plasmid DNA without adjuvant results in little or no antibody response even with booster injections in both primates and humans (Rodriguez and Whitton, 2000). It is thought that the DNA becomes localised and damaged by
enzymatic action and is thus eliminated. Safety concerns have restricted the development of adjuvants. Alum, introduced more than 70 years ago, it is an aluminium-based mineral salt which remains the only adjuvant approved by the U.S Food and Drug Administration. Although it has been used for many years, its mechanism of action is not fully understood. Previous studies by Allison (1979) suggested that the main mechanisms of adjuvants are thought to be associated with "the formation of depots of antigen" in tissue and stimulation of macrophages. However, more recent work proposes the contrary that alum does not form depots, but causes the upregulation of co-stimulatory signals on human monocytes (Gupta, 1998). Aluminium salts as adjuvants have also been approved in Europe including monophosphoryl lipid A (MPL), which has been evaluated as an adjuvant for allergen-specific immunotherapy (Crameri and Rhyner, 2006).

Toxicity is still one of the main concerns associated with experimental adjuvants and has led to their failure in clinical trials. Other properties that are essential for adjuvant development are biodegradability, stability, low cost, and application to a wide range of vaccines, as mentioned in the review by O'Hagan and colleagues (2001).

Adjuvants have been used to aid the efficiency of some vaccines by, increasing the immunogenicity of weak antigens, enhancing uptake of antigen; in tissues to produce prolonged exposure. Additionally adjuvants facilitate antigen uptake by antigen presenting cells and activation of macrophages, lymphocytes and lymph nodes.

The mechanism of action is complex and not fully understood, however this has not repressed considerable advances in adjuvant technology as understanding of the innate system develops and the key receptors of known adjuvants are identified. The mode of action of adjuvants falls into two subclasses, their association with antigen presenting cells or as a delivery system promoting antigen uptake (particulate system).

Small peptides are being studied by Lee et al. (2005) as potential adjuvants classified by their association with antigen presenting cells. A peptide analogue (20 amino acids) Trp-Lys-Tyr-Met-Val-Met (WKYMWm) was selected from combinatory libraries by functional screening. Three different DNA vaccines were used together with the selected peptide. All three vaccines stimulated the mouse bone marrow-derived dendritic cells, when combined with the peptide. The peptide
analogue was shown to enhance expression of CD80 which is the major co-stimulatory molecule for induction of antibodies and CD8 T cells. In addition the peptide stimulated phagocytosis in the mouse dendritic cell line and chemotaxis in human dendritic cells. The co-stimulation occurs for both CD8 T cells and CD4 T cells, but previous reports (Lang et al., 2002) have suggested that they are differential. Hence it is possible that CD8 T cells receive an enhanced surface expression from the CD80 receptor when the WKYMWm peptide is used when compared to the signal received by the CD4 T cells. This small peptide may work as an adjuvant for a DNA vaccine in humans by activating human dendritic cells.

Traditional vaccines such as bacterial toxoids and inactivated viruses contain most of the components that evoke strong immune responses, which modern vaccines lack. Therefore, the next generation of vaccines should incorporate these features. The spread of diseases such influenza, tuberculosis and HIV amongst others requires a sophisticated approach, entailing new and improved vaccine technology. It is predicted that by 2020 up to 25% of the population of southern Africa, Asia and former Soviet Union may die of AIDS (Hunter, 2002). Adjuvants form an essential part of this new technology and a better understanding of adjuvant properties and effects are crucial for the development of novel DNA vaccines.

### 1.5.1 Adjuvant particulate systems

Particulate systems which can act as adjuvant include emulsions, microparticles, liposomes and nanoparticles. Dependent on their size and surface properties, the particles are taken up by antigen presenting cells. The first oil-in-water adjuvant system (Syntex adjuvant) developed in the 1980’s, was formulated using squalane as a replacement for Freund’s adjuvant. Freund’s adjuvant was toxic but very effective; it contained water in mineral oil and traces of killed mycobacteria. However squalane did not have an acceptable safety profile until the removal of the additional synthetic factors thus lowering the toxicity level and forming MF59.

Liposomes are phospholipid vesicles that act as delivery systems for antigens and adjuvants. Liposomes formulated with MPL (monophosphoryl lipid A) have been used as vectors for vaccine targeting hepatitis A and influenza (Ambrosch at el., 1997). Synthetic systems also provide an adjuvant effect by having multiple copies
of antigen entrapped or absorbed into or onto a degradable polymeric system. The polymers used are polyesters, polylactide-coglycolides (PLG) and polylactide glycolic acid (PLGA) and have been used for many years without adverse effects. The entrapment of antigens was first achieved in the 1990s by several groups including Eldridge et al. (1991), Almeida and Alpar (1996) and O’Hagan et al. (1991) all demonstrating that microparticles exerted an adjuvant effect by inducing the cytotoxic lymphatic system in rodents. Further research into existing adjuvants, as well as potentially new adjuvants, will aid the development of synthetic systems. Traditional adjuvants that have been used in conjunction with microparticles were studied by Singh et al. (1998), where MF59 as systemic adjuvant was used in conjunction with microparticles.

To realise the potential of adjuvants will require focus on delivery systems that are site specific, especially as antigen presenting cell receptors are unveiled and their mechanisms are understood.

1.6 PARTICULATE DELIVERY SYSTEMS

Particulate systems composed of biodegradable polymers are part of the new vaccine technology. Particulates belong to a wide range of diverse delivery systems that encapsulate proteins or DNA, to increase uptake by target tissue. Such effect will be dependent on the type of particulate system and the surface composition of the particles.

1.6.1 Liposomes

Liposomes have developed immensely since they were first identified by Allison and Gregoriadis (1974) as potential immunological adjuvants for diphtheria toxoid. Phosphatidylserine (PS) used in liposomal formulations has shown an inhibitory effect on dendritic cell maturation. Hence recent work by Bondanza et al. (2004) showed that these PS liposomes containing Annexin V destroyed tumor cells by stimulating the specific immune system responses.

In the liposome structure most of the DNA is entrapped within the aqueous layers between the lipid bilayers (Perrie and Gregoriadis, 2000). The entrapment yield of DNA has been increased by using dehydration rehydration vesicles (DRV). DRV
liposomes with entrapped hepatitis B surface antigen have revealed optimal induced immunity in comparison to naked DNA, which is reliant on complexation of the DNA with the bilayers (Perrie et al., 2002). DRV liposome assisted enhanced expression in muscle tissue and lymph nodes. This substantiates the fact that liposomes initiate immune responses by aiding antigen uptake in the lymphoid tissues demonstrated by assessing effective uptake by antigen-presenting cells (APC) and transgene expression (Perrie et al., 2003).

1.6.2 Poly (lactide-co-glycolide) (PLGA)

PLGA is a well known polymer (figure 1.6) with low toxicity, and an excellent safety record. It is used for many applications such as bone implants, tissue engineering scaffolding (Langer et al. 1997), as well as a matrix for contraceptive implants. On administration into the body it undergoes hydrolysis forming non-hazardous biologically safe metabolites, lactic acid and glycolic acid.

\[
\begin{align*}
\text{CH}_3 & \quad \text{O} & \quad \text{CH}_3 & \quad \text{O} \\
\text{CH} & \quad \text{C} & \quad \text{O} & \quad \text{CH} & \quad \text{C} & \quad \text{O} \\
\end{align*}
\]

\[\begin{align*}
\text{H}_2 & \quad \text{O} & \quad \text{C} & \quad \text{O} & \quad \text{H}_2 & \quad \text{O} & \quad \text{C} & \quad \text{O} \\
\end{align*}\]

Figure 1.6: Basic structure of PLGA

PLGA has been used as a carrier system for DNA for the purposes of immunisation via the parenteral and mucosal routes (Jones et al. 1997). The process of encapsulation has an effect on the state of the DNA; it is seen to convert to a circular form, where initially it is supercoiled state. This can affect the biological activity. Nonetheless, the immune response actually increased in contrast to the control i.e. blank PLGA particles.

PLGA particles are formulated with a range of different surfactants that differ in their hydrophobicity (Singh et al, 2001), allowing scope to match surfactant properties required with the design of the DNA particles (Bramwell and Perrie, 2005).

The release profile of PLGA particles is influenced by the crystallinity, the extent of copolymerisation and the molecular weight of the copolymer: these parameters are critical for the biodegradation of the polymer. Although tailoring is possible the actual release kinetics from the PLGA particulate system is not always predictive. It
is hypothesised (McGee et al, 1994; Alpar et al, 2000) that PLGA particles have a pulsatile release system imitating a boost regime administration instead of continuous release. Therefore in addition to acting to produce a sustained release formulation of antigen, PLGA also elicits an adjuvant effect because of its particulate nature. This was verified by Jilek and co-workers (2004) using plain PLGA particles transfected with human dendritic cells. These showed upregulation of CD83 and CD86, whereas cationic PLGA particles upregulate CD86 only. Cationic PLGA particles are more proficiently taken up by dendritic cells and macrophages than negatively charged particles. Mollenkopf et al. (2004) demonstrated these using poly-lysine coated particles which were positively charged and BSA coated particles that were negatively charged. The surface characteristics play a significant role in particle uptake, so these properties can be manipulated to further aid their transport to target tissue.

1.6.2.1 Use of Water-In-Oil-Water (W/O/W) Emulsions to form microspheres

W/O/W emulsions have a secondary water phase after the primary o/w is formulated consequently the system is named a double emulsion (Ogawa et al., 1988). Chen et al. (2001) evaluated the relevance of the inner water-to-oil ratio, and assessed that it was the crucial factor affecting the morphology and release kinetics of the final particle formulation. They noticed microspheres with smaller inner volumes likely to collapse after vacuum drying. On the other hand when the volume of inner water increased significantly, more pores were observed on the surface of the particles. Other considerations are the choice of stabiliser used, mostly PVA (polyvinyl alcohol), in the external aqueous phase in PLGA particle formulation. This double emulsion technique was modified to produce nano-sized PLGA particles containing encapsulated DNA/dendron complexes, description of complexes in section 1.7.3.1.

Other emulsion strategies have been attempted, for example, a cationic emulsion formed by heating triglycerides, Lipoid E-80, stearylamine, α-tocopherol, poloxamer 188, glycerol, and double distilled water. Recently this cationic emulsion was used for delivery of M. tuberculosis Ag85B DNA with various cell lines including human monocytes-derived dendritic cells and Calu-3 cells from a bronchial cell line (Bivas-Benita et al., 2004). The results on this type of emulsion are very preliminary but
demonstrate that other methods are possible for complexation of DNA and may have completely different characteristics from the PLGA nanoparticles.

1.6.3 Emulsions

Freund's complete (CFA) adjuvant is generally regarded as the most effective adjuvant system. It is administered as a water in oil emulsion consisting of an aqueous solution of *Mycobacteria* and antigen emulsified into paraffin oil using Arlacel A as a stabiliser to form a water in oil emulsion. Unfortunately the inflammation produced is normally intense and can lead to abscess formation, therefore its use is not permitted in humans (Langer *et al.*, 1997). An alternative is Freund’s incomplete adjuvant (IFA) containing the same constituents as the parent emulsion, but not the *Mycobacteria* component making this adjuvant system less toxic.

MF59, an emulsion of squalene oil emulsified with Tween 80 and Span 85, has been granted a licence for human use and has been found to produce a humoral and cellular immune response against hepatitis, HIV and influenza (Singh *et al.*, 1998).

1.6.4 Gold particles

Gold particle formulations appear to be ideal for administration to the skin, directly accessible to the Langerham cells and the dendritic cells essential to induce the immune system. (Falo, 1999). Gene gun delivery has been the main mode of application for gold particles. Fynan and coworkers (1993) demonstrated that gold particles containing plasmid encoding hemagglutinin provided protective immunity against lethal influenza when inoculated via a gene gun in BALB/c mice. One interesting development has been the intradermal application of DNA-gold particles which have shown enhanced immunogenic responses. It has been theorised that gold particles have an adjuvant action due to antigen presenting cells that are the initiator of T cell responses (Zhang, 2003).

In this section we have depicted a variety of different particulate systems that have all been used for gene delivery. In relation to the work described in this thesis, dendrimers are the structures of interest, together with their encapsulation in PLGA.
1.7 DENDRIMERS

Dendrimers are hyperbranched structures inspired by nature, with the first dendrimer reports published in the early 1980s by the groups of Vögtle (1989), Denkewalter (1981), Tomalia (1985) and Newkome (1985). Since these pioneering studies were conducted, many hundreds of research groups have joined the field, leading to many advances in synthesis and application of these polymers. In the polymer field, dendrimers are recognised as the fourth major class in macromolecular structures (Svenson and Tomalia, 2005). This class can be divided into subclasses shown in figure 1.7 consisting of i) random hyperbranched polymers ii) dendrigraft polymers, iii) dendrons and iv) dendrimers.

Figure 1.7: (a) Dendritic polymers covalently bonded branched structures have crucial parameters such as: branching angles (a), rotational angles (b), repeat unit length (l) and branch cell multiplicity (N) to determine cargo-space properties in the interior lattice of a dendrimer. (b) Four major classes of macromolecular architecture, as traditional synthetic polymers are classified as: (i) linear, (ii) cross-linked (bridged) and (iii) branched whereas structural controlled polymers (IV) dendritic polymers (Random hyperbranched, dendrigrafts, dendrons and dendrimers). (Reproduced from Svenson and Tomalia, 2005).

The distinctive properties of dendrimers and dendrons are their well-defined structure as well as small molecular size with a low polydispersity in contrast to traditional polymers. In the past decade, over 5000 literature references on
dendrimers demonstrate the popularity of this unique subclass of polymers. Dendrimers are characterised by their ‘layers’ emanating from a central point. The numbers of these so called “generations” is defined from the central point i.e. the core (Go) to the surface (the periphery) of the structure. Dendrimer structures may be divided into three parts:

1) The multivalent surface, containing a large number of reactive sites;
2) The outer shell just beneath the surface of the dendrimer, defining the form of the structure and its character for instance the ridgity or flexibility;
3) The core enclosed by dendritic branches, the number dependent on the generation.

A dendron is a structurally controlled fragment of a dendrimer (see structure IVc in figure 1.7b) and are also known as partial dendrimers. A dendron can be divided into three different regions: the core, the branches, and periphery groups similar to the full dendrimer structure. The number of branching groups encountered upon moving outward from the dendron core defines its generation (G-1, G-2, G-3) containing less end groups compared to its parent dendrimer even at the highest generation.

These distinct regions of the dendron can be manipulated accordingly to the desired characteristics for drug or gene carrier systems. The dendrons must be tailored to achieve crucial criteria including low toxicity, translocation, tissue targeting, and permeability especially through cell membranes (Boas and Heegaard, 2004). In most cases, the nature of the periphery groups dictates the level of toxic effects. With cationic dendrimers for instance PAMAM dendrimers, the cytotoxicity increases with each generation of growth. The cytotoxic effect can be decreased by reducing the number of amino-terminated dendrimers (dendrons) (Malik et al., 2000; Jevprasesphant et al., 2003; Chen et al., 2004). In vivo toxicity correlates fairly well with in vitro toxicity. Toxicity studies in mice with certain dendrimers bearing cationic surface charges administered with doses above 10mg/kg revealed liver toxicity demonstrated by increased levels of alanine transaminase in serum and liver necrosis upon histopathological analysis (Neerman et al., 2004). Correspondingly, a family of noncharged polyester dendrimers showed lower toxicity (Padilla et al., 2002).
The wide variety of dendrimers is exemplified by the few shown in figure 1.8, where the three well known structures which are lysine based dendrimers (Sakthivel and Florence, 1998), polyether compositions (Fréchet, 1994) and PAMAM polyamidoamine (Tomalia, 1985).

Figure 1.8: Structures of the most commonly used dendrimers for drug delivery purposes (a) PAMAM dendrimers; (b) polyaryl ether dendrimers; (c) polylysine dendron; (d) Polyester dendrimer based on glycerol and succinic acid; (e) polyester dendrimer on glycerol and succinic acid and (f) dendritic polyglycerol. (Obtained from Gilles and Fréchet, 2005)

These dendrimers are just some of the few arrays of new assemblies with different structural functionalities. The relationship between these functionalities and the corresponding toxicity and biocompatibility is being actively researched (Malik et
al., 2000; Krishna and Jayaraman, 2003; Maszewska et al., 2003; Fuchs et al., 2004; Jevprasesphant et al., 2004).

1.7.1 Dendrimer carriers

Dendrimers have been used for a variety of applications. These include vaccine delivery, delivery of antitumor drugs (Battah et al., 2006; Nishiyama et al., 2005) and antibacterial drugs (Nagahori et al., 2002). Recently Shaunak et al. (2004) used a PAMAM dendrimer containing 64 peripheral carboxylic acid, the modified end groups producing molecules with novel biological properties. The group postulated that anionic dendrimer conjugates of aminosaccharides had defined immunomodulatory and antiangiogenic properties. The dendrimers were evaluated in rabbit models in order to prevent scar tissue formation after eye surgery. Histopathological examination of the eyes that were treated with dendrimer glucosamine showed minimal scar tissue formation compared to placebo treated animals. This was the first study that demonstrated targeting of proinflammatory mediators using these dendrimers. They were not toxic or immunogenic and which synergistically prevent scar tissue formation. This is one of the many examples of the expanding use of dendrimers, drawing attention to the importance of the structural design when applied to biological targeting. A large number of dendrimer libraries have been synthesised containing different assemblies, but lacking consideration of the biological target and without an application, so these remain compounds in a long list of dendrimers.

Dendrimer research has to an extent focused on the delivery of DNA as non-viral gene transfer agents aiding the transfection of DNA into the cell nucleus. Numerous reports have been published on the possible use of PAMAM, PPI or polylysine based dendrimers (Braun et al., 2005; Choi et al., 2006; Shah et al., 2000; Ramaswamy et al., 2003).
1.7.2 Dendrimers in vaccines

Small molecular weight peptides as previously mentioned have demonstrated adjuvant properties inducing a weak immune response against the peptide upon their injection into a recipient host. Dendrimers have emerged as valuable since they can act as multivalent carriers for antigenic substances by coupling to the antigenic molecule via the dendrimer peripheral functional groups. Further examples pioneered by Tam and co workers (2002) synthesised by stepwise peptide synthesis on branches of multiple antigenic peptide (MAP) or by segment coupling of peptide fragments. MAP constructs are asymmetrical dendrimers (figure 1.9) formed by successive addition of lysine residues which can then be coupled to small molecular weight antigen of interest.

![Figure 1.9](image)

**Figure 1.9:** Different constructs of MAP with varying designs all consisting of different peptides representing T- and B cell epitopes. Also showing some structures with additional fatty acids, either single straight chain or as specific tripalmitatecysteinyl structure (Boas and Heegaard, 2004).

MAP structures have been used by Nardin *et al.*, (2000); Nardin *et al.*, (2001) and others such as Sadler *et al.*, (2002) for producing peptide specific antibodies. Currently MAP-based malaria vaccines are being evaluated in phase I human trials (Boas and Heegaard, 2004). In this thesis, size and toxicity were primary areas of interest. Polylysine dendrons were synthesised and coupled to DNA containing the genetic sequence of a desired antigen.
1.7.3 Polylysine dendrons and dendrimers

In our laboratory, as well as in this thesis, studies have focused on polylysine-based dendrons and dendrimers. The lysine based dendrimers have been used for a wide range of particulate systems (figure 1.10) including dendron or dendrimer based emulsions, dendrisomes (Al-Jamal et al., 2005), dendriplexes (Ramaswamy et al., 2003), dendrimer derived nanoparticles (Singh and Florence 2005), dendriplexes encapsulated nanoparticles (Ribeiro et al., 2005), cationic dendrimer interacting with liposomes (Purohit et al., 2003).

**Figure 1.10:** A schematic diagram showing the variety of aggregates from primary dendron and dendrimers structures including dendrimer constructs, dendrimer nanoparticles (as well as absorption onto particles), dendrisomes, dendriplexes and their encapsulation into another polymeric system.

Dendrimers may form aggregates in aqueous media. The size and stability of these dendrimer aggregates is dependent on the packing characteristics of the individual building blocks which is related to the surface functionalities of the alkyl groups (Al-Jamal and Singh et al., 2005).
1.7.3.1 Dendriplexes

The complexes formed between cationic dendrons (figure 1.11) and polyanions are termed dendriplexes. Recent studies by Ramaswamy et al. (2003); Braun et al. (2004); Choi et al. (2006) have focused on the delivery of DNA as a non-viral gene therapy using unmodified amino-terminated PAMAM, PPI or polylysine dendrimers. When using polylysine structures the efficiency of condensation of DNA is dependent on the lipophilicity of the dendrons used in the formulation of these particles (Ramaswamy et al., 2003). The lipidic portions of the dendron move towards the DNA backbone through possible hydrophobic interactions. This has been visualised using a computer generated model shown in figure 1.12.

**Figure 1.11:** Model of lysine based-dendron structure used within the group and in the work described in this thesis.

The exact mechanism of binding of these host guest motifs has not been determined in detail, but it is based on acid-base interactions between the anionic phosphate moieties in the DNA backbone and the primary and tertiary amines in the dendron, which are positively charged under physiological conditions. It has been found by various groups including (Tang et al., 1996; Dennig and Duncan, 2002) that partial dendrimers, i.e. dendrons, are more suited for gene delivery than complete dendrimers of higher generations. Dendrons have a more flexible structure forming
more compact complexes with DNA, deemed to be more ideal for gene transfection in cells.

Not only do the cationic dendrons form aggregates with DNA, but also with other polyanionic molecules such as heparin. Complete association is dependent on the mass ratio between the dendrimer and unfractionalised heparin. The therapeutic potential of these dendrimer/heparin aggregates might be exploited in preventing angiogenesis in solid tumours (Kasai et al., 2002).

Figure 1.12: Computer generated model of dendriplexes using 32 bp segment of DNA A: (C_{10})_7 Lys(NH_2)_g dendron B: DNA C and D different view of the dendriplexes given by C.Ramaswamy, PhD thesis, University of London 2005.

Dendrisomes have the appearance and related properties of lipid vesicles, but with an apparently thicker bilayer membrane structure. They are reminiscent of cationic liposomes due to the structural conformation of the dendron used in the formulation. Lysine based dendrons and dendrimers used in our group have unique properties with low toxicity and high biopermeability. They can be manufactured using basic synthetic methods to yield a product of high purity.
1.8 ANTHRAX

Anthrax has become a vital subject because of recent bioterrorism threats. A heightened interest in anthrax pathogenesis, immunity, and vaccine development has ensued since. The only available vaccine relies heavily on concepts that date back to the early sixties. New vaccines are required that elicit protective immunity with a high degree of safety. These modern vaccines need to achieve more rapid onset of immunity with a limited number of doses which can be easily administered. This can only be achieved once a more detailed knowledge of the host immune system becomes available with the factors that account for protective immunity in humans.

The expression of anthrax is mediated by two secreted toxins: lethal toxin and edema toxin. Natural infection of anthrax is very rare and can only occur from exposure to spores from infected animals. The causative agent of anthrax is *B. anthracis*, the disease is only transmissible via their spores, which are very robust and the main route of infection in humans. These infective spores can be obtained from fermentation cultures and made in large quantities, and this is where the threat lies.

Knowledge of the binary sequence of the toxins of *B. anthracis* which are responsible for cell killing and host death is vital to design a new vaccine strategy. Two virulent determinants carrying two independent replicating plasmids, one of the plasmids (pXO1) encodes the genes necessary for the secreted toxin and the other (pXO2) for the formation of a capsule that prevents phagocytosis. The plasmid pXO1 encodes for protective antigen, PA, edema factor, EF and lethal factor, LF as well as the other compulsory genes. PA is recognised as the cell binding polypeptide which interacts with either EF or LF.

The complex of LF and PA is referred to as the lethal toxin. This toxin causes necrosis or apoptosis observed when human umbilical vein endothelial cells (HUVEC) were exposed to the lethal factor (Kirby, 2004). PA (protective antigen is named by the fact it induces antibodies against EF and LF providing immunogenicity against spore challenge and is the main active component of AVA (Ivins *et al.*, 1998). The lethal toxin plays a major part of the cause of the virulence associated with anthrax shown in figure 1.13. They paralyse the innate immune system by targeting various cell types like macrophages. Now that the principal mechanism of the disease along with its effect on the immune system is understood, modern
vaccines can be developed. These vaccines must be easily administered and produced in large quantities. There is an established vaccine which is anthrax vaccine adsorbed Biothrax (AVA).

![Diagram](image)

**Figure 1.13:** Depiction of the steps involved in anthrax toxin intoxication. Stage 1: PA binding to the extracellular receptor of a cell which becomes activated. A part of PA diffuses away called PA$_{20}$ in stage 2 while the rest oligomerises to form a heptamer. Stage 3b: EF and LF bind to the heptamer and becomes internalised by endocytosis and exposed to acidic pH of the endosome. This pH change triggers the mechanism that leads to symptoms of anthrax. However if anti-PA antibodies are present it stops the EF and LF binding thus anthrax symptoms do not appear depicted at stage 3a.
AVA is prepared by adsorbing filtered culture supernant of an attenuated strain to aluminium hydroxide as an adjuvant. The principal component is PA antigen with a small mixture of EF and LF. This vaccine is licensed in the UK to be given in 6 doses by subcutaneous injection. The safety of AVA has been called into question due to the recent allegations that the vaccine is linked to Gulf War syndrome. A review of the AVA vaccine was carried out by the Institute of Medicine with conclusions that the vaccine is safe and not associated with any systemic side effects (Sever et al., 2002). Although the overall conclusion pronounced it safe, the residual concerns over long term health affects remain. Thus, efforts have been made to develop a second generation anthrax vaccine which is safer and induces immunity.
1.9 AIMS OF THE WORK DESCRIBED IN THE THESIS:

The main aim was to develop and design a particulate system containing DNA that would provide for the DNA protection from degradation and elicit a sustained release mechanism. The application of this particulate system as a genetic vaccine with the plasmid DNA encoding the protein antigen in this case protective antigen (PA) to induce immunogenicity against anthrax. This involved various stages such as:

1) The synthesis by solid phase peptide synthesis and purification of cationic lysine based dendrons with some compounds containing lipidic chains.

2) Characterisation of the size and charge of the DNA/dendron complexes in terms of ratio of dendron:DNA.

3) Formulation of PLGA encapsulated dendriplexes to maximise payload and aid protection and release of DNA.

4) Toxicity analysis for both set of particulates,

5) *In vitro* study of DNA transfection using dendriplexes, PLGA-dendriplex particles containing pDsRed1-N1 (red fluorescent protein),

6) Evaluation of *in vitro* PA DNA uptake and antibody production when transfected as dendriplexes and PLGA-dendriplex particles,

7) *In vivo* study using both particulate systems investigating dose and number of administrations, by measuring anti-PA antibodies and toxin neutralisation assay to evaluate the efficiency of the vaccine.

The rest of the thesis is devoted to a description and discussion of the above topics.
CHAPTER TWO

DESIGN AND SYNTHESIS OF CATIONIC LIPIDIC LYSINE-BASED DENDRONS

2.1 INTRODUCTION

As described in the introduction to this thesis (Chapter 1), cationic lysine-based dendrons (partial dendrimers) with varying lipophilicity and peripheral functionality have a range of alterable physicochemical properties particularly of value in considering their use as vaccine delivery systems. The dendrons synthesized in this work and described in this chapter, are well defined small peptide based cationic polymers designed to complex with DNA (dendriplexes) thus forming nano-sized particles. Hence, the dendron must contain certain features enabling this complexation to occur. These include: i) water-solubility, also crucial for the dendriplexes designed for systemic administration; ii) biocompatibility, which should minimise cytotoxicity; and iii) biodegradability, which can circumvent problems related to polymer elimination. Amino terminated lysine dendrons were selected, as these compounds are cationic, water soluble and low generation and reported to have lower cytotoxicity than higher generation dendrons (Cloninger, 2002).

In this chapter, the method of synthesis (solid peptide synthesis) is explained and the dendron characterisation by mass spectrometry and NMR after purification by HPLC discussed.
2.2 DENDRON SYNTHESIS

2.2.1 Solid phase peptide synthesis (SPPS)

Peptide synthesis involves the formation of amide bond between a carboxyl group and an amino group of two amino acids. Amide bond normally required a large amount of energy therefore can be conducted at high temperature (160-180°C), which is quite in compatible with the presence of other functionalities (Montalbetti and Falque, 2005). Hence, activation of acid attachment of the leaving group to the acyl carbon of the acid, to permit the reaction with amino groups to form the peptide bond is necessary. The solid-phase procedure was first proposed by Merrifield in 1963 with the preparation of the tetrapeptide by successive addition of benzyloxy carbonyl amino acids to a polystyrene resin. The synthesis was undeniably successful, but as coupling or deprotection reaction was not brought to completion the tetrapeptide was contaminated with shorter peptides. By 1964, substantial changes to improve the efficiency of the technique were made by possible by using more acid-labile t-butoxycarbonyl derivatives as protective groups. Merrifield’s basic principles are now widely used in peptide chemistry, and used here to synthesise branched lysine-based dendrimers.

![Diagram](image)

**Figure 2.1:** The stepwise sequences involved in dendron synthesis during SPPS. This process begins with the selection of resin which is dependent on the methodology (Boc or Fmoc) and continues until the desired product (the dendron) is synthesised.
The principle of all solid phase synthesis is depicted in figure 2.1, the peptide is elaborated while attached to a stable solid bead. These beads are gels that swell but do not dissolve in the medium N,N-dimethylformamide (DMF). The addition of α-amino and side chain protected amino acid residues. The residues are either (Boc) tert-butoxycarbonyl protected or (Fmoc) fluorenylmethoxycarbonyl protected. The amino acid remains attached to the resin particle throughout the synthetic steps and is separated from soluble reagents from the solid support by filtrating and washing. The final step involves the cleavage of the desired product from the solid support. The side chain protecting groups are cleaved simultaneously with the cleavage from the resin.

The dendrons can be synthesised stepwise on the solid phase (divergent strategy) or, by ligation of prepurified fragment (convergent strategy). The divergent method, in which the growth of the dendron originates from the core site, is also shown in figure 2.2. This approach involves construction from the core and continues radially outwards by successive stepwise activation and condensation. This divergent approach is currently the preferred commercial route and used to synthesis the dendrons which this thesis. A second method pioneered by Fréchet and co-workers (1994) is the convergent growth process. The synthesis begins at what will be the periphery of the final macromolecule and proceeds inwards to the root of the dendron tree structure.

Divergent process:

Convergent process:

Figure 2.2: Illustration of the two methods in assembling a dendron during SPPS. Firstly the divergent growth occurs from the core to the surface of the dendron whereas the convergent method begins at the periphery and works inwards.
For successful synthesis of dendrons the reaction needs constant monitoring. This allows optimisation of the yields and minimises the products of side reactions. Monitoring this coupling reaction can be problematic when characterising the intermediates due to the limited analysis with on-bead methods. The most commonly used are colorimetric detection, coloured agents to follow the appearance or disappearance of a functional group is a classic method, adapted from TLC analysis. Modern techniques such as FTIR spectroscopy, have been developed that is sensitive and reliable to monitor SPPS.

2.2.2 Selection resin as solid support

Merrifield used polystyrene-divinylbenzene copolymer resin as the solid support. Divinylbenzene as the crosslinker on the bead aids insolubility, mechanical resistance and solvation. The solvation of gelatinous resins strongly influences the reactivity of the beads. These supports swell in most solvents and behave as gels. This gelatinous system, freely permeated by liquid media enhances access to the points of attachment to give a useful yield of peptide per unit volume. One of the main resins for Boc synthesis of peptide acids are the original Merrifield and improved PAM resin both based on polystyrene beads shown in figure 2.3. Gelatinous polymers other than these based on polystyrene have been employed in solid phase synthesis.

\[ \text{Figure 2.3: The resin consists of polystyrene beads (shown not to scale) functionalised with chloromethyl groups onto which the first amino acid of peptide is attached as shown above where A: Merrifield resin B: PAM resin. This resin is mainly used for Boc synthesis for small or medium sized peptides.} \]
The other resin available for Boc strategy and the most widely used is 4-methylbenzhydralamine (MBHA) resins (Marsueda and Steward, 1981). The resin selected as the solid support for the dendron synthesis is a polystyrene bead cross-linked with 1% DVB conjugated with a 4-methylphenyl-aminomethyl group.

![Chemical structure of MBHA resin](image)

**Figure 2.4:** Two other types of resins used in peptide synthesis are A: MBHA, mostly used for Boc chemistry and B: Wang resin selected for Fmoc strategy. The amino acid using MBHA resin is directly attached onto the amino group. The Wang resin is attached to the hydroxide group forming an ester linkage.

Other resins have now been developed like JandaJel solid support as an alternative to the traditional Merrifield resin for Boc chemistry (Moss et al., 2002). They claim increased swelling relative to the traditional resin affording a higher yield of pure peptide. Further investigation into the efficiency and the utility especially for dendron and dendrimers synthesis is required.

### 2.2.3 Protection/deprotection of amino acids

After the selection of methodology (Fmoc/Boc and resin) of the assembly, the protection strategy has to be chosen. Two double-protection strategies are commonly used in peptide synthesis: Boc (for N-\(\alpha\) amino groups) with benzyl (Bzl) for side chain protection (Anderson and McGregor, 1957) and Fmoc (for N-\(\alpha\) amino groups)
which is fully compatible with tert-butyl (t-Bu) based side chain protection (Carpino and Han, 1972) shown in figure 2.5. In the first strategy, Boc is removed with trifluoroacetic acid (TFA) and Bzl by HF, respectively the second Fmoc is removed by piperidine and t-Bu by TFA both methods cleaving the bond between the first residue and the solid support.

**Boc chemistry**

**Fmoc chemistry**

**Figure 2.5:** The protecting group strategies in SPPS for Boc and Fmoc chemistry (Novabiochem, 2005).
2.2.4 Activation and coupling

The process of coupling individual amino acids can be accomplished through the employment of the carbodiimide. The carbodiimides, primarily (DCC) dicyclohexylcarbodiimide, are the most widely used activating/coupling group reported by Sheenan and Hess (1955), selected because of its simplicity and for the apolar environment of polystyrene supports. All of the DCC and DCC-related derivatives are based on the formation of the symmetrical anhydride. The symmetrical anhydrides are very reactive and have been extensively used in solid phase peptide synthesis (Han and Kim, 2004). Essentially, the coupling regent promotes dehydration between the free carboxyl group of an N-protected amino acid and the free amino group of the C-protected amino acid resulting in an amide bond with precipitation of the by product N,N-dicyclohexylurea. This method, however is hampered by side reactions which can result in the formation of 5(4H-oxazolones) and N-acetylene. Consequently, new types of activating agents were developed, these including benzotriazolyloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP); (PyBOP) (Coste et al., 1990); 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3, 3-tetramethyluronium hexafluorophosphate (HBTU) (Knorr et al., 1989); and 2-(1H-Benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluoroborate (TBTU) (Gausepohl et al., 1992).

![Figure 2.6: Structures of coupling reagents available for peptide synthesis in order to suppress side reactions and racemisation in carbodiimide (Novabiochem, 2005).](image)
Fortunately, these side reactions can be minimised, if not eliminated by adding a coupling catalyst such as 1-hydroxybenzotriazole (HOBt) shown in figure 2.7 (Hudson, 1988).

**Figure 2.7:** The pathways of dicyclocarboimide coupling in the presence of HOBt leading to the same desired product but with reactive intermediates such as O-acylisourea, symmetrical anhydrides and HOBt ester of protected amino acid reducing the overall performance of the reaction.
In the synthesis of the lysine based dendron, HBTU was chosen as the coupling reagent (see Appendix 1). In coordination with a tertiary amine such as N,N-diisopropylethylamine (DIEA) are useful bases to ensure that the carboxylated group belonging to the N-protected amino acid remains in the anionic form. Hence this enables the reaction with the uranium cation to take place and then form a highly reactive intermediate acycloxyuronium, as depicted in figure 2.8.

Figure 2.8: HBTU coupling reactions showing the intermediate stages to achieve the final product. The coupling between the anionic carboxylate group and HBTU forms acyloyuronium a reactive intermediate. This intermediate reacts with HOBT therefore used as a catalyst to form HOBT ester or subsequently the second pathway forming a symmetrical anhydride. The anhydride can further react with HOBT(-OBt) and form the ester, illustrating the various pathways of protected amino acid.

In this work HBTU and HOBT were used as coupling agents with DIEA to minimise the side reactions that occur in Boc peptide synthesis (shown in figure 2.9).

Figure 2.9: General overview of Boc peptide chain synthesis used in this thesis, using MBHA resin as the solid support together with HOBT/HBTU coupling agents with DIEA.
2.2.5 Resin Testing assessing completion of coupling reaction

During the peptide synthesis process, testing the completion of coupling was done in our synthesis by using a coloured test called ‘Kaiser’ or ‘ninhydrin’ test. Named after Kaiser in 1970 who devised this colorimetric detection that is quick and easy to use during the coupling process. The resin beads change from a clear solution to a dark blue colour indicating the presence of free primary amino groups. The details of the Kaiser test is shown in appendix 1.

2.2.6 Cleavage from resin support for Boc chemistry

Cleavage of peptides for Boc-based resin and side chain deprotection require a strong acid. The most popular reagents are HF and trifluoromethane-sulfonic acid (TMSA). HF has many desirable characteristics for the deprotection of synthetic peptides and is the most frequently used (Sakakibora, 1971). On the other hand anhydrous HF is an extremely toxic, corrosive, and volatile liquid. The procedure must be carried out in compliance with strict safety specifications. Standard HF cleavage is conducted in a fume hood equipped with a scrubber is generally performed at temperatures of 0-5°C for a period of 30-60 mins. Controlling both the temperature and the time minimises side reactions during the cleavage process. These side reactions occur during the deprotection process and yields highly reactive carbonium ions via an $S_{N}1$ mechanism (Tam et al., 1983). Therefore to prevent the formation of carbonium byproducts alkylating side chains, scavengers such as p-cresol are employed. Under low-high procedure (Novabiochem) uses low concentrations of HF with a large amount of scavenger the cleavage mechanism changes from the usual $S_{N}1$ to $S_{N}2$ which doesn’t involve carbonium ion production. The drawbacks to the low high procedure are long cleavage time and large quantities of DMS therefore in our synthesis the standard HF procedure was performed. The peptide is precipitated by cold anhydrous ether to remove scavengers and separated by filtration and extracted by an appropriate percentage of glacial acetic acid and lyophilised. The product is re-suspended in double distilled water to remove residual acids and freeze-dried again.
2.3 EXPERIMENTAL

2.3.1 Materials

Reagents and chemicals used to synthesis lysine based dendrons are listed in appendix 2.

2.3.2 Methods

2.3.2.1 α-lipoaminoacids

Lipoamino acids are α amino acids with alkyl chains (attached where) as shown in figure 2.10. These therefore contain both hydrophobic and hydrophilic elements (Gibbons et al., 1990). Variation of structural components of the compound changes their physical properties e.g. lipophilicity is dependent on the length of the alky chains. Simultaneously these compounds possess polar and conformational behaviour maintaining their aqueous soluble properties. The structural features of the α-lipoaminoacids used in the synthesis of dendron dictate the behaviour of the lysine based dendrons. Lipidated peptide consisting of polyamide groups (4, 8 16 terminal lysines) were linked to the first generation lysine to three alkyl chains (C₁₄) and their interaction with liposomes was studied (Purohit et al., 2003). The lipophilic has been reported (Toth et al., 1999; Shah et al., 2000) to enhance the condensation of DNA (shown in chapter 3).

Peptide dendrimers comprised of amino acids with surface lipoamino acids chains have been used most extensively in biological applications. These lipidated peptides have profound advantages not only in drug delivery (Toth, 1991), but lubricants, detergents and in film formation (Gibbons, 1988).
The synthesis of an \( \alpha \)-lipoamino acid was first reported by Albertson, in 1946. Modifications have been made by Gibbons et al. (1990). The steps are shown in figure 2.11.

Two \( \alpha \)-amino lipidic acids were synthesised, \( \alpha \)-amino decanoic (C\(_{10}\)), \( \alpha \)-amino octanoic acid (C\(_{18}\)) depicted in figure 2.12 by using 1-bromohexane as (a1) and respectively 1-bromohexadecane (a2).
2.3.2.2 Protection of amino groups

Tertiary butocarbonyl (t-Boc) was chosen for protection of α-amino lipidic acids, because of the solubility properties in organic solvent and simplicity of removal procedure. Both compounds a1 and a2 were Boc protected as described by figure 2.13.
The resulting oil from the reaction illustrated in figure 3.3 was extracted with ethyl acetate after drying with anhydrous MgSO$_4$ and the organic layer was evaporated. The residue was titrated with cold acetonitrile and the desired product was filtered and dried to obtain compound c1 and c2.

**c1:** (C$_{15}$H$_{29}$NO$_4$)

**RMM:** 287.4

**Yield:** 80%

**MS:** 332 [M+2Na-H]$^+$ (100), 311 (18), 310 [M+Na]$^+$ (99), 254 (30), 232 (58), 188 (12), 186 (14), 142 (17), 57 (41).

**c2:** (C$_{23}$H$_{48}$NO$_4$)

**RMM:** 399.6

**Yield:** 75%

**MS:** 444 [M+2Na]$^+$ (77), 422 [M+Na]$^+$ (100), 366 (24), 344 (63), 300 (12), 254 (94).

2.3.2.3 **Coupling reactions of dendrons synthesised**

We designed and synthesized lipid-lysine dendrons with polycationic surfaces. Firstly the Boc-protected lipoamino acids were attached on the solid support (pMBHA resin), the number of additions of lipoamino acids and thus number of coupling reactions was dependent on the final structure of the dendron. Subsequent generations were built using lysine building blocks which were Boc-protected (Boc-Lys (Boc)-OH), resulting in several coupling steps to attain a final compound with seven lysine groups with eight terminal amino groups as shown in figure 3.4. All coupling was performed using HBTU and DIEA, using 3-fold molar equivalents for each generation of growth. After HF cleavage from the resin, the desired product was isolated and dissolved in 90% acetic acid and lyophilised. The compounds were purified by reverse-phase high performance liquid chromatograph using acetonitrile and water as solvents. The various dendron structures were confirmed by mass spectrometry and NMR.
Figure 2.14: Solid phase peptide synthesis of general dendron synthesised in this chapter
Our laboratory has established nomenclature to differentiate between the different compounds synthesised by the group given in Table 1. For example, \((C_{18})_3Lys_7(NH_2)_8\) denotes three \(C_{18}\) alkyl chains coupled to seven lysine branching units carrying eight terminal amino groups. This nomenclature was applied to all the dendrons synthesised in the chapter and throughout the thesis.

### 2.3.2.4 Mass spectrometry

More recently, electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI) MS have found widespread utility for peptide analysis. Both of the latter methods yield protonated molecules and, thus, provide direct molecular weight information. The mass spectra of the dendrons synthesised compounds are shown in appendix 3.

**Electro-Spray Ionisation Mass spectrometry (ES-MS):**

Electrospray mass spectrometry (ES-MS) was obtained using Finnigan Masslab Navigator quadropole mass spectrometer (\(N_2\) flow, 400 L/h; temperature, 150°C; cone voltage 25V; capillary voltage 3V).

**MALDI-TOF Mass spectrometry:**

The spectrum was obtained using Voyager-DE PRO MALDI-TOF (Biospectrometry Workstation, applied Biosystems).

### 2.3.2.5 High performance liquid chromatograph (HPLC)

Purification of cleaved dendron shown in table 1 was performed by RP-HPLC Hewlett Packard. All solvents used for the purification were HPLC grade. Analytical separation was carried out using \(C_{18}\) with 5 \(\mu\)m pore size and dimensions of 4.6 x 150nm reverse phase column. Following standard degassing techniques, particulate matter was removed from acetonitrile, increasing to 60% at 20 min. maintaining at this concentration for 20 min and decreasing steadily to 0% acetonitrile for 10 min at a constant flow of 1.2 ml min\(^{-1}\). The gradient was affected by two microprocessors-controlled Gilson 302 single piston pumps. Compounds were detected at 220nm with a Waters 486 tunable absorbance detector.
Table 2.1: Characterisation of dendrons synthesised with varying lipid chain number and length.

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>R</th>
<th>No of lipid Chains (n)</th>
<th>Terminal groups</th>
<th>Molecular formula</th>
<th>MW</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C_{10})_1(Lys)_7(NH_2)_8</td>
<td>NH_2</td>
<td>1 x C_{10}</td>
<td>8</td>
<td>C_{52}H_{106}O_{31}N_{16}</td>
<td>1083.55</td>
<td>74</td>
</tr>
<tr>
<td>(C_{18})_1(Lys)_7(NH_2)_8</td>
<td>NH_2</td>
<td>1 x C_{18}</td>
<td>8</td>
<td>C_{60}H_{122}O_{8}N_{16}</td>
<td>1195.71</td>
<td>82</td>
</tr>
<tr>
<td>(C_{18})_2(Lys)_7(NH_2)_8</td>
<td>NH_2</td>
<td>2 x C_{18}</td>
<td>8</td>
<td>C_{78}H_{157}O_{9}N_{17}</td>
<td>1477.08</td>
<td>80</td>
</tr>
<tr>
<td>(C_{18})_3(Lys)_7(NH_2)_8</td>
<td>NH_2</td>
<td>3 x C_{18}</td>
<td>8</td>
<td>C_{96}H_{192}O_{10}N_{18}</td>
<td>1758.45</td>
<td>85</td>
</tr>
<tr>
<td>(C)_0(Lys)_7(NH_2)_8</td>
<td>NH_2</td>
<td>0</td>
<td>8</td>
<td>C_{51}H_{103}O_{13}N_{18}</td>
<td>1174.91</td>
<td>77</td>
</tr>
</tbody>
</table>
Figure 2.15: Two HPLC chromatograms both obtained under the following conditions: 100μl (1mg/ml); C18 with dimensions of 4.6 x 150nm column; Solvent A: acetonitrile; Solvent B: Water at a constant flow rate of 1.0ml/min detected at 220nm. Attempt at purification of the dendron by collecting fraction at 2.354 and re-injected into HPLC illustrated by graph B.
The HPLC chromatograms (figure 2.15) depict two distinct peaks at 1.337 and 2.358 mins. The first peak is the impurities (i.e. small subunit peptides) and the second the dendron, which was extracted and analysed by mass spectrometry for all five dendrons synthesised. High performance chromatography is extensively used and widely accepted method for separation and purification and has been used by Islam et al. (2005) for PAMAM dendrimers.

2.3.2.6 NMR

The cationic based dendrons have been previously characterised and the concentration depended structural changes of the dendrons in solution were followed using $^1$H NMR spectroscopy (Zloh et al., 2005). The lysine units of the dendrons synthesised could be categorised as interior (Lys1, Lys2a and Lys2e) and exterior (terminal residues- Lys3aa, Lys3ae, Lys3ea, and Lys3ee) as illustrated in figure 2.16.

![Figure 2.16: The general structure of dendron (Lys)$_7$(NH$_2$)$_8$ synthesised depicting the interior and exterior lysine for example the term Lys 2a is used for the second lysine attached to the alpha (α) position of the first lysine whereas (e) denotes the ε position.](image)

The lysine components of the dendron can be divided, according to their environment in this case the interior branching and exterior residues. The interior lysine residues have amide protons involved in the formation of peptide bond compared to the exterior (or surface) residues which have amine protons.
Figure 2.17: $^1$H NMR spectrum of $(C_0)(Lys)_7(NH_2)_8$ in $D_2O/H_2O$ at room temperature.

Figure 2.18: $^1$H NMR spectrum of $(C_{18})(Lys)_7(NH_2)_8$ in $D_2O/H_2O$ at room temperature.
Four peaks were detected in the region of the $\alpha$ proton in the 1D spectrum (figure 2.17), two downfield peaks of three internal lysine $\alpha$ protons and two further upfield corresponding to four $\alpha$ proton. Therefore the spectrum of $(C)_{10}(Lys)_{7}(NH_2)_8$ relates to the typical spectrum observed for $(Lys)_7(NH_2)_8$ shown by the assignments of individual residues in Table 2.2. This is not the case for $(C)_{18}(Lys)_{7}(NH_2)_8$ and $(C)_{10}(Lys)_{7}(NH_2)_8$ dendrons, with lower ratios of $\alpha$ proton associated to the interior and exterior lysine residues which becomes more prominent as the lipidic chain increases, leading us to believe free hydrocarbon chains must be associated within the dendron aggregates.
However the assignments of the lipid chain peaks correlates to the structure for both $C_{18}$ and $C_{10}$ dendrons illustrated by Table 2.3.

**Table 2.3**: Assignments of $^1$H NMR spectra of lipid chains for $C_{18}$ and $C_{10}$ dendron.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>H-1</th>
<th>H-2</th>
<th>H-3</th>
<th>H4-H9</th>
<th>H4-H17</th>
<th>H10</th>
<th>H18</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{18}$</td>
<td>1.91</td>
<td>1.72</td>
<td>1.52</td>
<td>-</td>
<td>1.27</td>
<td>-</td>
<td>0.87</td>
</tr>
<tr>
<td>$C_{10}$</td>
<td>1.91</td>
<td>1.72</td>
<td>1.47</td>
<td>1.26</td>
<td>-</td>
<td>0.86</td>
<td>-</td>
</tr>
</tbody>
</table>

These dendron molecules are complex structures making the NMR assignment difficult for these compounds. The results obtained in this $^1$H NMR spectra for the three dendrons illustrates the discrepancies in the appearance of peaks as the hydrocarbon chain increases leading to the speculation that even with extraction and purification by HPLC, defects in the structure, incomplete reactions especially in relation to the longer hydrocarbon chain dendrons leads to changes in peak areas which are easily detected by NMR.
CHAPTER THREE

FORMULATION AND DESIGN OF DENDRIPLEX PARTICLES

3.1 INTRODUCTION

In this chapter a selection of lysine-based dendrons complexed with DNA ("dendriplexes") were characterised by various biophysical methods, in order to investigate the relationship between their structural properties and transfection efficiency. Therefore we have conducted an extensive study of the optimisation of formulations of the dendron/DNA particles and their biophysical characterisation with the goal of establishing a correlation between these physical properties and effective gene delivery. The colloidal properties measured included size, morphology and zeta potential as a function of the charge ratio of dendron to DNA (N/P ratio). Diameters were obtained by standard dynamic light scattering and size and morphology observed by transmission electron microscopy. Dynamic light scattering has been the most frequently used technique (Braun et al., 2005) and provides a measure of volume type size distribution of the particle suspension. The relative affinities of DNA/dendron interactions were determined by ethidium bromide dye displacement, together with a measure of the release of the DNA when challenged with anionic compounds (such as heparin).

The DNA is complexed rather than delivered as naked DNA, as it has been shown to produce low levels of gene expression in all major organs (Liu et al., 1995). Specific carrier delivery systems are required which have low cytotoxicity and are biocompatible with biological systems. The majority of gene therapy trials use viral vectors, which are more efficient yet plagued by safety concerns (Roy, 2001). Hence
despite the lower efficiency, non-viral vectors still attract attention due to their advantageous safety profile. Our work has focussed with the use of dendrons as DNA carrier systems i.e. complexes of DNA. These dendrons where synthesized as has been described in chapter 1. The size of these synthetic complexes plays a vital role in gene transfer. Plasmids of ($10^3$-$10^4$ base pairs) have effective hydrodynamic diameters in excess of 100 nm and a highly negative surface charge density (Ledley, 1996). The large size limits their transport through tissues, thus cationic polymers by complexation reduce the overall size of the DNA condensates and promote localised delivery due to their nano-sized complex structures. Recently, ultrafine silica nanoparticles with surfaces functionalised with cationic-amino groups, have been shown not only to bind and protect DNA from enzymatic degradation but also to express encoded proteins (Kneuer et al., 2000). Another study has examined the particle size of DNA condensates produced with polylysine, by varying the size from dp (degree of polymerization) 30 to 1500, it was found that low molecular weight polylysine, condensed DNA into small particles (20-60 nm in diameter) and was also less toxic to cells in culture than high molecular weight polylysine (Wolfert et al., 1996). The size of the dendriplexes is vital in terms of uptake and release of DNA in cells, in order to be able to pass through the nuclear pore (~ 25 nm in diameter) and gain access to the actual nucleus to express the selected proteins.

The dendriplexes are formed by condensation, which has received considerable attention in the development of gene delivery vehicles (Golan et al., 1999). The dendrons cause localised bending or distortion of DNA at a critical extent of charge neutralisation, which facilitates the formation of these compact nanostructures. DNA can under different conditions condense into toroids and short rods of varying sizes, which is dependent on the concentration or length of the DNA (Golan et al., 1999). Commercial polylysine has been used to condense DNA, but not only does the polymer have a distribution of molecular weights but toxicity becomes an issue. Dendrons are unique synthetic polymers with a highly branched structure that can be modified according to their biological applications (Niederhafner et al., 2005). Consequently, their size and structure are highly controllable and their molecular weight distribution is generally very narrow. Taking into account these factors, lysine dendrons are attractive materials for the design of gene carriers.
Using a group of structurally related dendrons, the effects of vehicle constituents and the preparation technique on the pDNA complexation properties were systematically examined prior to in vitro transfection studies.

3.2 EXPERIMENTAL

3.2.1 Materials
Ethidium bromide (Promega, Southampton UK), Heparin (Sigma Chemical, USA), Agarose (Promega, Southampton UK), Brilliant Blue R250 (Sigma Aldrich, UK), 3.05 mm copper grid for TEM (Taab, UK). Materials used in dendron synthesis were discussed in chapter 2.

3.2.2 The plasmid DNA construct:
The plasmid DNA chosen was pDsRed1-N1 (Clonetech, USA), which encodes for a red fluorescent protein (figure 3.1). DNA pDsRed1-N1 has been optimised for high expression in mammalian cells, and can be easily detected. The red fluorescent protein produced when plasmid is expressed, is found throughout the cytoplasm, in addition to the nucleus. Therefore this causes the whole cell to fluoresce brightly red, detectable under a fluorescence microscope using a rhodamine filter. The pDsRed1-N1 plasmid was amplified by transformation into DH5-α competent Escherichia coli cells (Clonetech) according to standard procedures (Sambrook & Russell 2001, Molecular Cloning, a laboratory manual, 6ed. Cold Spring Harbor Laboratory press). The initial bacterial stock was grown overnight at 37°C in 10 ml Luria Broth media containing kanamycin (LB media: 1 % w/v tryptone (Oxoid, UK), 0.5 %w/v yeast extract (Merck, Germany), 1 % w/v NaCl (Sigma Chemicals, USA) in water, 50 μg/ml kanamycin (Sigma Chemicals, USA)), was further amplified in 1 L of Luria broth containing kanamycin (50 μg/ml), for 6-7 h. The bacteria were pelleted using centrifugation (5000 rpm for 20 min, Beckman J6) and Qiagen EndoFree Plasmid Mega Kit was used according to manufacturer’s protocol.
Figure 3.1: A schematic overview of the vector pDsRed1-N1 containing a kanamycin and neomycin resistance segment, used for selectively culturing the bacteria containing the plasmid. The size of the plasmid is 4700 base pairs.

3.2.2.1 Determination of plasmid concentration by U.V. absorbance

U.V. absorbance readings were taken using a Beckman DU650 spectrophotometer together with micro-cell cuvettes with a volume capacity of 100 µl (Beckman, UK). The concentration was determined by measuring the absorbance at 260 nm and 280 nm. Dilution of DNA was required to be within the working range for Beer-Lambert's law to apply when calculating the DNA concentration. The purity of the DNA can also be assessed using the absorbance ratio at 260 nm and 280 nm. The calculated ratio of $A_{260}$ over $A_{280}$ for pure DNA should fall within the range of 1.75 - 2.10. Measurement of U.V. visible absorbance is the simplest technique for direct measurement of proteins and nucleic acids, therefore it was also used to determine the concentration of plasmid in the dendriplex samples. A standard curve was prepared using varying concentrations of plasmid or dendriplex samples with known concentrations of plasmid.

An agarose gel was primed, by preparing the samples which were loaded into the wells by using a gel loading buffer (Eppendorf, Germany) and the gel was run at 90 V for 3 h. To determine the size of the bands detected in the samples, standard DNA markers were used. These markers were loaded onto the gel; DNA marker XVII (Boehringer Mannheim, Germany) is a 500 base pair (bp) ladder, which ranges from 500 bp to 5000 bp. DNA marker II (Roche Diagnostics, Germany) ranges from 564 to 23,130 bp. After 3 h the gel was exposed to U.V. light, and the stained restriction fragments appeared as fluorescent orange bands on the gel.
3.2.3 Dendriplex formulation

Charge ratios between dendron (molar positive charge) and plasmid DNA (molar negative charge) were calculated. A charge ratio 2:1 refers therefore to 2 positive charges (from dendron) per 1 negative charge (from plasmid) calculated from the molecular weight of the dendrons and the charge ratios between the dendron and the DNA. The concentrations were calculated for each selected ratio (0.5:1, 1:1, 2:1, 5:1, 10:1). Individual dilutions were performed for DNA and dendron at each charge ratio, calculated as shown below:

\[
\text{Amount of dendron} = \frac{\text{Weight of DNA}}{\text{RMW of nucleotide}} \times \frac{\text{Charge ratio}}{\text{Number of charges}} \times \text{MW of dendrons}
\]

The dendriplexes (figure 3.2) were formed by addition of equal volumes of DNA and dendron while stirring. These complexes were allowed to equilibrate for 30 mins prior to analysis. Complexes were prepared in double distilled de-ionised water unless otherwise noted.

![Figure 3.2: Schematic representation of positively charged dendriplex formation after condensation of DNA with a specific dendron.](image)

3.2.4 Size measurement

The size of the particles was determined by Photon Correlation Spectroscopy (PCS) using a Malvern 4700c sub-micron particle analyzer. Particles move randomly in Brownian motion and it is from this that PCS determines the mean particle size (the z-average diameter) based on the intensity of light scattered and the width of
distribution the (polydispersity index). The size of a particle is calculated from the diffusion coefficient, by using the Stoke-Einstein equation:

\[ d(H) = \frac{kT}{3\eta D} \]

\( d(H) \), is the hydrodynamic diameter; \( k \), Boltzmann’s constant; \( T \), absolute temperature; and \( D \), diffusion coefficient.

The diameter, measured by PCS, is a value that reflects how a particle diffuses within a fluid and is the known as hydrodynamic diameter. The upper limit for the size measurement is dependent on the density of the sample and is determined by the inception of sedimentation of the sample. The lower limit is determined by the concentration of the sample. Consequently the range that the PCS can determine is between 50 nm and 3 μm measured at a fixed angle of 90°. All samples were measured after calibration of the instrument with Duke Scientific nanosphere standard polystyrene 204 ± 6 nm in diameter.

3.2.5 Zeta potential measurement

The zeta potential of the dendriplexes was measured with a Malvern Zetasizer 3000 (Malvern instruments, Malvern, UK). Particles when migrating through a liquid medium under the influence of an applied electric field, are attracted towards the electrode of the opposite charge. Viscous forces tend to oppose this movement until equilibrium is reached, when the particles move with a constant velocity. The technique is known as laser Doppler velocimetry (LDV).

The particle velocity is dependent on a number of factors, the strength of electric field, the viscosity of the medium and the zeta potential. The velocity of the particle in a unit field is referred to as its electrophoretic mobility (Malvern instruments technical notes). Zeta potential is related to electrophoretic mobility by the Henry equation:

\[ U_E = 2 \varepsilon z f(Ka) / 3\eta \]

where \( z \) is the zeta potential; \( U_E \) is the electrophoretic mobility; \( \varepsilon \), the dielectric constant; \( \eta \) viscosity; and \( f(Ka) \) Henrys function.
The zeta potential measures the charge at the intersection between the particle and the Stern layer and not the surface potential. Before the samples were studied it was calibrated with a zeta potential transfer standard with a known potential of -50 mV ± 5 mV. The sample was diluted by adding 9 ml of PBS (MilliQ System, Millipore, USA) to 1 ml of nanoparticle solution.

3.2.6 Transmission electron microscopy (TEM)

To visually examine the dendriplex structure, TEM was employed. Specimens were prepared by depositing a small droplet of complex suspension on a copper grid (Gilder, Grantham, UK). The samples were stained with 1 % uranyl acetate and viewed under a Philips 201L electron microscope.

3.2.7 Ethidium bromide displacement assay

Quantitation of ethidium bromide (EtBr) intercalation between DNA base pairs was determined by fluorescence spectroscopy (Perkin Elmer luminescence spectrometer LS50B). The plasmid DNA (10 μg) in double distilled nuclease free water containing EtBr (500 ng/ml). The resultant fluorescence emission (at excitation and emission wavelengths of 516 and 598 nm, respectively) was measured and taken to represent 100 %. Dendron was then added incrementally, the sample mixed and the decreased fluorescence was allowed to stabilise prior to reading. The fluorescence was corrected at each dilution and expressed as a percentage of emission intensity attributed to that for DNA alone (Sorgi et al. 1997). Adaptations of the assay were made to examine the differential effects of anionic polymers (heparin) upon the displacement of dendron from DNA binding. Specially, dendron: DNA complexes equivalent to charge ratio 5:1 were prepared in distilled water and fluorescent signal arising from co-incubation with EtBr was allowed to stabilise. This complex was then challenged by incremental additions of various ratios of heparin. Differential displacement of dendron from DNA binding following anion challenge was also examined by gel electrophoresis.

Dendriplexes were prepared by mixing in double distilled de-ionised water to a final plasmid concentration of 10 μg/ml and subjected to agarose gel electrophoresis.
(0.8 % ethidium bromide included for visualisation) for 2 h at 5 V/cm. Images were obtained using a U.V. transluminator and a Gene Genius Syngene (Bioimaging systems, Cambridge) with the settings adjusted to avoid signal saturation. Band integration and background correction were obtained using Molecular Analyst software version 1.1.

3.2.7.1 Gel electrophoretic retardation

The dendriplexes were pulse-spun at 13500 rpm in a benchtop microcentrifuge followed by brief vortexing to ensure full mixing of the solutions. The complexes were then re-spun and incubated at room temperature for 30 min. Loading buffer (3 μl) was added and the samples were re-spun. Samples were loaded (10 μl) into a 0.8 % agarose gel in TAE (containing 1 μg/ml EtBr) and electrophoresed at 3.5 V/cm for 2 h using TAE as running buffer. Gels were photographed under U.V. transillumination prior to staining for polymer for 40 min followed by an overnight destain. Agarose of molecular biology grade (Promega, Southampton, UK) was used for gel studies, these gels were run as described above (section 3.2.7). However additional polymer staining with 1 % w/v solution of brilliant blue R250 in 50/10/40 methanol/glacial acetic acid/distilled water was conducted. Destaining was carried out with a 10/10/80 mixture of these solvents.
3.3 RESULTS AND DISCUSSION

3.3.1 Characterisation of dendriplexes

The mean hydrodynamic diameter and zeta potential of the complexes were determined as described. The results are shown in figure 3.3. At low molar charge ratios of dendron to DNA (the N/P ratio), large aggregates (>1 µm) were formed for all lysine based dendrons employed in this study. In general, ratios above 2:1 were required to form stable compact particles with a narrow size distribution. Lipidic dendrons rather than non-lipidic formed particles at high charge ratios (range 4-10) over a narrow size distribution of 82 nm to 85 nm in diameter. These findings were verified and compared to the complexes with C₀ (no lipidic chain) dendron even at elevated molar charge ratio, which had a broader more heterogeneous particle size distribution than the lipidic dendrons. Ferrari et al. (2001) made similar observations with lipoplexes varying in polymer alkyl chain length and headgroups, and finding vehicle-dependent effects on the degree of accessible pDNA. The larger the number of branched groups in the polyethyleneimine structures the smaller the particle size, as verified by Türk and co-workers (2004). The length and the number of lipidic chains of the dendron greatly influence the efficiency of condensation of DNA. These complexes are driven by the electrostatic attraction between the cationic dendron and the negatively charged phosphate backbone of DNA, complemented by any hydrophobic interactions. Therefore, the hydrophobic interactions of the lipid based dendron with the DNA, aids the formation of narrow and homogeneous particle size, as reflected in the data in figure 3.3 A and B.

The size distribution is one of the most important properties of polycation-plasmid DNA complexes, and care has to be taken with the interpretation of the data. There are a variety of particle size analysis techniques, and the dispersion process and shape of the particle makes the analysis more complex. Three types of mean data can be obtained from the PCS technique, intensity, volume, and number diameters, the selection as to which value to use dependent on the purpose of the measurement. In our case, the larger diameter size of the particles was required, not just at the smaller end of the size scale represented by the number values illustrated in figure 3.4A. Three profiles are shown for 0.5:1 C₁₀ dendriplexes depicting quite similar trends, with two adjoining peaks. However the volume values relate to the larger distribution
of particles than the other two methods (Rawle, Malvern Instruments). Therefore the mean volume size values were used to compare the various dendriplex formulations. The particle size distribution provides additional information about the homogeneity of the dendriplexes, which in most cases is not discussed in the literature where the mean values are usually quoted. Clarification in the method of analysis must be provided as different size outcomes can be assumed from different laboratories. This makes it difficult to compare values of different particulate systems, especially when comparing the data and relating it to the efficiency of gene transfer.

The 0.5:1 molar charge ratio has a broad size distribution shown in figure 3.4 for all sets of particles formulated, a higher volume of particles larger than 1 μm was found for Co dendriplexes, that was less prominent for the other dendriplex formulations. The size distribution was expected, due mainly to the low charge ratio below the point of neutralisation. Two distinct size populations are observed for the 2:1 ratio (figure 3.5), the largest at approximately 800 nm; slight variations of these size populations were dependent on the lipophilicity of the dendron, indicating that the DNA was not fully condensed. The lack of condensation of DNA by all dendrons was equal or lower than 2:1 charge ratio. Meanwhile the particle size for the 10:1 ratio, all fell below 100 nm in diameter, a size which is considered to be appropriate for transfection (Vijayanathan et al., 2002). This was shown by figure 3.6, where the C18 dendriplexes formed one peak at about 90 nm in diameter. The size of the dendriplexes decreased as the number of C18 chains increased, a result emphasised by the clear shift of the particle distribution peak of the (C18)3 dendriplexes towards the lower end of the hydrodynamic size range.

In addition the zeta potential (figure 3.9) verified the differences in size distribution of the various dendriplexes with different charge ratios (N/P) from approximately -30 mV at 0.5:1 ratio, to +30 mV at 10:1 ratio. Differences were dependent on which dendrons were used in the particle formulation. Positive values were observed only when the N/P ratio was higher than 1, except for the C0 dendriplexes where the ratio increased to above 2. This corroborated with the previous size data on the state of condensation, again emphasising the importance of lipophilicity of the dendron on the formation of compact dendriplexes.
Figure 3.3: A) Mean hydrodynamic diameters of dendriplexes formulated with a series of dendrons and B) polydispersity index of the complexes presented as a function of charge ratio. The data are the mean of at least three separate measurements with error bars representing the standard error.
Figure 3.4: Comparison of particle size distributions for 0.5:1 molar charge ratio dendriplex formulations using different dendrons. A) the \((C_{10})_{2}\) dendriplexes comparing the three types of mean hydrodynamic size values. B) two sets of \(C_{18}\) dendriplexes, and C) \(C_{6}\) dendriplexes all were prepared in double distilled de-ionised water, containing 10 \(\mu\)g of DNA. Note the difference in the y-axis scale of the results in A).
**Figure 3.5:** Comparison of volume particle size distribution for 2:1 dendripplexes, where graph A) shows data for the C_{18} dendripplexes with varying alkyl chains numbers and graph B) a comparison between C_{6}, C_{10} complexes.
Figure 3.6: The volume particle size distribution for 10:1 dendriplexes, the graph A compares the size population of the C₁₈ dendriplexes (C₁₈), and (C₁₈)₃) were prepared in double distilled de-ionised water, containing 10µg of DNA.

At a 10:1 DNA to (C₁₈)₃ dendron charge ratio, monodispersed toroidal structures were observed by transmission electron microscopy (TEM) and their mean size (approximately 85 nm) was in accordance with the values found using dynamic light scattering (figure 3.8B). This was consistent with the magnified TEM version of the same sample, containing some rod shaped dendriplexes but with the majority compact spherical particles. The morphology of the 0.5:1 ratio complex was not identical to that of the 10:1 ratio, with irregular aggregates ranging from 250 nm to above 500 nm. When compared to the particle size distribution graph in figure 3.4B depicting a wide size population and the mean values in figure 3.3A, the TEM showed the sample of the dendriplex population was at the lower end of the size range.
Figure 3.7: The effect of molar charge ratio and medium on the apparent particle size of C18 dendriplexes, A: complexes formed in water or diluted into Opti-MEM. B: A stability assessment over 28 days of three sets of C18 dendriplexes containing varying number of chains formulated in double distilled water. Mean ± S.D. (n=3). Dendriplexes were stable in distilled water over the 28 days without any significant change in particle sizes, however in Opti-MEM changes were observed near neutralisation point between DNA and dendron.
Figure 3.8: Morphological structure of (C<sub>19</sub>) dendriplexes, all containing 10 µg of DNA. A) Electronmicrographs of dendriplexes at 0.5:1 molar charge ratio showing both toroidal structures and irregular aggregates of dendron and DNA; B) dendriplexes at a 10:1 ratio demonstrating compacted toroid DNA particles; C) magnified 10:1 dendriplexes and D) 10:1 dendriplexes incubated in Opti-MEM for 5 h.
Figure 3.8D illustrates the change in morphology of the 10:1 dendriplexes formed spherical to larger aggregates when incubated in Opti-MEM, which was the media normally used for transfection studies and contain all the essential supplements, without phenol red. The graph in figure 3.7, verified the particle size growth which is clearly sensitive to charge ratio, the greatest increase occurring at or above the 3:1 charge ratio. Other studies by Pouton and co-workers (1998) using polylysine-DNA complexes confirm instability to electrolyte challenge when these complexes were diluted in Opti-MEM. They observed that this phenomenon was also sensitive to charge ratio, with the greatest growth at 1:1 ratio, suggesting that this stoichiometric ratio is close to the point of neutralisation between the DNA and polylysine. Aggregation is of course linked to changes in the electrical double layer, leading to reduced charge repulsion between particles. They further indicate that the size of the complexes less than 100 nm in diameter may not play a significant role in the mechanism of transfection in vitro. Other groups such as Braun et al. (2005)
characterised PAMAM dendrimers and DNA complexes and revealed no obvious correlation with tranfection efficiency.

### 3.3.2 Release of DNA from dendriplexes

From the previous findings we can conclude that in order to form compact stable complexes, high molar charge ratios must be utilised, but the release of active DNA from these dendriplexes can be evaluated using gel electrophoresis. Gel electrophoresis is still the most direct and sensitive approach to examine the stability of plasmid DNA in a gene delivery formulation. The dissociation of DNA from the dendriplexes in the presence of an anionic counter species, in this case heparin is depicted below in figure 3.10.

![Figure 3.10: Results of 0.8% w/v agarose gel electrophoresis and EtBr staining of (C18)3 dendriplexes decomplexed with heparin. Lane 1: DNA marker II, lane 2: pure plasmid, lane 3: 10:1 dendriplex, lane 4 - 7: 10:1 dendriplex with incremental addition from 0.2 – 0.8 units of heparin, and lane 8: DNA marker XVII.](image)

Figure 3.10 firstly revealed no bands for the dendriplex alone, the lack of fluorescent staining of DNA on an agarose gel containing ethidium bromide, a known DNA intercalator that only fluoresces upon DNA complexation, suggested that dendriplexes are fully condensed structures.

Molecular modelling by Zinselmeyer and co-workers (2002) of polypropylenimine dendrimer/DNA complexes, demonstrated that the polypropylenimine dendrimer was
able to bind an entire helical turn of DNA molecules, therefore minimising the potential contact points preventing intercalation of ethidium bromide (EtBr).

**Figure 3.11:** A schematic diagram of the intercalation of ethidium bromide into a DNA molecule. Ethidium bromide increases the spacing of base pairs and distorts the regular sugar-phosphate backbone, which allows fluorescent tracking of DNA and assessment of stability by electrophoretic gel. Reproduced from Watson *et al.* Molecular Biology of the gene, Benjamin/Cummings Publishing, 2003.

With addition of heparin in lanes 4-7, the DNA dissociated from the dendron enabling the EtBr to intercalate with the released DNA. When comparing these bands with the intensity of naked DNA in lane 2, it shows that not all the DNA was separated from the dendriplexes. The agarose gel was further analysed by densitometry where the DNA release from the dendriplex was quantified, that showing approximately 58% of DNA was released when 0.2 units of heparin was added to the complex. This did not significantly increase even when the concentration of heparin was elevated from 0.2 to 0.8 units, which indicated that maximum decomplexation had been reached.

In an effort to quantitatively assess DNA condensation, EtBr was added prior to complexation with incremental addition of structurally different lysine based dendrons, therefore measuring the percentage of quenching of fluorescence of EtBr.
until a certain polycation mass was reached, forming complexes more resistant to disruption following challenge with anionic counter species.

Figure 3.12: A) Ethidium bromide exclusion assay, relative fluorescence of dendron-DNA complexes in double distilled water is expressed as the percentage, following incremental addition of dendron, of the initial fluorescence of ethidium bromide (500 ng/ml) in the presence of pDsRed1-N1 (10 μg). B) demonstrates the anionic displacement of various structurally different dendrons from DNA. Dendriplex with 5:1 charge ratio were challenged by incremental addition of heparin and relative fluorescence was measured. Data represented as mean ± S.D., n=3.
Figure 3.13: Gel electrophoresis retardation results for pDsRed1-N1/ (C<sub>n</sub>), dendron complexes, where the lower image shows fluorescently labelled plasmid migration and the upper image illustrates the same gel following staining for polymers. The anodic and cathodic ends are marked with + and −, respectively, and the loading wells are marked by arrows. In lane 1: DNA marker and lanes 2-9 contain 0.25, 0.5, 0.75, 1.0, 2.0, 5.0, 10:1 dendron :DNA respectively, whereas lane 10 contains naked DNA (2 μg) and lane 11 dendron alone.
Figure 3.14: Gel electrophoresis retardation results for C₉ dendriplexes, where the lower image shows fluorescently labelled plasmid migration and the upper image illustrates the same gel following staining for polymers. The anodic and cathodic ends are marked with + and -, respectively and the loading wells are marked by arrows. In lane 1: DNA marker and lane 2-9 contain 0.25, 0.5, 0.75, 1.0, 2.0, 5.0, 10:1 dendron :DNA respectively, whereas lane 10 contains dendron alone and lane 11 naked DNA (2 µg).
Figure 3.15: Agarose electrophoretic gel depicting the retardation results for \((C_{10})_1\) dendripplexes, where the upper image shows the staining for dendron and the lower illustrates fluorescently labelled plasmid migration of the same gel. Lane 1 contains DNA marker; lane 2 naked DNA (2 µg) and lanes 3-9 contain various complex ratios as follows: 0.25, 0.5, 0.75, 1.0, 2.0, 5.0, 10: 1 (dendron: pDsRed1-N1 DNA) respectively.
Many similar studies have been conducted for different polymers using this methodology such as Trubetskoy et al. (1999); Wiethoff et al. (2003) and Tokunaga et al. (2004). Maximal DNA condensation was represented as a plateauing signal between 10 - 15 of relative fluorescence, was achieved at a charge ratio of 3:1 for all C_{18} dendron (3 to 1 alkyl chains) together with C_{10} dendron whereas a ratio of 4.5:1 and 6:1 for C_{0} dendron, polylsine respectively (figure 3.12A). The corresponding IC_{50} values of 1.0, 1.1, 1.4, 1.6 for C_{18} dendron (3 to 1 alkyl chains); C_{10} dendron including 2.15, 2.3 for C_{0} dendron and polylsine. IC_{50} is defined as the polycations:DNA charge ratio at 50% relative fluorescence. It was clear from the IC_{50} values, and the mass of polycations required to achieve maximal condensation, that the dendron:DNA complexes display dissimilar DNA condensation behaviour. The (C_{18})\textsubscript{3} dendron appeared to induce DNA condensation more readily than the polylsine and the C_{0} dendron, this corresponded to the interpretation of previous size and zeta potential data.

To further probe for differential properties of the dendron-DNA complexes, we adapted the spectrofluorimetric EtBr exclusion assay to assess the stability of the respective complexes to challenge with anionic species. In this case, heparin was used, as in the gel electrophoresis study. Specifically, preformed 5:1 dendriplexes were challenged with heparin as illustrated in figure 3.12B. The graph illustrates the change in relative fluorescence as a function of the incremental addition of heparin, expressed as the molar ratio of negative charge, derived from anion counter species, to the positive charge contributed by the dendron condensing agent.

The addition of heparin resulted in a continued gradual increase in fluorescence until it reached approximately 80%, where the signal plateaud between 5:1 and 6:1 anion:cation molar ratio for all dendriplexes studied. Slightly greater recoveries in relative fluorescence (implying greater dissociation of the dendriplex under heparin challenge) were observed with (C_{18})\textsubscript{1} dendriplex and (C_{10})\textsubscript{1} dendriplex, than the (C_{18})\textsubscript{3} dendriplexes. Taken in its entirety the spectrofluorimetric EtBr exclusion and anionic displacement data would support the hypothesis that (C_{18})\textsubscript{3} dendron interacted with pDsRed1-N1 DNA, and that the stability of the resulting complexes were distinct.

Agarose gel electrophoresis was used to monitor the interactions of dendrons and DNA, visually revealing much detail of the biophysical properties of the complexes.
The standard agarose gel procedure was adapted in order to stain for the polymer, this technique has been used previously by Jones et al. (2000). As mentioned previously, DNA bands become slightly dimmer due to the loss of fluorescence of EtBr near complete complexation. As the zeta potential data revealed, the dendriplexes at this point changed from negative to positively charged particles, which are than more likely to migrate to the cathodic side. By staining the gel for excess dendron we were able to visualise the migration of the complexes. In our case there was a clear indication of migration, especially when the fluorescent bands disappear above 1:1 charge ratio. Both (C_{18})_3 (figure 3.13) and (C_{10})_1 (figure 3.15) dendriplexes have similar patterns, as the concentration of dendron in the complexes (higher N/P ratio) increased, a higher shift towards the cathodic side was observed. However the retardation results for C_0 dendriplexes, shown by figure 3.14, contrast with the trends seen for the other dendriplexes, with the highest mobility only at ratios above 2:1 indicating that hydrophobic interactions of the lipidic dendrons aid the formation of compact nanostructures.

3.4 CONCLUSION

This study of five related lysine-based dendrons show that even a relatively small difference in structural composition of the polymer can have profound effect on the efficiency of condensation of DNA. This was clearly illustrated by the (C_{18})_3 dendriplexes particularly with stoichiometric ratios above 5:1. The overall strength of interaction had great impact on the colloidal properties of the dendriplex shown by the size and zeta potential results, together with the electrophoresis gels and morphology evaluated by TEM. The overall stability of these dendriplexes in transfecting medium such as Opti-MEM, was evaluated. The sizing results and TEM micrographs appear to show significant alteration as the ratio of dendron to DNA was varied. These appear to be greatest up to the point where the amount of the dendron becomes equal to that of the DNA. With this biophysical data of the various dendriplex formulations, the next step involved *in vitro* assessment of gene transfer.
CHAPTER FOUR

FORMULATION AND DESIGN OF DENDRIPLEX-PLGA PARTICLES

4.1 INTRODUCTION

This chapter discusses the encapsulation of DNA in poly(lactide-co-glycolide-acid) (PLGA) nanoparticles, a process which may be advantageous in controlling DNA release and its protection against degradation when administered as a non-viral gene delivery carrier. One facet of the work was to condense the therapeutic DNA with the dendron as shown in chapter 3, and further to encapsulate the complex in PLGA nanoparticles, as these biodegradable particles have been shown to be potent antigen delivery systems (Jilek et al., 2004). We hypothesise that the dendriplexes instead of naked DNA offer the distinct advantage. These include circumventing the degrading effects on DNA during the PLGA formulation, facilitating rapid delivery to target antigen presenting cells and provide an additional adjuvant effect observed by particulate systems (Jiang et al., 2005).

PLGA polymers based on lactic and glycolic acid are thermoplastic polyesters which are being extensively investigated for various therapeutic applications especially for drug, vaccine, and gene delivery (Panyam et al., 2003). Lei Jang and co-workers have investigated the potential of cationic PLGA and cetyltrimethylammonium bromide (CTAB) with DNA containing proteins against hepatitis B adsorbed onto the surface of such particles, the results show them to be promising candidates to evoke immunogenicity and enhanced immunoprotection.

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Prior to *in vivo* testing, the double emulsion methodology was optimised to produce dendriplex-PLGA particles less than 1 μm in diameter. Size significantly affects the level of cellular uptake in most cell lines, only submicron size particles being efficiently taken up (Panyam *et al.*, 2003). The dendriplex-PLGA particles of varying formulations were characterised with regard to their physicochemical properties, encapsulation efficiency and release of DNA.

4.2 MATERIAL AND METHODS

4.2.1 Materials and DNA

Poly(lactide-co-glycolide) (PLGA) (50:50), Medisorb DL 2A, (Mw 15,200, with carboxylic end groups) purchased from Alkermes. Poly(vinyl alcohol) (PVA) of MW 13000-23000 (87-89 % hydrolysed) was supplied by Sigma-Aldrich. The plasmid DNA, pDsRed1-N1 (Clonetech, USA), encoding for a red fluorescence protein was as described in chapter 3, section 3.2.2. All other chemicals were of analytical grade.

4.2.2 Radiolabelling pDsRed1-N1 DNA

To label the plasmid DNA with $^{35}$S dCTP, a modified nick-translation technique previously described (El Ouahabi *et al.*, 1997) was used (Figure 4.1). Briefly, plasmid DNA (10 μg/50 μl) was prepared from a stock solution (1 μg/μl) to be within the working range 20-200 ng/μl in TE (Tris-HCl, EDTA) buffer. The reaction mixtures of nucleotide/buffer containing 50 mM Tris-HCl pH 7.8, 10 mM MgCl$_2$, 2-mercaptoethanol, 100 mM of each dATP, dGTP, dTTP and enzyme solution (0.5 units/μl DNA polymerase I, 10 pg/μl DNasel) and 250 μCi $^{35}$S dCTP were added to pDNA. Unincorporated radiolabelled nucleotides are removed using gravity-flow chromatography with a maximum volume of 100 μl, prepacked with Sephadex G-50 DNA grade in distilled water with 0.15 % Kathon CG/ICP Biocide (Figure 4.2). These conditions allow the incorporation of labelled nucleotide into the plasmid DNA without significant alteration of plasmid integrity, as assessed by agarose gel electrophoresis (Figure 4.3).
Figure 4.1: Schematic diagram of DNA radiolabelling methodology using a nick translation kit. Step A involves an enzyme DNA polymerase activity acting in the 5' → 3' direction. Removal occurred in the adjacent 5' terminus. Step B includes the addition of $^{35}$S nucleotides sequentially to the 3' end of a nick within a DNA duplex.
Figure 4.2: Sephadex G-50 DNA grade filtration profiles of $^{35}$S DNA. Eluent fractions (20 µl) were collected and radioactivity measured. The graph illustrates two peaks: the first represents the radiolabelled DNA and the second, the unincorporated radiolabelled nucleotides. The fractions from the first large peak were collected and assessed by agarose gel electrophoresis.

Figure 4.3: Agarose gel electrophoresis of $^{35}$S DNA (10 µg) of both radiolabelled and non-radiolabelled DNA was applied to 0.8% agarose and run at a constant voltage and detected by U.V lamp. Lane 1 contains DNA marker, lane 2: naked DNA (4.7 kbp), and, lanes 3-9: radiolabelled fractions of DNA, respectively.

4.2.3 Dendriplex preparation

An aqueous stock solution of 1 mg/ml of each dendron was made and subsequent volumes were removed with a concentration of 10 µg of DNA. The DNA and dendron were separately diluted in 250 µl PBS mixed, and allowed to stand for 15 mins as described in chapter 3. Dendriplex hydrodynamic diameter and zeta potentials were measured using a Zetasizer 3000 (Malvern Instruments, Malvern, UK).
4.2.4 Scanning electron microscopy

The dendriplexes were characterized using a Philips CM 120 BioTwin transmission electron microscope; samples were stained with 1 % uranyl acetate.

4.2.5 Dendriplex-PLGA formulation

The dendriplexes were separately encapsulated by a double emulsification method using a poly (vinyl) alcohol (PVA, 3 % w/v, Fisher, USA) solution added to PLGA (50:50) dissolved in DCM forming a primary emulsion after homogenisation (13,500 rpm), as shown in figure 4.4. This o/w emulsion was added to a solution of PVA (1.25% w/v) and further homogenised (10,000 rpm) to form a w/o/w emulsion. The PLGA particles were harvested by centrifuging at 20,000 g at 15 °C and subsequently washed, then lyophilised to permit long term stability.

Figure 4.4: Schematic diagram of the methodology for dendriplex and dendriplex-PLGA particles formation. The dendriplexes formed by mixing the DNA and dendron. These particles were added to 3% w/v of PVA. The surfactant solution containing the dendriplexes were homogenised with PLGA (50:50) in DCM forming a primary o/w emulsion. A second concentration of 1.25% w/v PVA was homogenised with the primary o/w emulsion giving a w/o/w emulsion.
4.2.6 Encapsulation efficiency

Radio-labelling pDNA allowed quantification of the encapsulation efficiency of the dendriplexes in PLGA particles. The particles were formulated as stated, they were harvested by centrifugation, washed to remove excess PVA and aliquots of the supernatant collected. Supernatant radioactivity was measured by scintillation counter. The lyophilised particles were dissolved in DCM and incubated for 30 min. The radiolabelled DNA was extracted by forming organic-water phases and aliquots from the aqueous layer were taken and radioactivity measured.

4.2.7 Release of DNA from dendriplex-PLGA particles

DNA release from different nanoparticle formulations was determined in PBS (5 ml) (pH 7.4) at a dendriplex-PLGA concentration of 2 mg/ml. The vials were incubated at 37 °C under gentle shaking and periodically samples were withdrawn and replaced by fresh PBS. The DNA concentration in the supernatant was quantified as described in the encapsulation method.

4.2.8 Cell culture

Aliquots from the release study containing DNA extracted from PLGA-dendriplex particles were incubated with CHO-K1 cells and Caco-2 cells grown on coverslips, details in 5.2.8. Cell were maintained in DMEM supplemented with 1 % non-essential amino acids, 10 % FCS and penicillin (50 µg/ml) incubated in standard cell culture conditions.

4.2.9 X-Ray Photoelectron Spectroscopy(XPS)

XPS determines the chemical composition of a surface using photoelectric effect. The PLGA-dendriplex particles were irradiated with X-ray photons and electrons are emitted from the particles if the photon is of sufficient energy.
The kinetic energy of these photoelectrons is measured by the analyser to determine the elemental composition of the surface of the particles.

**Figure 4.5:** Schematic diagram of XPS methodology depicting the depth of sample surface analysis.

Samples were measured and analysed by Dr Roger Nix, Queen Mary, University of London using XPS scienta ESCA 200.
4.3 RESULTS AND DISCUSSION

4.3.1 Optimisation of multiple w/o/w emulsion method

The water in–oil in water (w/o/w) emulsion technique is a well established method to prepare PLGA nanoparticles used by various groups such as Julienne et al., 1992; Scholes et al. (1993); Hausberger et al. (1995); Hsu et al. (1999) and Jain, (2000). The method is best suited to encapsulate water soluble drug-like peptides and in our case DNA, unlike the o/w method which is best for water insoluble drugs like steroids. The o/w technique produces microparticles larger than 1 μm, whereas the double emulsion method can be modified to formulate nano-sized particles. Several process parameters can influence the particle size distribution, such as homogenisation rate, concentration of surfactant and the amount of polymer. These parameters were varied and individually investigated to optimize the method. This was done by selecting criteria for the size, size distribution, morphology, and stability of the PLGA particles.

The emulsifier employed in the preparation of PLGA particles was polyvinylalcohol (PVA), commonly used since it allows the formation of particles of relatively small size and uniform size distribution. The mechanism of PVA’s action has been proposed to be due to the interpenetration of PVA and PLGA molecules during nanoparticle formulation (Hsu et al., 1999). The hydrophobic part of PVA penetrates into the organic phase and remains entrapped into the polymeric matrix of the nanoparticles. Elevated PVA concentration of the continuous phase could lead to an increase in PVA molecule density at the o/w phase of the emulsion droplet. Therefore resulting in the formation of a stable emulsion, with a small and uniform droplet size. The mean size of the nanoparticles decreased from 815 to 576 nm with an increase in the PVA concentration in the external aqueous phase. The range of PVA concentration from 0.25 to 1.5% w/v, shown in Table 4.1.
Table 4.1: Effect of PVA on the physicochemical characteristics of blank PLGA nanoparticles.

<table>
<thead>
<tr>
<th>PVA concentration (%w/v)</th>
<th>Dynamic Viscosity of PVA (mPa.s)</th>
<th>Mean particle size (nm) *</th>
<th>Polydispersity index **</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.783</td>
<td>815 ±54.2</td>
<td>0.27</td>
<td>-20.3 ± 1.06</td>
</tr>
<tr>
<td>0.5</td>
<td>0.810</td>
<td>777 ±31.3</td>
<td>0.23</td>
<td>-19.3 ± 0.40</td>
</tr>
<tr>
<td>1.0</td>
<td>0.922</td>
<td>655 ±29.9</td>
<td>0.19</td>
<td>-16.4 ± 0.32</td>
</tr>
<tr>
<td>1.5</td>
<td>1.103</td>
<td>576 ±22.8</td>
<td>0.16</td>
<td>-12.9 ± 0.50</td>
</tr>
</tbody>
</table>

* determined by PCS ** PCS value

Previous reports by Scholes and colleagues (1993) corroborate this decrease in particle size when the PVA concentration of the aqueous phase is increased. This change in size was probably due to an increase in the stability of the emulsion when the PVA concentration was augmented. As the PVA concentration increases the surface layer of PVA increases, causing greater repulsion between the particles and forming smaller particles with a lower polydispersity index.

The homogenisation step was the determining factor in obtaining submicron particles. The emulsification of the oil and water by mechanical shear creates small droplets with diameters less than 500 nm for the primary emulsion, overall producing smaller particles (Figure 4.6).

Figure 4.6: Scanning electron micrographs of blank PLGA particles, where the primary o/w emulsion was homogenised at two different speeds A: 10,000 rpm, B: 13,500 rpm, the overall mean sizes being 465 nm and 350 nm respectively.
The concentration of the PLGA polymer had no clear effect on the overall size of the particles, except for a slight increase in zeta potential due to the uncapped carboxyl end groups of the polymer (Zambaux et al., 1998). Therefore with the optimised method established (section 4.2.5), the next step was the addition of the dendriplexes to the PLGA formulation preparation.

4.3.2 Characterisation of PLGA-dendriplex particles

The dendriplexes were added to the primary PVA solution, to investigate any change in morphology, transmission electron micrographs (TEM) were taken of the \((\text{C}_{18})_3\) dendriplex at 10:1 ratio in 3 % w/v PVA, depicted in Figure 4.7. The TEM illustrated no change in the size of the particles which have a mean size of 85 nm.

![Image of TEM micrograph](image)

**Figure 4.7:** Transmission electron micrograph of \((\text{C}_{18})_3\) dendriplexes at 10:1 ratio in 3 % w/v PVA, the mean particle size of 85 nm, equivalent to the size of dendriplexes in distilled water shown in chapter 3, fig. 8b. It seemed that the PVA coated the dendriplex (see text).
Figure 4.8: The relationship between the mean particle size of PLGA particles containing 10 μg dendriplexes (n = 3) after nanoparticles were washed with distilled water, lyophilised and re-suspended prior to measurement. The decrease in mean size from ~ 800 nm to values between 200-500 nm for the C_{10}−C_{18} dendriplex containing particles was clear.

Table 4.2: Zeta potential (mV) characteristics of dendriplex-PLGA particles.

<table>
<thead>
<tr>
<th>Molar charge ratio of dendriplexes</th>
<th>(C_{18})_1</th>
<th>(C_{18})_2</th>
<th>(C_{18})_3</th>
<th>(C_{10})_1</th>
<th>(C_0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5:1</td>
<td>-14.3 ± 3.2</td>
<td>-15.3 ± 2.1</td>
<td>-16.1 ± 1.7</td>
<td>-13.2 ± 1.5</td>
<td>-12.2 ± 0.8</td>
</tr>
<tr>
<td>2:1</td>
<td>-15.5 ± 2.5</td>
<td>-19.6 ± 2.3</td>
<td>-18.8 ± 1.1</td>
<td>-14.9 ± 0.2</td>
<td>-13.7 ± 0.2</td>
</tr>
<tr>
<td>5:1</td>
<td>-21.2 ± 1.9</td>
<td>-22.7 ± 0.7</td>
<td>-20.5 ± 0.6</td>
<td>-21.1 ± 0.4</td>
<td>-18.6 ± 1.1</td>
</tr>
<tr>
<td>10:1</td>
<td>-25.3 ± 1.6</td>
<td>-23.9 ± 0.5</td>
<td>-22.6 ± 0.3</td>
<td>-21.9 ± 0.2</td>
<td>-19.9 ± 0.4</td>
</tr>
</tbody>
</table>

However, the PVA did seem to accumulate around the dendriplexes almost forming a secondary coat around the particles. Oupicky and co-workers (2000) established PVA’s ability to condense DNA by the ethidium bromide displacement method, supporting our suggestion that PVA forms a secondary coat around the dendriplexes, and therefore aiding the encapsulation process of the dendriplexes into the PLGA particles.
The hydrodynamic sizes of both dendriplexes before and after encapsulation were measured (the size characterisation of the dendriplexes in described in chapter 3). To summarise the previous results, the data show that the molar ratio was important in the condensation process. A 10:1 C_{18} dendron:DNA ratio enabled complexes of less than 80 nm diameter to be formed (polydispersity, 0.26). The lipophilicity of the C_{18} dendron enhances the compaction and uniformity of the complexes. At a 2:1 ratio of all five dendrons the apparent particle size of the dendriplexes was over 800 nm but this significantly decreased at the 5:1 molar ratio. The DNA was not fully condensed at the 2:1 ratio as evidenced by the size and the zeta potentials from approximately -30 mV at 0.5:1 ratio, to +30 mV at 10:1 ratio. The differences were dependent on which dendrons were used in the particle formulation.

The size of the dendriplex-PLGA particles (Figure 4.8) displayed a similar trend, size decreased as dendriplexes with increasing charge ratio were encapsulated. The hydrodynamic size of PLGA-dendriplex particles were strongly related to the efficiency of DNA condensation. Generally an increase in PLGA-dendriplex particle size was observed (at N/P >2), and in comparison to the primary dendriplexes where an increase in size may reveal higher encapsulation efficiencies. There was little disparity in the size and size distribution of PLGA nanoparticles containing dendriplexes before and after lyophilization (data not shown). The zeta potentials (Table 4.2) of the PLGA-dendriplexes were approximately -15 mV at neutral pH with no significant difference in relation to the molar ratio of encapsulated dendriplexes.

Quantification of the DNA encapsulated in the PLGA-dendriplex particles was attempted firstly by extraction with organic solvent followed by aqueous extraction of DNA. Secondly, the concentration of DNA was determined by U.V. spectrophotometry illustrated by the U.V. scans from 200 to 400 nm in figure 4.9 of (C_{18})_3 dendron and (C_{18})_3 dendriplexes respectively. The scans show that the dendrons do not absorb at 260 nm, which indicated that any free dendron would not interfere with the absorbance of the dendriplexes, and therefore allowing for the quantification of the DNA in the PLGA particles. However, PVA (the surfactant used in the PLGA formulations) absorbed at 260 nm as shown in figure 4.9C, which may result in elevated absorbance readings and thus higher encapsulation efficiencies. Non-radioactive methods were not used to quantify the encapsulation
efficiency and release of DNA in the PLGA particles, the alternative method was radiolabelling the DNA.

Figure 4.9: Two U.V. scans from 200 nm to 400 nm where scan A depicts (C_{18})<sub>3</sub> dendron and B represents (C_{18})<sub>3</sub> dendriplexes, illustrating that the dendron does not absorb at 260 nm unlike the dendriplexes, C: depicts the absorbance at 260 nm of different PVA concentrations.

4.3.3 Encapsulation and release of DNA from PLGA-dendriplex particles

The C<sub>18</sub> dendriplexes-PLGA particles were used for the encapsulation (Table 4.3) and release study. Cationic C<sub>18</sub> based dendriplexes were more efficiently encapsulated than native DNA with a size population of less than 1 μm and at a 10:1 ratio had a mean size of 451.6 ± 2.4 nm. The difference in size population between the PLGA-dendriplex (10:1 and 2:1) particles and encapsulated with native DNA were further exemplified by figure 4.10.
Table 4.3: Encapsulation efficiency and mean size of PLGA particles containing C\textsubscript{18} dendriplexes.

<table>
<thead>
<tr>
<th>Molar charge ratio of encapsulated species</th>
<th>DNA encapsulation % w/w</th>
<th>Mean diameter (nm)</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:1</td>
<td>15.6</td>
<td>451.6 ± 2.4</td>
<td>0.291</td>
</tr>
<tr>
<td>2:1</td>
<td>13.4</td>
<td>733.5 ± 7.2</td>
<td>0.391</td>
</tr>
<tr>
<td>DNA alone</td>
<td>9.9</td>
<td>998 ± 4.34</td>
<td>0.521</td>
</tr>
</tbody>
</table>

Figure 4.10: Plot of percentage particles in class over a range 200-1299 nm. $^{35}$S radiolabelled DNA was mixed with varying ratios of (C\textsubscript{18})\textsubscript{3} dendriplexes entrapped in PLGA, washed, and lyophilised. The particles were dissolved in DCM and incubated for 30 mins; distilled water was added and mixed.
Figure 4.11: a) Cumulative release of radiolabelled DNA from PLGA particles containing entrapped DNA or (C_{18})_{3} dendrplexes with varying ratios of DNA/dendron in isotonic PBS, pH 7.4.

b) The release of DNA for these systems, but normalised to a standard DNA content and particle size (that of the PLGA particles containing uncomplexed DNA).
The release rate of radiolabelled DNA from the encapsulated dendriplexes at 2:1, 10:1, and native DNA particles was determined (Figure 4.11A). An initial burst effect was seen in the first 24 h, followed by a slower controlled release for all three sets of PLGA particles. Luu et al. (2003) measured the release of DNA from a porous scaffold using PLGA: an initial burst effect was also found, but between 18 and 36 % DNA depending on copolymer content was released after 15 mins in aqueous suspension.

In our study, the particles containing DNA alone released 27 % of DNA over 9 days, yet approximately 55 % of DNA was released from the particles containing a 10:1 dendriplex. When different DNA loads and particle sizes were taken into account, as shown in figure 4.11B, the particles containing the uncomplexed DNA had a higher rate of release compared to the dendriplexes. The integrity of the DNA was assessed by electrophoresis (no data shown), no bands were observed suggesting that the dendron was possibly still complexed with the DNA when released from PLGA particles. A second method of evaluation was the transfection of the aliquots taken from the release profiles that contained the DNA released from the PLGA particles, into CHO-K1 cells, as shown in figure 4.13.

The morphological changes of the particles during dissolution were observed by SEM (Figure 4.12). The 10:1 C₁₈ dendriplex-PLGA particles which had a hydrodynamic diameter size of 510 ± 12 nm, changed shape and morphology after 24 h with a concave appearance, others crumbling. The surface of the particles changed with increasing porosity after 48 h, until finally they ruptured. Gebrekidan et al. (2000) observed convective diffusion of pDNA through the porous structure of PLGA particles with complexed pDNA/PLL, and was released in a sustained fashion. The burst effect could be due to the porous nature of the PLGA around the dendron explaining the change in morphology.

The addition of lipidic dendrons condensed DNA, aiding efficient encapsulation into PLGA particles. The PLGA nanoparticles provide a system which could protect DNA from degradation and provide sustained release of DNA.
Figure 4.12: Scanning electron micrographs showing a gradual degradation of PLGA particles containing dendriplexes. A and B: 10:1 C18 dendriplex-PLGA particles at two different magnifications at the initial time point; C: after 24 h in PBS, D after 48 h.

Figure 4.13: Transfection of CHO-K1 cells with aliquots taken from release study at the 24 h time point containing DNA released from PLGA-C18 dendriplex particles. Panel A: photomicrograph of CHO-K1 cells under light microscope and B depicts the expression of red fluorescent protein encoded in the plasmid DNA by cells under fluorescent microscope. The expression of released DNA from the study confirmed the activity and integrity of the DNA during the unravelling of the particles.
Figure 4.14: XPS (surface analysis) of PLGA particles containing 2:1 and 10:1 dendriplexes.

X-ray Photoelectron spectroscopy (XPS) is a sensitive technique capable of quantifying the elemental composition of the outermost 1-10 nm of a sample surface (Demathieu et al., 1999). By using this technique it was possible to evaluate if dendriplexes were present at the particle surface (figure 4.14). The evaluation of the surface by XPS showed no phosphorus peak and nitrogen peak from DNA and the dendron, therefore suggesting the absence of dendriplex on the surface of the PLGA-dendriplex particles for both the 2:1 and 10:1 ratios. This verified that the DNA complexes were encapsulated into the PLGA matrix, with little or none remaining on the surface of the particles.
4.4 CONCLUSION

A range of dendriplex formulations were successfully encapsulated into PLGA particles. Both the release and encapsulation efficiency were dependent on the molar charge ratio of dendron to DNA in the dendriplexes. Therefore the primary characteristics of the dendriplexes (chapter 3) greatly influence the overall physicochemical properties of the PLGA particles. For synthetic polymers such as PLGA, the integrity of the DNA can be affected by the degradation of the polymer to lactic acid and glycolic acid. However in our case a secondary protection system was utilised, the dendron providing not only protection but also sustained release. The formulations were used in animal studies, as discussed in Chapter 7.
CHAPTER FIVE

IN VITRO STUDIES ON DENDRIPLEXES AND PLGA-DENDRIPLEX PARTICLES

5.1 INTRODUCTION

In vitro gene expression of pDsRed1-N1 DNA complexed with an array of assemblies of dendrons at various molar charge ratios, encapsulated in PLGA particles is the main theme of this chapter. Before transfection studies were commenced, the toxicity of the dendrons must be considered beforehand. Thus the cell line, incubation time, and assay must be selected to determine the level of cytotoxicity, with knowledge of the dendrons intended use as gene delivery systems. Toxicity is defined as a measure of non-specific unnecessary harm a drug or polymer may elicit towards cells and other tissues (Duncan and Izzo, 2005), in our case involving the dendron alone, dendriplexes and PLGA-dendriplexes. Simple in vitro assays such as measurement of cytotoxicity against Caco-2 and CHO-K1 cell lines with incubation times of 24 h, which is the maximal incubation time when measuring the gene expression in the cell lines stated, involves assessment of the cell viability measured by 3-(4,5-dimethythiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay was used to assess preliminary formulations prior to in vitro evaluation using PA DNA (chapter 6) and further in vivo testing (chapter 7).

These cytotoxicity tests have been adopted by many workers (Roberts et al., 1996; Malik et al., 2000; Jevprasesphant et al., 2003, and Klajnert et al., 2006) all having widely documented that dendrons as well as dendrimers containing amine termini exhibit concentration and especially in the case of dendrimers generation-
dependent cytotoxicity. Dendrons have a smaller number of amine functionalities on
the surface than their parent dendrimer, further emphasising the rational in dendron
selection, and when complexed to DNA the complexes are less cytotoxic, mainly due
to the overall reduction in the charge of the particles (Brazeau et al., 1998).

Condensed dendron/DNA complexes bind non-specifically to the cell surface,
often due to the negative charge of the cells electrostatically interacting with
positively charged complex, and are then subsequently taken up by cells by
absorptive endocytosis. Once dendriplexes are taken up by the cell and released from
endosomes into the cytosol, the complexes must undergo two processes. Firstly there
is migration to the nucleus through the cytoplasm and secondly, the DNA must
disassemble from the dendriplex before it can be transcribed (Panyam et al., 2002).
The failure to overcome these two barriers often results in low gene expression and
may be due to a number of factors, such as ineffective endosomal release,
degradation in the cytoplasm or limited migration to the nucleus. Cornelis and co­
workers (2002) observed the degradation of naked DNA microinjection in the
cytoplasm of HeLa cells under the confocal microscope, with a typical half life of
between 50 to 90 min. Transport of the transfecting DNA into the nucleus is still a
matter of debate. Suggestions that the nuclear import of DNA was facilitated during
mitosis were based on correlation between the frequency of cell divisions at or near
to time of transfection for the final transgene expression stage (Wilke et al., 1996).

It is generally accepted that a part of delivery of non-viral vectors occurs through
an endocytic process although the amount of DNA uptake from carrier vehicles such
as dendriplexes and PLGA-dendriplexes is unknown. The secondary protection of
the DNA provided by PLGA particles results in the sustained release of DNA and
subsequent sustained gene expression has been shown by Hedley et al. (1998). In
vivo work conducted by Labhasetwar et al. (1999), involved the use of an osteotomy
model which were dosed with PLGA nanoparticles containing reporter plasmid DNA
also illustrated sustained expression, 5 weeks after surgery.

In this chapter we focus on the cytotoxicity, efficiency of uptake and gene
transfection of both dendriplexes and PLGA-dendriplexes, they will be compared
and their differences highlighted.
5.2 MATERIAL AND METHODS

5.2.1 Materials

Dimethylsulfoxide (DMSO) (VWR International Ltd), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich), Trypan blue solution (0.4%), Dulbecco’s modified Eagle medium with Glutamax-I (DMEM) (Invitrogen), OptiMEM-1 serum free medium, penicillin solution (Invitrogen), non-essential amino acids solution 100x (NEAA), foetal calf serum (FCS), phosphate buffered saline (PBS) (pH 7.4, 10X), Hank’s balanced salt solution (HBSS), trypsin/EDTA (1X) (Gibco Invitrogen Corporation, UK), Costar Transwell polycarbonate cell culture inserts (12 mm diameter, growth area 1.13 cm², pore diameter 3.0 μm), 96- and 12-well plates (Corning Costar Corporation, USA), Optiphase ‘safe’ scintillation cocktail (Perkin-Elmer, UK), slides 76 x 26 mm (Menzel-glaser), Coverslips 22 x 22 mm (Scientific laboratory supplies LTD), Vectashield (Vector laboratories Inc), ammonium chloride (BDH Lab supplies), paraformaldehyde (Sigma-Aldrich), polylysine (Sigma-Aldrich). Materials used for radiolabelling DNA were described in chapter 3.

5.2.2 Preparation of dendriplexes and encapsulated dendriplex particles

Dendriplexes were prepared as described in chapter 3, section 3.2.3. All five dendrons, as synthesised in chapter 2 were used for the complexation with pDsRed1-N1 DNA unless specified. PLGA-dendriplex particles were prepared by the double w/o/w emulsion as shown in chapter 4, section 4.2.5. Only the 10:1 molar charge ratio primary dendriplex formulation was used for encapsulation.

5.2.3 Cell culture

P3X63Ag8 cell line (mouse myeloma, derivative of plasmacytoma in BALB/c mice), Chinese hamster ovary cells (CHO-K1) and colon adenocarcinoma cells (Caco-2) were purchased from ECACC (European Collection of Cell culture). Cells were maintained in DMEM supplemented with 5 % non-essential amino acids, 10 % FCS and penicillin (50 μg/ml) at 37 °C, 5 % CO₂, in 95 % relative humidity. All three cell
lines were passaged at 3-4 day intervals in a 10:1 dilution in tissue culture flasks (75 cm² growth area). The passage occurred when cells were 80% confluent, the medium removed and cells washed with HBSS and digestion with trypsin/EDTA. The cells were detached from the tissue culture flask using 5 ml of warm trypsin/EDTA for 1 min, 4 mls was aspirated leaving 1 ml of cell suspension containing trypsin/EDTA. The tytpsinization was stopped by adding 9 ml of serum containing DMEM. The cells were centrifuged for 5 min at 1000 rpm at 4 °C, the pellet collected and resuspended in 10 ml of serum containing medium.

5.2.4 MTT assay measuring cell viability

The MTT assay is a standard colorimetric method for measuring cellular proliferation by the amount of yellow MTT oxidised to purple formazan which is quantified spectrophotometrically. This oxidation takes place when mitochondrial reductase enzymes are active, and thus conversion is directly related to the number of viable cells. The production of purple formazan in cells treated with the cytotoxic drug is measured relative to the production in the control cell, hence elucidating the cytotoxicity of the drug (Mosmann, 1983).

The cells were seeded into 96-well plate at a density of 100,000 cells per well using a cell suspension assessed by trypan blue exclusion method. Repeated media change every second day after seeding. After three days the cells were incubated with 100 μl of dendron solution (0.2-2.5 mg/ml) in DMEM for 4 h at 37 °C. The plates were tilted and dendron solution was aspirated and fresh media (180 μl) was added including 20 μl of MTT solution (5mg/ml) incubated with cells for 8 h. The plates were wrapped in aluminium foil due to MTT photosensitivity. The plates were tilted to aspirate media without touching the cells. DMSO (200 μl) was added to each well then placed on a rotary shaker for 20 min to solubilise the crystals as depicted by figure 5.1. The absorbance was read at 550 nm and 660 nm with untreated wells as the control. The results were expressed as the percentage viability (n = 5; ± S.D) using:

\[
\text{% viability} = \left( \frac{A_{550} - A_{630}}{A_{550} - A_{630}} \right) \text{of treated wells} \times 100
\]

\[
\left( A_{550} - A_{630} \right) \text{of untreated wells}
\]
A Cells seeded and grown in 96-well plate for 3 days

B Addition of test material to allocated wells and none to the control

C 1) Removal of testing material
2) Incubation with 0.5 mg/ml MTT solution
3) Solubilisation using DMSO
4) Measurement of optical density at 550 nm and 630 nm

Figure 5.1: Outline of protocol of MTT assay, divided into three main steps: A) seeding and growth of cells; B) treatment stage of cells and C) the processing stage. The photograph of an original 96-well plate shows in the highlighted black box are the untreated wells (control) and in the red box are the treated wells, viable cells give dark blue formazan product. Damaged cells show reduced or clear colour demonstrating no mitochondrial dehydrogenase activity.
5.2.5 $^{35}$S-DNA uptake study across Caco-2 monolayer

Caco-2 cells were seeded into 12 well transwells at a density of $1.5 \times 10^5$ cells/cm$^2$ of which 0.5 ml was added to the apical side of the Costar Transwell cell culture insert and the confluent monolayers (21-28 days) were used for transepithelial transport studies. The monolayers were used when TEER was in range of 600-1000 ohmsxcm$^2$. The cell culture medium in both apical and basolateral chambers was replaced with serum free transport medium, OptiMEM, prior to initial experiment commencing. This medium was selected as it contained essential supplements cells require without serum components. The red coloured component of DMEM causes quenching of the radioactivity of $^{35}$S-DNA therefore OptiMEM without phenol red was utilised.

Figure 5.2: Representation of transwell plate containing Caco-2 monolayer cultivated on top of uncoated polycarbonated membrane. These cells were grown for 21 days to allow the formation of a thick continuous monolayer. The transport of dendriplexes and PLGA-dendriplexes across this monolayer was assessed.
Dendriplexes at various molar charge ratios containing $^{35}$S-DNA and PLGA-
dendriplex particles at 10:1 ratio were added to the basolateral chamber and cells
were incubated in a humidified atmosphere at 37 °C. At time points 0, 15, 30, 60,
120, 240 min cells were rinsed twice with PBS to remove any precipitated complex
and lysed with hypotonic solution (450 µl) together with lysis medium (50 µl).
(Hypotonic solution: 0.03 % magnesium chloride in 0.24 % HEPES buffer, lysis
solution: 5 % ethylhexadecyldimethylammonium bromide in 3 % glacial acetic acid).
The lysed cells were assayed in LS6500 Multi-purpose Scintillation Counter
( Beckman, USA) using Optiphase ‘safe’ scintillation cocktail (9 ml). The results
were expressed as % $^{32}$S DNA cumulative transport ($n = 3; ± SD$).

5.2.6 TEER permeability

The integrity of the monolayer was monitored by measurement of the potential
difference between the apical and basolateral side of the Caco-2 monolayer following
transepithelial transport of $^{35}$S DNA in dendriplexes and PLGA-dendriplex particles.
At specified time points, the transepithelial electrical resistance (TEER) was
measured with specially designed electrodes (World Precision Instruments, USA).
The TEER was expressed as transmembrane resistance (ohm x cm$^2$) after subtraction
of the intrinsic resistance of the control (from cell-free inserts).

5.2.7 In vitro gene expression

A red fluorescent protein expressing plasmid (pDsRed1-N1) was amplified to
sufficient quantities in *Escherichia coli* and purified with a Qiagen Maxi-prep kit
(Qiagen, USA) and complexed with various assemblies of dendron at specific charge
ratios. PLGA containing dendriplexes at 10:1 molar charge ratio were utilised in the
transfection studies.

For the transfection studies, 6-well plates containing polylysine coverslips (section
5.2.7). In each well, cells were seeded at a density of $60 \times 10^3$ cells per well in 2 ml
of DMEM supplemented with foetal calf serum. These wells were then incubated at
37 °C in 5 % CO$_2$ for 24 h. Afterwards, the medium in each well was replaced with
1.5 ml of Opti-MAX (without FCS and antibiotics). The wells were kept in CO$_2$
incubator for 1 h, and then 500 µl of transfection solution (dendriplexes and PLGA-dendriplex particles) were placed in each well and left in the incubator at the usual conditions for up to 2-8 h. The media containing complex solutions were then replaced with fresh medium supplemented DMEM. Transfections were followed by fluorescence microscopy with a rhodamine filter (Nikon microphot FXA).

5.2.8 Polylysine coverslips

Glass coverslips were firstly acid washed using 1 M HCL for 1 h, rinsed in sterile distilled water and left in 70 % ethanol for a further 1 h. Each coverslip was individually removed from the ethanol and left to dry on tissue paper. In order to sterilise them, they were baked at 220 °C in dry oven for 2 h. Once at room temperature each coverslip was added into 6-well plate and 1 ml of polylysine (50 mg/ml) was added on top of the coverslip and left overnight. The remaining polylysine was removed and coverslip was washed with sterile water to remove excess polylysine.

5.2.9 Confocal microscopy

Cells grown on coverslips were treated with dendriplexes and PLGA-dendriplexes as described in section 5.2.6. At stated times post-transfection, cells on the coverslip were washed twice with PBS and fixed in 4 % paraformaldehyde for 2 mins at 4 °C. Cells were then washed twice in PBS and 1 ml of ammonium chloride (50 mM) in PBS was added to each well for 8 mins to stop the reaction of the paraformaldehyde. Afterwards the cells were washed twice with PBS, once with sterile water, and mounted in Vectashield, mounting medium for fluorescence with DAPI. Imaging data were collected using inverted Zeiss LSM 510 Meta Confocal laser scanning microscope (Zeiss, Jena, Germany) and processed using Adobe Photoshop. The excitation wavelength used was 488 nm.
5.3 RESULTS AND DISCUSSION

5.3.1 Cytotoxicity of dendrons, dendriplexes, and PLGA-dendriplexes

Ideally novel polymeric biomaterials should be biocompatible especially with consideration as a drug delivery carrier system, \textit{in vitro} and blood compatibility behaviour are two essential aspects in biomaterial screening. To define cytotoxicity of dendrimers and dendrons, it is appropriate to use physiologically significant incubation times. In our case, 24 h was selected as the maximum time used for the transfection studies. Cells were treated with dendron alone as well as equivalent concentrations of dendron in the complexes (dendron:DNA). Malik \textit{et al.} (2000) studied the cytotoxicity of cationic PAMAM dendrimers of generations 1-4 and found that even lower generations were cytotoxic and displayed IC$_{50}$ values similar to poly(L-Lysine). Therefore a ranking of polycation cytotoxicity: poly(ethylenimine) = poly(L-Lysine) > diethylaminoethyl-dextran > PAMAM generation 3, was established with correlation between their MTT measurements assessing cell viability and changes in cell morphology (Duncan and Izzo, 2005).

The effects of diverse lysine-based dendrons synthesised here were examined on the viability of Caco-2 and CHO-K1 cells. Both these cell lines have different histological compositions; the Caco-2 cells are intestinal epithelial cells and the CHO-K1 are derived from Chinese Hamster ovary cells. Caco-2 cells contain p-glycoprotein, a transmembrane protein, and a known energy-dependent drug efflux pump which is over expressed in Caco-2 cells, therefore making these cells much more sensitive for cytotoxicity assessment (Bromberg and Alakhov, 2003). In additional, the cytotoxicity evaluation was conducted for the CHO-K1 cells prior to the \textit{in vitro} testing of PA-dendriplexes and PLGA-PA dendriplex particles in chapter 6.

Cell viability was plotted versus dendron concentration as shown in figure 5.3, for a range of lipidic dendrons and one non-lipidic (C$_{6}$) dendron, all possessing the same number of NH$_2$-terminal groups. The cationic surface charge was proposed as the main source of toxicity particularly for the higher generation dendrimers. Previously this concept was verified and it has been shown that cytotoxic effects of non-lipidic dendrons was dependent on the generation from (Lys)$_7$(NH$_2$)$_8$ to (Lys)$_{31}$(NH$_2$)$_{32}$ and also on the concentration of the dendrons (Ramaswamy PhD thesis, 2005). The alteration of the cationic dendrons with lipid chains was likely to shield the positive
charge of the dendron surface in line with a decrease in the cytotoxicity as shown by the results in figure 5.3 for both cell lines.

**Figure 5.3:** Cytotoxicity of lipidic dendrons: the effect of the five dendron assemblies on the viability of Caco-2 cells (A) and CHO-K1 cells (B) after 24 h incubation time using a concentration from 0.2 mg/ml to 2.5 mg/ml. The cell viability was determined by MTT assay. (Mean ± SD, n=5)
Table 5.1: The effect of lipid modification on the cytotoxicity of lysine-based dendrons as determined by IC₅₀ (mean, n = 5)

<table>
<thead>
<tr>
<th>Dendrons</th>
<th>IC₅₀ mg/ml of Caco-2 cells</th>
<th>IC₅₀ mg/ml of CHO-K1 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C₁₀)₁(Lys)₇(NH₂)₈</td>
<td>&lt; 1 mg/ml</td>
<td>&lt; 0.7 mg/ml</td>
</tr>
<tr>
<td>(C₁₈)₁(Lys)₇(NH₂)₈</td>
<td>&gt;&gt; 1 mg/ml</td>
<td>0.8 mg/ml</td>
</tr>
<tr>
<td>(C₁₈)₂(Lys)₇(NH₂)₈</td>
<td>&gt;&gt; 1 mg/ml</td>
<td>1.0 mg/ml</td>
</tr>
<tr>
<td>(C₁₈)₃(Lys)₇(NH₂)₈</td>
<td>&gt;&gt; 1 mg/ml</td>
<td>1.5 mg/ml</td>
</tr>
<tr>
<td>(C₀)(Lys)₇(NH₂)₈</td>
<td>0.5 mg/ml</td>
<td>0.6 mg/ml</td>
</tr>
</tbody>
</table>

Although the cell viability decreased at higher concentrations of dendron in both cell lines, a marked decrease in cytotoxicity of the C₁₈ dendron was noted when the number of lipid chains was increased. It is important to note that using C₁₀ dendron and non-lipid C₀ dendron had slightly higher cytotoxicity than the C₁₈ dendron. We could conclude that the lengths of carbon chains in the core influenced the toxicity to both cell lines. D’Emanuele and co-workers (2003) have corroborated this finding when investigating the impact on toxicity with Caco-2 cells using surface modified cationic PAMAM dendrimers and found that surface modification on (G₂, G₃, G₄) PAMAM with either lauroyl chains or polyethylene glycol (PEG), markedly reduced toxicity (Jevprasesphant et al., 2003). Previously the dendrons exhibiting the maximum toxicity showed the greatest reduction in surface tension (Ramaswamy PhD thesis, 2005).

From Table 5.1, illustrating the IC₅₀ values for all five dendrons tested in two different cell lines, the CHO-K1 cells seem to show a greater differentiation in cytotoxic sensitivity to the dendrons than the Caco-2 cells, but both show similar trends. The minimum IC₅₀ for the dendrons was found to be 0.5 mg/ml and 0.6 mg/ml for (C₀) with Caco-2 and CHO-K1 respectively. Whereas the IC₅₀ for the C₁₈ dendron with Caco-2 cells were above 1 mg/ml even with increasing the number of lipid chains, this was not observed for the CHO-K1 cells. These observation clearly illustrates the viability in cytotoxic behaviour of the dendrons in different cell lines.

Based on the IC₅₀ values obtained the dendrons can be ranked dependent on their toxicity as follows:

(C₁₀)₁(Lys)₇(NH₂)₈ = (C₀)(Lys)₇(NH₂)₈ > (C₁₈)₁(Lys)₇(NH₂)₈ > (C₁₈)₂(Lys)₇(NH₂)₈ > (C₁₈)₃(Lys)₇(NH₂)₈
It has been reported that complexation of polycations and DNA reduces the cytotoxicity when compared to free dendron particularly with dendritic polylysine and lipidic dendrons (Shah et al., 2000; Takahashi et al., 2003). In this study, all five dendrons were complexed with 2 μg of pDsRed1-N1 DNA to determine the effect of free and complexed dendron on CHO-K1 cell viability after an incubation of 24 h (figure 5.4). The results illustrate that cell viability depends upon the molar charge ratio of the dendriples. It was likely that at the 0.5:1 ratio, the DNA was not fully condensed (as shown in Chapter 3 by the particle size, zeta potential data including electron micrographs), and therefore the 0.5:1 dendriples exhibit more of a cytotoxic effect due mainly to the overall positive charge of the uncomplexed dendron. However with the 10:1 ratio, in particular for the C₀ dendron as complexes with DNA, the toxicity was reduced in contrast to the 0.5:1 charge ratio. Schätzlein and co-workers (2005) have also observed an increase in IC₅₀ values for polypropyleneimine dendrimers complexed with DNA from 3:1 to 5:1 ratios particularly with generations 3 and 4.

![Figure 5.4](image-url)

**Figure 5.4:** Cytotoxicity of dendriples at various molar charge ratios on CHO-K1 cells after 24 h incubation time using 2 μg DNA/well. Cell viability was determined by MTT assay. (Mean ± S.D., n = 5)
Furthermore the result from the MTT assay shown in figure 5.5 reveals that plain dendrons show higher cytotoxicity against CHO-K1 cells than the equivalent dendron and DNA complex, even after 24 h incubation. Both the Caco-2 and CHO-K1 cell lines were tested, but there was no significant difference in cell viability when these dendriplexes were evaluated.

Phenylalanine-modified dendrimers examined (Kono et al. 2004) as potential gene carriers were also evaluated from the viewpoint of cell toxicity. The viability of the cells were measured by treatment with G4 dendrimer, Lipofectin® and SuperFect® and also their complexes with DNA. The dendrimer itself was also found to be cytotoxic when compared to the commercial products. However, when complexed to DNA the cell viability increased dramatically. This was more apparent with complexes with Superfect and Lipofectin. In our study, Lipofectin and SuperFect were complexed with DNA (data not shown) and cell viability was
comparable to the dendriplexes, ~ 95% even after 24 h incubation. These results have been found to correspond with those acquired from Kono and colleagues (2004).

The cell viability in the presence of PLGA particles at 50 μg/well for both C_{18} and C_{0} dendriplexes (figure 5.5) were above 95 %, even after longer incubation times and was considered to be non-toxic compared to their counterparts. PLGA is an ideal polymer for encapsulation of dendriplexes, this is due to the overall negative surface charge which is not observed by other polymeric cationic nanoparticles (Kwon et al., 2005).

In figure 5.6, a comparative study was conducted using 10:1 and 20:1 charge ratio dendriplexes to verify whether these dendriplexes would have an enhanced cytotoxic effect. One of these two ratios may later be considered for transfection studies and finally in vivo testing. At N/P higher than 10, the cell viability was compromised and revealed a decrease in cell number which became more prominent as the DNA in the complexes was increased. This corresponds well to findings by Maksimenko et al. (2003).

![Figure 5.6](image)

**Figure 5.6:** Cytotoxicity of dendriplexes at 10:1 and 20:1 molar charge ratio by varying concentration of DNA complexed with (C_{18})_{3} and (C_{0}) respectively at 24 h incubation. CHO-K1 cell viability was measured by MTT assay. (Mean ± S.D., n = 5)
5.3.2 $^{35}$S DNA uptake study across Caco-2 monolayers

Epithelial intestinal cells when cultured on microporous membranes to form an impermeable cellular barrier of polarized cells connected by tight junctions. Hence various studies have been conducted to investigate the mechanism of dendrimer transport (D'Emanuele et al., 2003; El-Sayed et al., 2003). However, our aim was to measure the uptake of radiolabelled DNA in dendriplexes and in PLGA-dendriplex particles at different molar charge ratios. Therefore, this method was adapted and formulations containing the radiolabelled DNA were added to the basolateral chamber. At regular intervals Caco-2 cells were lysed and the radioactivity measured by scintillation counter.

It was found that following the incubation of monolayers with dendriplexes at the 10:1 charge ratio, there was enhanced uptake when compared to the 0.5:1 ratio and naked DNA which had similar rates of uptake (figure 5.7). The transport of naked DNA appeared to saturate after approximately 50 min, while uptake of PLGA-dendriplex particles and the 0.5:1 dendriplexes appeared to plateau after 125 mins. In contrast, the 5:1 and 10:1 dendriplexes continued to penetrate through the Caco-2 monolayer. These preliminary results correspond to the characterisation of the colloidal properties of the dendriplexes and enhanced sustained release illustrated as by the PLGA-dendriplex particles. The TEER of the monolayers was measured (figure 5.8) and illustrated no change during incubation with each formulation.

Carlisle and co-workers (1999) have investigated cellular uptake and gene expression of the synthetic vector formed by self-assembly of DNA with an oligolysine peptide forming complexes. Cell uptake was measured using $^{32}$P-DNA over a period of 3 h and 7% became cell associated after 3 h, this was 10 fold increase compared to the polylysine/DNA controls. In our study at 3 h (180 min), 15 % became cell associated for both naked DNA and PLGA-dendriplex particle (figure 5.10) while the uptake of the 5:1 and 10:1 dendriplexes was much higher (figure 5.9). The differences relate to the concentration of DNA radiolabelled, in our case we used 2 µg and Carlisles group used 50 ng not including the disparity in the biophysical properties of the complexes and the methodology (cell density). The main conclusion found was that the results correlated well with the transfection activity of the complexes, following measurement of the expression of $\beta$-galactosidase transgene.
Figure 5.7: Uptake studies of percentage cumulative transport of $^{35}$S DNA in dendriplexes at different molar charge ratios including PLGA-dendriplex particles across Caco-2 monolayers as a function of time. (n=3; ± S.D.)

Figure 5.8: Effect of DNA:dendron complexation ratio and PLGA-dendriplexes on the TEER of Caco-2 monolayers as a function of time. (n=3; ± S.D.)
**Figure 5.9:** Individual graphs from the uptake studies of % DNA transport as a function of time (min) depicted in Fig. 5.7, integrating the data (calculation of area shown in black) to obtain the amount of transport of $^3$S-DNA in Caco-2 cells for each dendriplex composition (0.5:1, 5:1 and 10:1).
**Figure 5.10:** Individual graphs of %$^{35}$SDNA cumulative transport as a function of time for naked DNA and PLGA-dendriplexes integrating the results in order to obtain the amount of transport across Caco-2 cell monolayer.
Complexes with C18 dendron

![Bar graph showing total amount of ^32SDNA transport in Caco-2 cells (%) for various formulation sets.](image)

**Figure 5.11:** Total amount of ^32SDNA transport in Caco-2 cell monolayer of naked DNA or complexed with (C18) dendron at various ratios of 0.5:1, 5:1, 10:1 and including encapsulation in PLGA-dendriplex particles.

The integration of the results from figure 5.9 and 5.10 to establish the graph illustrated in figure 5.11, confirms the previous findings. Complexes equal to or above a 5:1 charge ratio form compact structures which increase the transport of DNA in cells, but not to the extent we were expecting due to the unpredicted initial transport of naked DNA.

This methodology was utilized for *in vitro* studies of complexes containing PA DNA using an alternative cell line, CHO-K1 cells to evaluate the disparity of penetration and transport of these complexes containing PA DNA (different plasmid DNA construct) as described in Chapter 6. Further studies were conducted to assess the efficiency of transfection and gene expression of both dendriplexes and PLGA-dendriplex particles.
5.3.3 In vitro transfection and gene expression of dendriplexes and PLGA-dendriplex particles

Gene transfer using dendriplexes or PLGA-dendriplex particles as non-viral vectors is a promising approach for safe delivery of therapeutic DNA in genetic or acquired human diseases. The transfection efficiency of these formulations may vary with different parameters such as the colloidal properties and molar charge ratio of these transfection agents, as depicted in figure 5.11, by the amount of plasmid DNA, incubation time for transfection, and by the selection of cell lines. Previous collaborative studies carried out by Dr. H. Bayele at University College London used dendrons contained three \( C_{14} \) lipidic chains as opposed to the usual \( C_{18} \) chains and these were complexed with DNA and transfection efficiencies were noticed (figure 5.12).

![Figure 5.12](image)

**Figure 5.12:** Transfection efficiencies of \((C_{14})_{7}(\text{Lys})_{7}(\text{NH}_2)_8\) dendron in CHO-K1 cell line: CHO-K1 were transfected with complexes containing 5 \( \mu \)g luciferase DNA and various amounts of dendron. The cells were harvested and luciferase assays were performed after 48 h and gene expression was expressed as RLU/mg of protein. (Redrawn from Bayele et al., 2005).

The transfection ability increased by approximately 300 fold as the dendron concentration complexed with DNA doubled from 2.5 to 5 \( \mu \)g/well. The transfection efficiency of the other cell lines were assessed and transfection variability was observed, which may be due to the differences in cell membrane architecture.
Figure 5.13: Transfection of Caco-2 cells using pDsRed1-N1, a plasmid including a gene that encodes a red fluorescent protein delivered by four diverse formulations, panel A: 10:1 \((C_{18})_3\) dendriplexes; B: PLGA- \((C_{18})_3\) dendriplex \((10:1)\) particles; C: DNA and lipofectin; and D: 5:1 \((C_{18})_3\) dendriplexes. All formulations contained 12 µg of DNA. Red dots represent Caco-2 cell expressing red fluorescent protein, only expressed once transfected and transcription of the protein had taken place. Arrows denote cells with no expression (black areas). The most efficient delivery system where gene expression was highly apparent for the 10:1 \((C_{18})_3\) dendriplexes (panel A) which decreased significantly at the 5:1 charge ratio (panel D).
Clearly from the photomicrographs in figure 5.13 illustrating the expression of RFP (red fluorescent protein) in cells shown as dots detected under a fluorescence microscope, the 10:1 charge ratio of the dendriplexes enhanced the gene transfer of DNA in Caco-2 cells. This was still the case even when comparing the expression achieved by the 5:1 dendriplexes (panel D) to the commercially available transfecting agent Lipofectin® (panel C), the dendriplexes had higher transfection efficiency but the extent of expression was dependent on the molar charge ratio utilised to formulate the dendriplexes.

Other groups have found improved transfection at various charge ratios, for instance 2:1 for PLL (MW 23 K), 10:1 for PLL (MW 30K)/DNA (Lee et al., 2002) and 200:1 for PAMAM and conjugates with cyclodextrin (Kihara et al., 2002), highlighting the significance the carrier polymer properties play in transfection. The expression of PLGA-dendriplex (10:1) (panel B) was low particularly in contrast to the 10:1 dendriplexes, and perhaps could be due to the an incubation time of 8 h. Previously the release study (chapter 4 section 4.3.3) depicted a maximal release at 24 h and was additionally assessed by confocal microscopy. Bielinska and colleagues (2000), incorporated PLGA polymer membranes coated with DNA/dendrimer complexes to evaluate the ability to transfect cells and observed limited expression at a charge ratio of 10:1 and incubation time of 3 h using fluorescent microscopy, these results coincided with the results obtained from the confocal in figure 5.19.

It was difficult to visualise the gene expression of 0.5:1 charge ratio dendriplexes with a standard fluorescent microscope, mainly due to the background fluorescence from outside the focal plane affecting the signal to noise ratio resulting in unclear photomicrographs. With confocal microscopy, the laser and filtering system reduce this noise. In this study, Wiz-GFP (green fluorescent protein) was encoded in the plasmid DNA with same amount of base pairs as the PA plasmid DNA used in chapters 6 and 7. The fluorescence distribution inside the cells was analysed using confocal microscopy. Localisation of GFP expression varied between cell lines as shown by figure 5.14 of CHO-K1 and P3X63Ag8 cells treated with 0.5:1 (C_{18})_{3} dendriplexes. The CHO-K1 cells treated with 0:5:1 (C_{18})_{3} dendriplexes had overall transgene expression throughout the cell, whereas the P3X63Ag8 cells illustrated localised expression at the nucleus. The general trend of increased GFP expression of over 55 % in cells treated with the 10:1 (C_{18})_{3} dendriplexes (figure 5.16) can be compared to less than 5 % for the 0.5:1 (C_{18})_{3} dendriplexes.
Figure 5.14: Visualisation of GFP expression of DNA (12 µg) complexed with (C₆₃)₃ dendron at 0.5:1 charge ratio by confocal microscopy. The above panels show differential interference contrast images which correspond to the fluorescent image (left panel) of the same slide with the cells (right panel) and below the integration of the two micrographs. Two sets of three micrographs are depicted above comparing the gene expression in CHO-K1 cells (A) and P3X63Ag8 cells (B) illustrating the variation of DNA transfection between the cell lines. Red arrows denote expression in the nucleus of the cell, and the black arrow expression throughout CHO-k1 cell.
Figure 5.15: Confocal microscopic images of P3X63Ag8 cells treated with (C\(_{10}\)\(_1\)) dendriples at 10:1 charge ratio containing 12 μg of DNA with GFP incubated for 8 h. Two sets of differential interference contrast images (explained in figure 5.14) and panels are shown investigating the overall gene expression (A) and individual cellular distribution (B) of the P3X63Ag8 cell line. Red lines denote expression in the nucleus of the cell, panel B illustrates cellular division which has been linked to higher DNA uptake. Both panels A and B depict low expression in comparison to 10:1 (C\(_{18}\)\(_3\)) dendriples in Fig. 5.16.
Figure 5.16: Confocal microscopy of GFP expression of DNA (12 μg) delivered as a complex with the (C_{18})_3 dendron at the 10:1 charge ratio and incubated for 8 h with P3X63Ag8 cells. Two sets of differential interference contrast images (explained in figure 5.14) and panels are shown investigating overall gene expression (A) and individual cellular distribution (B) of the P3X63Ag8 cell line. The (C_{18})_3 dendron at the 10:1 charge ratio had GFP expression over 55% in contrast to 5% for 0.5:1 charge ratio for the same dendriplex composition. On a cellular level the expression is mostly observed on the periphery of the cell denoted by the blue arrows.
Figure 5.17: Confocal images of P3X63Ag8 cells treated with dendron alone (control) (A) and (C₀) dendripexes at 10:1 charge ratio with GFP DNA (panel B). Two sets of differential interference contrast images (explained in figure 5.14) and panels are shown investigating overall gene expression. The GFP expression was mainly observed on the surface of the cells denoted by orange arrows, with an overall expression of 21% for (C₀) dendripexes.
Figure 5.18: Confocal images of P3X63Ag8 cells treated with 12 μg of GFP DNA alone. The above panels show differential interference contrast images which correspond to the fluorescent image (left panel) of the same slide with the cells (right panel) and below the integration of the two micrographs. Naked DNA expression was less than 10% illustrated by the confocal photomicrograph above.

Dendriplexes formulated with the C₀ dendron (figure 5.17B) had cell associated fluorescence clusters in the periphery of the cell membrane, with minimal fluorescence in the cytoplasm of the cell and suggested that the C₀ dendriplexes may not have been efficiently internalised across the membrane. This is consistent with the notion that lipophilicity of the dendron may aid cellular entry via the cholesterol pathway has been suggested by Manunta and co-workers (2004). These findings coincide with the results from the uptake study in figure 5.11 and may explain the differences between the C₀ dendron and C₁₈ dendron when used as DNA carriers. It should be noted that dendron alone (control) (figure 5.17A) illustrated no fluorescence and naked DNA (figure 5.18) produced minimal expression. The CHO-K1 cells treated with PLGA-dendriplex particles (figure 5.19), demonstrated that incubation time has a significant effect on transgene expression with maximal transfection at 24 h which coincides well with the release data in chapter 4 section.
Figure 5.19: Confocal image of CHO-K1 cells treated with PLGA-dendriplex (10:1) particles containing GFP DNA incubated for 2 h (A) and 24 h (B) time points. Two sets of differential interference contrast images (explained in figure 5.14), and panels are shown investigating overall gene expression. Panels A and B illustrated the influence incubation time had on GFP expression for the PLGA-dendriplex particles.
5.4 CONCLUSION

In this chapter, the biological properties of dendriplexes and encapsulated dendriplexes, in particular transfection, gene expression and cytotoxicity of dendrons alone, complexed with DNA and encapsulated into PLGA particles were studied. The dendrons synthesised in this thesis contained only 8 NH$_2$- terminal groups, accounting for the reduced toxicity compared to that normally found with higher generation dendrimers. The cell viability results showed a relationship between lipophilicity of the dendrons and their toxicity, which decreased significantly when complexed to DNA. However differences in gene expression were observed. These were predominantly higher for the C$_{18}$ dendriplexes in comparison to the non-lipidic C$_{0}$ dendriplexes. Additionally the molar charge ratios of the dendriplexes above 5 (N/P) increased the uptake and transfection of DNA due to the nano-sized compact structure of the dendriplexes. The transgene expression of PLGA particles was dependent on incubation time illustrated by low expression at 2 h with a dramatic increase after 24 h, this was consistent DNA release data as described in section 4.3.3. Further *in vitro* studies were conducted and can be found in Chapter 6, using the PA-dendriplex and encapsulated into PLGA particles, assessing the level of antibody production.
CHAPTER SIX

EXPRESSION OF PLASMID PROTECTIVE ANTIGEN-DENDRON AND PLGA COMPLEXES IN CELLS

6.1 INTRODUCTION

In this chapter, the measurements of \textit{in vitro} expression of PA in dendriples and PLGA-dendriples at low and high dose were attempted by adapting the original ELISA method used to measure the level of anti-PA antibodies in the \textit{in vivo} studies. Both sets of particles, the dendriples and the PLGA-dendriplex particles were formulated and characterised with consideration for future \textit{in vivo} treatments. Therefore, specific factors such as particle size, charge, toxicity, ratio of dendron:DNA, stability and morphology were investigated and formulations were selected according to their colloidal properties prior to \textit{in vitro}. \textit{In vitro} studies were also conducted to provide a further insight into PA binding to cellular receptors and links to antibody production as well as to draw a parallel with animal work.

The bioassays used were dependent on the selection of cell lines, each cell line having different characteristics, including variations in cell surface proteins, and morphology (cell membrane rigidity) causing some cells lines such as the CHO-K1 line to be more susceptible to toxins. Studies by Escuyer and Collier (1991) have indicated that the receptor to which PA binds, is a ubiquitous protein expressed at high levels on cell surfaces on CHO-K1 (Chinese hamster ovary cells). Most of the cellular analyses assessing protein binding to PA and mediated toxin internalisation, have been conducted using receptor-deficient CHO cell lines (Scobie \textit{et al.}, 2003). In our work the CHO-K1 and P3X63Ag8 cell lines were used to conduct transfection
studies with the dendriplexes and the PLGA-dendriplex particles, both containing PA DNA. For the control, DNA without PA was used in both cases. The majority of the reported work aimed at treating or preventing anthrax have centred on PA, predominantly for its ability to mediate the intoxication pathway (Casadevall, 2002; Pitt et al., 2001; Little et al., 1997). These findings have led to an increased focus on the development of specific antibodies that neutralise the toxin effect of the binary proteins associated with anthrax.

Reliable bioassays capable of assessing \textit{in vitro} potency are limited to two main assays, the rubidium release assay and lethal toxin mediated killing assay. The rubidium release assay measures the release of radioactive rubidium through pores formed in the membrane of CHO cells by PA heptamerisation (Milne and Collier, 1993). The variability of the assay may be due to multiple factors, apart from the safety issues in the use of radioactive isotopes, does not make the rubidium assay reliable. In comparison, the lethal toxin assay is more relevant assay for measuring intoxication of cells based on PA binding to LF eliciting the toxic effect (Hanna et al., 1993). This assay has normally been used to measure antibody mediated neutralisation which will be assessed in the animal study in chapter 7. Other groups, such as that of Kasuya et al. (2005), examined expression and secretion of anti-PA antibody in cells by Western blot analysis and lethal toxin challenge. While Gaur et al. (2002) used PA DNA containing GFP (green fluorescent protein) in the construct upon transfection of cells and examined this by fluorescence microscopy. Recently, Zmuda et al. (2005) developed a method incorporating a chemiluminescent ELISA technique, quantifying PA-mediated cellular intoxication \textit{in vitro}. Therefore the ELISA method may be adapted for biological cellular assessment.

In this work, we demonstrated transfection of P3X63Ag8 cells with PA dendriplexes and PLGA-PA dendriplexes, by determining production of antibody in the supernatants by adapted indirect ELISA on rPA coated plates. This was not the first attempt to use this technique; other groups such as Sawada-Hirai et al. (2004) have also incorporated this method using a specific vector to identify positive clones in transfected cells. The control was DNA without PA, in both formulations as in the \textit{in vivo} study. Attempts at verification of this technique by radiolabelling PA DNA for each set of formulations (dendriplexes and PLGA-dendriplex particles) were made to evaluate the reliability of the results from the ELISA technique. Cytotoxicity measurements were also conducted using CHO-K1 cells, a standard method in
evaluating the binary process forming the toxin which effects cellular viability measured using standard MTT assay.

6.2 EXPERIMENTAL

6.2.1 Materials

DNA encoding protective antigen (PA83) cloned into the eukaryotic expression plasmid pCMV/myc/ER containing a signal sequence for fusion protein secretion required for cellular expression was used. The control plasmid was pCMV/myc/ER vector DNA without the PA83 insert. The PA DNA construct is shown below in figure 6.2. All the materials used will be indicated in each section of the methodology.

6.2.2 Dendron Synthesis

The two cationic (lysine)-based dendrons selected for biological assessment as shown in figure 6.1.

![Dendron Diagrams](image)

Figure 6.1: Two lysine based dendrons \((\text{C}_0\text{Lys})_7\text{(NH}_2)_8\) MW 1174.91 \((\text{C}_0\)) and \((\text{C}_18\text{Lys})_3\text{(NH}_2)_8\) MW 1758.45 \((\text{C}_{18}\)) both containing 16 amino groups and 7 lysine groups attached to the core with 0 or 3 hydrocarbon chains.

6.2.3 DNA constructs

The PA DNA and the control DNA were a gift of Dr U Hahn (Hahn et al. 2004) and generously provided by the National Institute of Biological Standard and Control.
Ampicillin resistance gene
Kpn
pSecTag2B-PA 7301bp
PA 83
Apa
SV40 promoter and origin

**Figure 6.2:** Diagrammatic representation of PA DNA vaccine plasmid. Plasmid map shows vaccine pSecTag2B-PA containing PA 83 (83-kDa) with genes for ampicillin selection. The PA gene was amplified with new restriction sites added at the end and cloned into Kpn I and Apa I sites.

### 6.2.4 Formulation of dendriplex and PLGA-dendriplex particles
The dendriplex formulations possess different molar charge ratios of the dendron:PA DNA complexes. Both PA DNA and dendron were complexed as specified in section 3.2.3. A secondary formulation of encapsulated dendriplexes was also utilised, namely the PLGA-PA dendriplexes, formulated using the double emulsion method described in section 4.2.5. These dendriplexes and the PLGA-dendriplex particles were previously characterised with pDsRed1-N1 (DNA containing red fluorescent protein ~ 4.3 Kbp), but not with PA DNA (7.3 Kbp).

### 6.2.5 Characterisation of dendriplex and PLGA-dendriplex particles
The sizes of the PLGA-dendriplex and dendriplex particles, which are all below 1 μm in diameter, were determined by Photon Correlation Spectroscopy (PCS) using a Zetasizer 3000 (Malvern Instruments, Malvern, UK, He-Ne laser, 90° angle of measurement) after dilution of samples. The average of three measurements was used and results expressed as the Z-average (nm) ± S.D and zeta potential (mV) ± S.D. The details of size and zeta potential of the systems are specified in Chapter 3.

### 6.2.6 Electron microscopy
Cryo-transmission electron microscopy (TEM) was used to visualise the aqueous dendriplex samples in double distilled water, using a Philips 201L transmission electron microscope at 100kV with a 1% aqueous uranyl acetate stain (negative
stain). Lyophilised PLGA-dendriplexes samples were visualised using scanning electron microscopy (SEM).

### 6.2.7 P3X63Ag8 cell line establishment

P3X63Ag8 cells (mouse myeloma, derivative of plasmacytoma in BALB/c mice) purchased from ECACC (European Collection of Cell culture) were plated at an initial density of 1 x 10^5 cells/well into 6 well plates (Sigma) containing selected media supplemented with 1 % non-essential amino acids, 10 % dialysed FBS (Foetal Bovine serum, Invitrogen) and penicillin (50 µg/ml) at 37 °C, 5 % CO_2, in 95 % relative humidity. Cells were maintained in an incubator under standard tissue culture conditions (37°C, 5 % CO_2). After 24 h, transfected cells were transferred to 10 cm sterile dishes with supplemented DMEM. These cells were incubated for a week until the cells were 80 % confluent. Control samples were dendriplexes and PLGA particles containing the DNA without PA insert. Several factors were analysed such as cell culture media, well size, and time of incubation, to investigate if they had an effect on the final ELISA readings. The media under evaluation were DMEM, GM, and F-6, while the well size attempted were 96, 12, and 6 well plates for initial transfection. Incubation time varied from 10 to 48 h with both sets of PA particles. The only problem was the high volume of supernatants for transfer into 96 well plates for the ELISA readings. Mini spin columns were used to concentrate by ion exchange chromatography purified antibodies from the cell supernatant, according to the manufacturer’s instructions (Proteus). The final volume produced from these protein G mini plug columns was less than 1 ml. The concentrated supernatants containing antibodies were collected and used to quantify anti-PA antibodies by an indirect ELISA method.

#### 6.2.7.1 Protein G mini spin columns

The Proteus G kit is designed for simple, rapid antibody purification from cell culture supernatant and replaces lengthy chromatographic methods. The Proteus antibody purification kit used incorporated a pre-packed resin Mini plug containing immobilised recombinant protein G resin into the barrel of the Proteus spin column. There are several steps specified by the manufacturer, as illustrated by the flow diagram below.
The columns are equilibrated using a buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4) spun at 5000 rpm for 1 min, repeated twice.

The supematant from P3X63Ag8 cells was loaded into the column and spun at 2,600 rpm for 6 mins.

The column was washed with binding buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4) to remove unbound contaminants by centrifuging for 1 min at 4500 rpm.

Elution of bound IgG with 0.5 ml of elution buffer (0.2 M glycine/HCl, pH 2.5) and 65 μl of neutralisation buffer (1 M Tris/HCl, pH 9) to bring pH of the sample to approximately pH 7.5.

This protocol was repeated for each transfection study including both dendriplexes and PLGA particles in order to concentrate the cell supernatants to conduct the ELISA.

6.2.7.2 Indirect ELISA method

Concentrated supematant from P3X63Ag8 cells were analysed by ELISA for specific antibody expression, in this case PA antibodies. All washing steps were carried out with PBS containing 0.05 % polysorbate (Tween) (PBST); all incubation steps were carried out at room temperature, unless indicated otherwise. The 96 wells of high-binding protein microtiter plates (MaxiSorp™, Nunc) were each coated with 100 μl 5 μg/ml of PA in a 0.05 M carbonate buffer at pH 9.5 (at 4 °C overnight). The wells were washed and blocked with 200 μl/well of 2 % Marvel (skimmed milk powder) in PBST for 2 h. The supematant of cells were diluted starting with 2-fold dilution, with further dilution across the plate and incubated for 3 h. The wells were washed extensively with PBS/Tween and PA antibodies were detected by goat anti-human IgG-HRP (Sigma) incubated for 2 h. The plates were developed as described in chapter 7, section 7.2.8.
6.2.8 Radiolabelling PA DNA

PA DNA (12 μg) radiolabeled with $^{35}$S dCTP, was prepared by using the nick translation technique previously described (El Ouahabi et al., 1997), in figure 4.1. Plasmid DNA integrity was assessed by agarose gel electrophoresis (data not shown). Radiolabeled PA DNA was separated from unincorporated radiolabelled nucleotides using gravity-flow chromatography columns with a maximum volume of 100 μl, prepacked with Sephadex G-50 DNA grade in distilled water with 0.15 % Kathon CG/ICP Biocide. The radiolabelled DNA was used to formulate the dendriplexes and PLGA-dendriplexes using the optimised methods described in section 5.2.2. Costar transwell plates (12 mm diameter, growth area 1.13 cm$^2$, pore diameter 3.0 μm) were used where CHO-K1 cells seeded with $1 \times 10^6$ cells with 0.5 ml of Optimax (Invitrogen) (serum free media) in the chamber. The CHO-K1 cells were incubated in standard cell culture conditions, grown for 3 days until 80 % confluent. The cell culture medium was added to both apical and basolateral chambers of the transwell plates. The lysed cells in the apical chamber were assayed at selected time points (0, 30, 60, 120, and 240 mins) by liquid scintillation counting as described in section 5.2.5.

6.2.9 Cytotoxicity assay

A CHO cell suspension (100,000 cells/ml) added to 60 well of a 96-well plate (Sigma) was incubated at 37 °C in a 5 % CO$_2$ environment for 24 h before use. The medium was removed and replaced with 75 μl of selected medium and 20 μl of test solution i.e. (PA, dendriplexes, PLGA-dendriplex) incubated for 24 h.

LF (Lethal Factor, List biological 172B) was diluted to a final concentration of 0.1 μg/ml, 75 μl of LF to each well and allowed to incubate for 1 h at 37 °C. After this pre-incubation, the medium was aspirated and the MTT protocol was followed as in section 5.2.4. The results were expressed as the percentage viability ($n=10; \pm$ S.D), calculated using the formula below.

$$\%{viability} = \left( \frac{A_{570} \text{ of cells treated with LF} - \text{treated with samples}}{A_{570} \text{ of cells treated with LF and medium}} \right) \times 100$$
6.3 RESULTS AND DISCUSSION

6.3.1 Characterisation of PA dendriplexes and PLGA-PA dendriplex particles

Preliminary studies showed that DNA complexes with cationic lysine-based dendrons, charge neutralisation promoting condensation of DNA into compact particles (see section 3.3.1). Cells take up these compact particles more or less efficiently via a number of natural processes, such as by adsorptive endocytosis, pinocytosis and phagocytosis. When forming these dendriplexes, the extent of condensation between the dendron and DNA is dependent on the molar charge ratio as shown by the particle size data in figure 6.3. The charge ratio is defined as the ratio of nitrogens (from the dendrons) to phosphates (from DNA) i.e. the N/P ratio. Both graphs A and B (figure 6.3) have similar trends, as the molar charge ratio increases (i.e. higher concentrations of dendron) the mean particle size decreases, which correlates with the preliminary formulation studies. At the lowest molar charge ratio (0.5:1) in both graphs, the mean size is over 1 μm, or higher for the C₀ dendriplexes. Therefore when low amounts of dendron were used to complex DNA around electroneutrality, then these particles were large with a high polydispersity, as illustrated in figure 6.4. Aggregates with two size distributions for both the 0.5:1 and 2.1 ratio were seen, and the particles were not fully condensed. However at a ratio of 5:1 (a higher N/P ratio), for both dendrons, the mean particle size decreased, and was independent of the presence of PA or control DNA. Small, positively charged particles were also verified by the change in zeta potential.

It has been suggested by Mislick and Baldeschwieler (1996) that polyplexes with a positive zeta potential will interact with cell surfaces of varying types because of the presence of negatively charged cell surface proteoglycans. Both dendriplexes above 5:1 ratio are positively charged, however a difference in diameter was observed. This was less than 200 nm for C₁₈ dendriplexes, whereas for the C₀ dendriplexes were greater than 400 nm in size. This confirmed the previous particle size data (shown in section 3.3.1) suggesting that lipophilicity aids condensation of DNA, forming more compact particles. Svenson and Tomalia (2005) observed a sharp maximum in structure-activity relationships, and based this upon on the hydrophobic nature of the amino-terminated (G = 3) PAMAM dendrimer. They have also suggested that the hydrophobic character of the dendron influenced DNA binding and transport, in particular in the enhancement of cellular gene transfer. The morphology of the both
dendriplex particles are shown in figure 6.5 (A, B and C). The micrographs substantiate the particle size data depicting small dense spherical compact structures. Magnification in panel C, shows that the dendriplex was approximately 80 nm in size.

Figure 6.3: Histograms of graphs illustrating the effect of molar charge ratio (dendron:DNA) on the apparent particle size and zeta potential (ZP) of dendriplexes. Graph A: C_{18} dendriplexes with two concentrations of DNA (12 μg and 50 μg) for both PA and control DNA; graph B: C_{6} dendriplexes with the formulation conditions shown. The results demonstrate the effect of molar charge ratio on the size and zeta potential of both dendriplexes which was independent of DNA concentration used in the complexes.
Figure 6.4: Percentage size distribution of C₁₈ and C₆ dendriplexes containing 50 μg of PA DNA at A) 0.5:1 and B) 2:1 charge ratio by photon correlation spectroscopy. Graph A and B depict that the 0.5:1 and 2:1 ratio have two distinct size populations, therefore illustrating a heterogeneous size population not shown by the other molar charge ratios such as the 5:1 and 10:1 ratios.
Figure 6.5: Cryo-transmission electron microscopy (TEM) of dendriplexes with a 1% aqueous uranyl acetate stain (negative stain) demonstrated in panel A: C_6 dendriplexes (50 µg PA DNA); B: C_{18} dendriplexes with equivalent DNA; and C: magnified C_{18} dendriplex particle. Scanning electron microscopy (SEM) was performed for lyophilised PLGA-dendriplex particles illustrated by panel D: encapsulated with C_{18} dendriplexes (13 µg of PA DNA); and E: encapsulated with C_6 dendriplexes with equivalent DNA concentration.
Figure 6.6: The overall effect molar charge ratio of dendriplexes, on the mean particle size of dendriplexes encapsulated into PLGA particles. The zeta potential of these PLGA particles was assessed and plotted here. The characterisation of the dendriplexes was evaluated in order to select the formulation with optimal colloidal properties for in vivo and further in vitro studies. The 20:1 molar charge ratio had larger particles, over 450 nm, whereas the 10:1 were smaller, with a narrow polydispersity. Both displayed negative zeta potentials.

The magnified TEM of the 50 μg PA 10:1 C_{18}-dendrplex (figure 6.5C) might show complexation within the particle that is, of the dendron molecules around the PA DNA used in both the in vitro and in vivo studies, but the micrographs can be difficult to interpret. The aim of the characterisation was to select the potential particles for the animal study. The PLGA particles were formulated only at the 10:1 ratio for cellular transfection and assessment of antibody production, as at a 20:1 ratio, larger particles were found as shown in figure 6.5. The 10:1 molar charge ratio for both the dendriplexes and the PLGA particles was selected for cell transfection and animal immunisation testing. The advantages of the 10:1 ratio was that there was lower concentration of dendron, the particle size was 400 nm as opposed to a size greater than 500 nm in diameter as found for the 20:1 ratio, according to the PLGA formulation results and the zeta potential data. Maksimenko et al. (2003) found the optimal ratio for all carriers was 10. They discovered that if it was lower gene expression decreased, linking the deficient uptake to reduced cell surface binding of the polyplexes. Therefore, with contemplation for in vitro testing and systemic delivery in animal work characterisation, it is obligatory to understand the possible
processes involved in cellular transfection and particle behaviour within different biological fluids.

6.3.2 DNA uptake study of PA dendripexes and PLGA-PA dendriplex particles

The transfection efficiency of these particles was evaluated by radiolabeling the PA DNA using the nick translation kit as described in the in vitro preliminary studies in section 5.3.1. The main purpose was to measure the rate of uptake CHO-K1 cells of PA DNA complexed in the optimised particles into, but in addition, to correlate the expression of PA and antibody production using the ELISA technique prior to testing these formulations in animals. In figure 6.7, graph A exemplifies the uptake profiles of five formulations incubated at various time points. The 0.5:1 C_{18} dendriplexes and naked PA DNA have similar rates of DNA uptake and are particles with the slowest uptake. The physicochemical properties of the particles have a significant effect at the cellular level. The poor PA DNA binding at the 0.5:1 ratio and the resultant colloidal instability is illustrated by a polydispersity value above 0.3 and wide size distribution as depicted in figure 6.4A. Other studies by Olbrich et al. (2001) investigated the efficiency of solid lipid carriers containing cationic modifier as a transfection agent. They varied the ratio of lipid to DNA and found a relationship between gene expression and \((N/P)\) ratio increase. Incubation with uncomplexed DNA, however did not result in detectable levels of gene expression. Tokunaga et al. (2004) found similar results for the transfection efficiencies of liposome complexes and polyplexes.

Furthermore the PLGA particle had a sustained uptake. At the 25 min time point less than 2% of PA DNA uptake from the encapsulated PLGA particles in comparison to the 0.5:1 dendriplex where 7% uptake was observed. This dramatically increased after 50 min which coincides with the PLGA-dendriplex particle release data (figure 4.10). Therefore the PLGA-dendriplex particles overall had a slightly higher uptake than the naked DNA as shown in graph B (figure 6.7) depicting the area under the curve i.e. the total amount of uptake. It is hypothesized that the transfection is mediated by the physical approach of DNA loaded PLGA particles towards the cell surface. This might explain the sustained uptake observed particularly up to the 30 min time point together with the fact that the particles are
five times larger than the dendriplexes (Ogris et al., 2001). In relation to immunological response, Jilek et al. (2004) used DNA-loaded PLGA and PLA particles to evaluate the stimulation of dendritic cells by the expression of specific surface markers and the secretion of IL-12 and TNF-α. They found PLGA particles upregulated both CD83 and CD86, which was not the case for PLA particles. The polymer composition and the rate of degradation of the carrier itself may play an important role to induce stimulation of dendritic cells. All these factors are linked to the rate of release of DNA; we have shown that our PLGA formulation unravels to release dendriplexes which has to then undergo decomplexation. It seems, therefore that the PLGA-dendriplex particles provide a secondary release system.

Our data represents clear evidence that complete condensation hence improved DNA carriage, is detrimental to gene transfer. The highest total uptake by the 10:1 dendriplexes, which are positively charged small compact particles. The uptake for the 10:1 dendriplex plateaued at 120 mins which may suggest that PA DNA uptake is saturable. The binding of cluster of dendriplexes onto the surface of CHO cells may in fact result in retention on the surface and inability to gain entry into the cells. This was also suggested by Choi et al. (2005) when DNA-assembled nanoclusters of the FITC-conjugated G5 PAMAM dendrimers were evaluated in vitro.
Figure 6.7: Uptake studies in CHO cells of radiolabeled $^{35}$S dCTP PA DNA alone and complexed with the C$_{18}$ dendron at various molar charge ratios (0.5:1, 5:1, and 10:1), and PLGA-C$_{18}$ dendron dendriplexes. A: The percentage uptake of radiolabelled PA DNA over 4 h and B: shows the area under the graph from graph A, for the dendriplexes at different charge ratio and encapsulated dendriplex particles equivalent to the total amount of uptake for each formulation.
6.3.3 Antibody production by P3X63Ag8 cells transfected with PA dendriplexes and PLGA-PA dendriplex particles

In the case of PA DNA, the exact receptors involved in cellular binding and uptake have been suggested to be at least partially proteinaceous, with attempts by Escuyer et al. (1991) to extract and purify these proteins. Furthermore, Scobie et al. (2003) demonstrated that human capillary morphogenesis protein 2 (CMG2) is expressed at the surface of CHO cells, binds PA and supports intoxication of these cells. However, in 2005 the same authors (Scobie et al. 2005) also reported that their previous findings did not hold true for cells that were engineered to express tumour endothelial marker 8 (TEM8). TEM8 receptor expression has been documented to be found in the epithelium of lung, intestine, and skin, the three routes of entry in anthrax infection. Therefore we can speculate that dendriplexes and naked DNA may bind to different receptors, but that they enter mammalian cells via receptor mediated endocytosis, like most bacterial toxins and particulate systems.

Now with some concept of transfection ability of PA DNA in the particle carrier systems (in our case, dendron and dendron complexes in PLGA) attempts were made to correlate these results with antibody production by using the P3X63Ag8 cell line, a mouse myeloma derivative of plasmacytoma in BALB/c mice (Köhler et al., 1976). These cells are the most commonly used myeloma cell line from BALB/c hydridomas expressing the IgG1 antibody. The cells were grown in the usual manner in DMEM containing 2 mM glutamine and 5% foetal bovine serum as mentioned in the methodology. In the ELISA assay, detection of antibodies produced by transfected cells was measured; firstly the control DNA and naked PA DNA titres were assessed. The results in figure 6.8 clearly indicate the distinction between O.D. levels from transfected cells with naked PA DNA and the control DNA. This antibody expression trend continued even with serial dilution of cell supernatant, verifying the performance of this bioassay making it desirable from a validation standpoint due to the simple format and sensitive antibody detection technique.

Lastly, excellent repeatability and reproducibility was indicated by the narrow standard deviations that were observed when ideal preparations of transfected cells using PA dendriplexes and PLGA encapsulated with PA dendriplexes were tested on individual assay plates. These results indicated that the modified ELISA assay possessed the necessary biological relevance and performance characteristics to be
considered as a potency-indicating antibody assay produced from CHO cells transfected with various formulations of PA dendriplexes and PLGA-PA dendriplex particles.

**Figure 6.8:** ELISA titre graphs of transfected P3x63Ag8 cells measuring antibody production A: cells transfected with 12 μg of Naked PA; B: cells transfected with 12 μg of control plasmid without PA. Each plot represents the mean optical density of antibodies from cell supernatants at different dilutions binding to antigen. Individual coloured bars represent independent experiments. A statistically significant difference of antibody production between transfection with PA and the control was shown statistically by one way ANOVA.
The media used in the growth of cells and transfection processed was assessed as seen in figure 6.9A. Dulbecco’s modified eagle serum (DMEM) containing 5 % foetal bovine serum was selected as it provided the nutrients for cell growth (Köhler et al., 1976) i.e. higher antibody production. Growth in different media was attempted, such as F-12K without calcium, to investigate the histology and growth of cells together with antibody production. DMEM medium was used throughout the cell immunisation procedure as well as an incubation time of 24 h for both dendriplexes and PLGA-dendriplex particles.

The transfection with PLGA particles, as illustrated in figure 6.9B, showed that the PLGA-C18 dendriplexes had higher IgG expression than PLGA-C0 dendriplexes both containing 12 µg of PA DNA. Whereas the PLGA-control dendriplex particles produced no antibody expression relative to the naked control DNA transfection (figure 6.8B). This trend was not only depicted for PLGA particles but also for the unencapsulated dendriplex particles as shown in figure 6.10. In this case, the high dose (50 µg) for both dendrons at the 10:1 and 20:1 ratios was evaluated for gene expression and to explore if there was a possible link with antibody production. These results demonstrate that antibody production was dose dependent, but more so at the 10:1 ratio for both dendriplexes formulated. However, the C18 dendriplexes had much higher antibodies titres, even at the higher dilution ratio for both the 10:1 and 20:1 N/P ratio systems (figure. 6.10B). These ELISA results correlated well with the previous uptake studies using radiolabelled PA DNA, and both depicted low gene transfer for the naked PA DNA compared to the 10:1 dendriplexes. This suggested that as more DNA was taken up by the cells, especially by the C18 dendriplexes due to increased lipophilicity, the reporter gene was expressed therefore releasing the PA antigen lending to antibody production by the B cells from the hybrid cell line selected.

These antibodies have been shown (Casadevall, 2002) to be a source of immunotherapeutic reagents. Immunotherapy has been used to mitigate the effect of lethal toxin with a combination of antibiotics as is now seen as a viable alternative and so a number of companies are working on mab (high-affinity monoclonal antibody) anti-PA preparations, some of which are undergoing clinical trails already.
Figure 6.9: Graphs of ELISA titres A: evaluating the effect of the medium (DMEM, GM, F-12) on cell supernatant transfected with 12 µg of PA DNA and the control DNA; and B: transfection with various formulation of PLGA with 10:1 ratio dendriplexes containing 12 µg of DNA. The PLGA-dendriplexes demonstrated enhanced antibody production when compared to the PLGA-control dendriplexes and naked DNA in various media, more so for the PLGA-C_{18} dendriplex particles than for the PLGA-C_{0} dendriplex particles.
Figure 6.10: Two ELISA data sets shown A: two formulations (20:1 and 10:1) of \( C_0 \) dendriplexes containing either 50 \( \mu \)g or 12 \( \mu \)g of PA DNA alternatively with control plasmid transfected with cells. The supernatant collected and antibody was measured. For graph B, the same procedure was undertaken, except for the use of \( C_{18} \) dendriplexes. Antibody production was dose dependent shown by both dendriplexes, but the \( C_{18} \) dendriplexes produced higher titre values signifying elevated antibody production particularly at the 10:1 molar charge ratio.
These observations were validated using the standard cytotoxicity study normally used to assess the binary process of intoxication by the main anthrax proteins (Kushner et al., 2003; Alileche et al., 2005; Paddle et al., 2006). The cytotoxicity assay was modified to assess uptake of dendriplexes and PLGA particles. The above papers confirm that the main binary model of PA and LF causing anthrax intoxication of cells follows a series of steps. One of the first steps involves the proteolytic activation of the 83-kDa version of PA which is part of the DNA construct, and hence eukaryotic transcription must be necessary to release the antigen (Chauhan et al., 2002). Once released, cleavage of PA is a necessary step in processing the anthrax toxin, and PA is unable to bind to either EF or LF unless this cleavage has occurred as discussed in Chapter 1.

However with the PA dendriplexes and PLGA particles, the mechanism of gene delivery may slightly alter due to the physicochemical properties of the particles. Manuta et al. (2004), have reported that dendriplexes may operate via the cholesterol pathway, which is suggested to play a role in endocytosis. This may in turn help clarify the reason why the C18 dendron (containing 3 lipidic chains), i.e. containing a partial hydrophobic nature is a highly efficient DNA carrier, as shown in the preliminary studies in chapter 5. The other scenario may simply be due to the attraction of the cationic charge dendriplexes to the negatively charged cell membrane. The complex can then be internalised into the cell via receptor mediated endocytosis. Acidification of the endosome causes the release of the protein-DNA complex into the cytosol and the reporter gene PA is expressed. Kusher et al. (2003) reports that the antigens are processed endogenously via the MHC-I pathway and intracellular processing (TAP-dependent). The phagocytosis pathway, in comparison demonstrates that PA antigens complexed with particles are processed in the endosomes rather than in the proteosone as shown in figure 6.11.
Figure 6.11: Schematic representation of the possible mechanism of PA DNA dendrplex. Particle uptake may occur or naturally by electrostatic attraction to the cell surface (1a) by endocytosis (2) in conjunction with the cholesterol pathway. The classical MHC-I pathway requires protein expression within the cell, followed by secretion into the cytosol (3). Surface proteins via class I MHC are synthesised in the rough endoplasmic reticulum (RER) and the antigen is processed intracellularly (TAP-dependent) (4). The other possibility the antigen could be processed within the endosome during acidification rather than proteosome. Alternatively, the particles can degrade on the outside of cells and then “loaded” as peptides in empty pockets on the cell surface (1b).

Both naked DNA and both dendrplex formulations encoding antigens as vaccines enable the antigens to enter into the classic MHC-I pathway and help to simulate the humoral response more than cell-mediated responses.

In attempts to evaluate that the antigen encoded in the PA DNA was released from the dendriplexes and PLGA particles, cell viability was measured once the CHO-K1 cells were transfected with both sets of particles. The assumption was that the antigen would be released and delivered to the surface to bind to LF, and that this would cause intoxication as illustrated in figure 6.11. For each treatment a constant concentration of LF was added to the cells after transfection and the viability was assessed. Although this assay, which uses a visual subject end-point, was not performed in a manner to yield highly quantitative data, as would be obtained by the measurement of cyclic AMP (Zmuda et al., 2005), the assay did demonstrate that
these formulations inhibited the cellular elongation response of CHO cells in the presence of lethal toxin. The graph shown in figure 6.12 depicts the differences in viability between the dendriplexes and the encapsulated PLGA dendriplexes, with no significant difference between the two dendrons used in the formulations.

**Figure 6.12:** Percentage of live CHO cells after transfection with dendriplexes and encapsulated PLGA dendriplexes as the concentration of DNA content in each formulation increased. After transfection, 0.1 µg/ml of LF was added to the cells incubated and cell viability measured by MTT assay. The un-encapsulated dendriplexes showed a lower number of live cells when compared to the PLGA particles. Statistical assessment demonstrated a significant difference between the PLGA particles and dendriplexes.

Furthermore naked DNA was assayed, resulting in very low percentage of live cells ~ 26 %, which only increased slightly with higher PA DNA concentrations. This suggested that the DNA may have degraded outside the cells and released the PA antigen which could then begin the intoxication process. The differences in level of cell cytotoxicity can have several possible explanations, including the release rate of DNA from PLGA particles, charge properties which differ from the cationic dendriplexes themselves. These PLGA particle properties may reduce cellular binding to the cell surface. Hence, PLGA may release the antigen at a slower rate and
as PA requires complexation with LF to commence the cellular intoxication, cell viability is higher than the dendriplexes.

From the *in vitro* data obtained from all three assays, the 10:1 dendriplexes and encapsulated into PLGA seem promising candidates for testing in animals. They showed higher uptake than naked DNA, and antibody production particularly with increasing PA DNA content in the complexes. Therefore this nano-sized, cationic dendron-PA particle composition may provide an efficient DNA delivery system as a prospective genetic vaccine against anthrax.

**6.4 CONCLUSION**

PA83 encoded in plasmid DNA has been reported to induce immunogenicity against anthrax (Leppla *et al.*, 2002). The PA DNA can be delivered into the cytosol of mammalian cells in order to improve its efficiency using dendrons as carriers. Higher molar charge ratios (10:1) are required to fully condense PA DNA into compact nano-sized particles as well as the PLGA-dendriplexes. Three types of assays were conducted. Radiolabelled PA DNA uptake study, modified ELISA, and a cytotoxicity assay analysing expression of PA in cells. All assays verify that complexation with dendrons aids stability and uptake of PA DNA, with significant differences in terms of antibody production when compared to naked DNA. PA dendriplex delivery to the cell surface may be different to naked DNA, well-known to have low gene expression *in vitro* because of immediate degradation, substantiated by the differences observed in uptake studies and illustrated by limited antibody production. The dendriplexes have enhanced gene expression and antibody production due to the cationic nature of the complexes improving uptake of these particles. Further *in vivo* studies for both PA dendriplexes and PLGA-dendriplex particles, at the optimal molar charge ratio of 10:1, will be conducted to evaluate the ability of direct priming in the induction of immune responses.
CHAPTER SEVEN

IN VIVO STUDIES USING DENDRIPLEXES AND PLGA-DENDRIPLEXES CONTAINING PLASMID PROTECTIVE ANTIGEN AGAINST ANTHRAX

7.1 INTRODUCTION

This chapter describes the development and evaluation of an effective vaccine against anthrax using two forms of delivery systems; dendriplexes, and PLGA-dendriplex particles containing PA DNA, tested by in vivo immunological studies. The DNA vector in the dendriplexes in both formulations is an alternative to vaccination with proteins or peptide antigens. The recombinant plasmid DNA from the strong human cytomegalovirus contains a fragment of protective antigen genes (see section 6.2.3). PA is the most important antigen required for specific immunity to anthrax (Leplla et al., 2002). It was first cloned by Ivins and Welkos (1986) and was shown to provide protective immunity. PA is a long molecule at 0.21nm with four distinct domains. Each domain is required for the intoxication process as describe in Chapter 1. Domain 1 is a proteolytic activation site, whereas domain 2 is a β barrel cone containing a flexible loop. This loop is thought to be involved in transmembrane pore formation in cell membranes. Domain 3 aids protein-protein interaction and domain 4, found at the carboxy-terminal end of the molecule, assists binding to cellular receptors. The selection of the region of PA is vital to elicit immunodomain epitopes required in producing specific antibodies. In our studies,
PA DNA containing domain 4 could be used as potential candidates for toxicity neutralisation by interfering with PA binding to its host receptor. The second plasmid DNA is the control without the antigen, the two vector constructs are described in Chapter 6.

Vaccine development efforts have focused on well-characterised genetic vaccines with truncated derivatives of PA. The limitations of AVA have raised interest in the new generation of vaccines. The main disadvantage of AVA is due to its "dirty" preparation (containing small amounts of EF and LF) which may explain the side effects that develop after being administered with the AVA vaccine (Wang and Roehrl, 2005). Strategies designed to produce purified components together with reducing the doses and number of immunisations are required to improve compliance (Xie et al., 2005). These new vaccines must be tested on a suitable animal model. A number of models have been investigated including mice, guinea pigs, and rabbits. In our case mice were used as the primary candidate to investigate anti-PA antibody production. Susceptibility to the toxin component, i.e. lethal factor varies in mouse strains: A/J and BALB/c strain are very susceptible compared to the C57/BL strain. The A/J strain lacks a functional Hc gene encoding for complement component C5. The C5 derived peptides are important chemoattractants for macrophages and neutrophils during inflammation. The absence of these factors results in a delay in the arrival of macrophages at the site of infection therefore retarding the immune response. The lethal factor plays a critical role in anthrax pathogenesis, which varies in its manifestation with different strains of mice (Welkos et al., 1986). There are evident stages of pathogenesis where the first stage is the interaction of spores with macrophage and the second, later macrophage interaction with lethal toxin after infection is established. Lethal toxin causes death in BALB/c, A/J and C57BL/6J strains, but the timing of the pathological event is somewhat different as BALB/c and A/J contain sensitive macrophages which die more rapidly (Welkos and Friedlander, 1988). Hence due to this susceptibility of A/J and BALB/c and their ability to protect them against challenge with unencapsulated anthrax strains, they were selected as potential models for initial screening of dendriplex anthrax vaccine.

The DNA was complexed with poly-lysine dendrons to form small compact particles as described in Chapter 3. These particles were characterised with the PA DNA investigating the morphology and size. Two dendrons were used in the in vivo study, the first dendron (C₀), containing seven lysine groups and 8 amino groups and
no hydrocarbon chains attached to the core and the second dendron, which contained three C_{18} chains as shown in Fig. 7.1.

![Chemical structure of dendrons](image)

**Figure 7.1** The simplified chemical structure of two cationic (lysine)-based dendrons (L=lysine) (C_{60}(Lys)_{7}(NH_{2})_{16} MW 1174.91 (Co) and (C_{18})_{3}(Lys)_{7}(NH_{2})_{8} MW 1758.45 (Cis) both containing 16 amino groups and 7 lysine groups attached to the core and have either 0 or 3 hydrocarbon chains.

The small synthetic peptide Trp-Lys-Tyr-Met-Val-o-Met demonstrated enhanced surface expression of CD80 receptor on mouse bone marrow-derived dendritic cells, which is one of the main co-stimulatory signals for the induction of immune responses with various types of co-delivered DNA (Lee et al., 2005). The peptide enhanced IFN-γ ELISPOT response in a dose dependent manner, further indicating that this small peptide contains adjuvant properties. Other groups such as Dasgupta et al. (2003) have assessed L-Lysine HCl as a possible adjuvant in tuberculosis vaccination with heat killed *Mycobacterium tuberculosis* (MTB) and Bacille Calmette Guerin (BCG). When lysine boosts were given to the primary group of mice, the antibody titres were significantly higher (about 1.2 fold) than the control. They proposed L-lysine HCl as a probable adjuvant in vaccination due to its biocompatible nature in comparison to the licensed adjuvant, alum, which is more hazardous. As the compounds used in this thesis are small peptide poly-lysine based polymers these may have similar adjuvant properties.

These dendriplexes were further encapsulated into PLGA particles to further protect the DNA from degradation and control the manner of DNA release from these particles. Apart from these advantageous aspects, PLGA particles represent a different form of immune adjuvant (Xie et al., 2005). These particles have improved uptake and processing by antigen presentig cells (Mize et al., 2000; 2003; Singh et al., 2004). This chapter examines whether the PA dendriplexes and PA dendriplex PLGA particles increase the speed and magnitude of anti-PA antibodies and their
neutralising effect when challenged with anthrax, especially in comparison to naked PA DNA.

7.2 EXPERIMENTAL

7.2.1 Materials
DNA encoding the full length of *B. anthracis* protective antigen (PA83) was cloned into the eukaryotic expression plasmid, pCMV/myc/ER containing a signal sequence for fusion protein secretion. A negative control was used where the pCMV/myc/ER vector DNA was without the PA83 insert. All the materials used will be indicated in each section of the methodology.

7.2.2 Synthesis of C\textsubscript{18} and C\textsubscript{0} dendrons
The structures of the two polylysine-based dendrons used in the animal study, (C\textsubscript{0}(Lys)\textsubscript{7}(NH\textsubscript{2})\textsubscript{8} (C\textsubscript{0}) and (C\textsubscript{18})(Lys)\textsubscript{7}(NH\textsubscript{2})\textsubscript{8} (C\textsubscript{18}). Details of their solid peptide synthesis and their purification can be found in Chapter 2.

7.2.3 Preparation of PA DNA and control dendriplexes
A 10:1 molar charge ratio (dendron:DNA) was used in both sets of dendriplexes as indicated in section 3.2.1, as this specific stoichiometric ratio condenses the DNA more efficiently to produce small particles with a narrow polydispersity.

7.2.3.1 Low dose study
The dose administered was 1 \( \mu \)g of DNA in 20 \( \mu \)l of PBS per mouse, therefore for a group of five mice the concentration of PA DNA per group was 5 \( \mu \)g of DNA in 100 \( \mu \)l of PBS. After three boosts of injections, the total dose of DNA per study was 15 \( \mu \)g/group. Total concentration of DNA for particle synthesis for all three groups was 45 \( \mu \)g/group and altogether 0.5 mg of DNA was used to formulate the low dose dendriplexes.
7.2.3.2 High dose study

Each group consisted of five mice, each mouse required a dose of 50 μg/20 μl, and so for the whole group, 250 μg/100 μl of PA DNA was required. Three booster injections (at time points of 3, 6 and 9 weeks) were given, resulting in a total PA DNA for the entire study of 750 μg/group. The total PA DNA for particle synthesis was 3.6 mg approximately 1.2 mg (360 μg/300 μl) of PA DNA per group. The C\textsubscript{18} PA dendriplexes were prepared as described in figure 7.2 below:

![Flow diagram explaining C\textsubscript{18} PA dendriplex formulation.](image)

**Figure 7.2** Flow diagram explaining C\textsubscript{18} PA dendriplex formulation. This methodology was used for both set of dendriplexes, but the concentration of dendron adjusted according to the molecular weight of each dendron at a 10:1 molar charge ratio.

7.2.4 Preparation of PLGA/PA dendriplexes and PLGA/PA DNA

The PLGA particles were formulated by the double emulsion method, explained in section 4.3.1. Three sets were prepared for the animal study. These included, PA DNA-C\textsubscript{18}-PLGA, PA DNA-C\textsubscript{0}-PLGA, and PA DNA-PLGA particles. Encapsulation efficiency (see section 4.3.3) for the 10:1 dendriplex in PLGA was 15.6% w/v and for DNA-PLGA it was 9.92% w/v. These figures were used to calculate the concentration of DNA per dose. The minimal amount of PA DNA required was ~ 15 μg/mouse/injection and therefore, 225 μg/group.

7.2.5 Animals

Two different mouse strains the BALB/c and A/J (16-20 g) were used for the low dose study, and for the high dose study only BALB/c mice were used. All mice were
female and aged between 8-10 weeks at the beginning of the investigation. The dendriplex samples were directly injected intramuscularly into the thigh as a suspension in PBS using Hamilton syringe, while the lyophilised PLGA samples were re-suspended in PBS one hour prior to administration, also by Hamilton syringe.

7.2.6 Experimental design and dosing
In total three main studies were undertaken shown in scheme 7.1. Firstly the low dose dendriplexes were administered, followed by the high dose dendriplexes and finally by the PLGA/dendriplexes. Each dose was administered by intramuscular injection into the thigh.

Experiment 1: Low dose C₁₈ dendriplexes
Animals: 10 BALB/c and 10 A/J mice
Dose: 1 μg/20 μl
Route: I.M
Time points of booster vaccinations: 3, 6, 9 weeks
Control: DNA without PA protein/C₁₈ dendron

Experiment 2 Low dose C₀ dendriplexes
Animals: 10 BALB/c and 10 A/J mice
Dose: 1 μg/20 μl
Route: I.M
Time points of booster vaccinations: 3, 6, 9 weeks
Control: DNA without PA protein/C₁₈ dendron

Experiment 3 High dose C₁₈ dendriplexes and C₀ dendriplexes
Animals: 15 BALB/c mice
Dose: 50 μg/20 μl
Route: I.M
Time points of booster vaccinations: 3, 6, 9 weeks
Control: DNA without PA protein/C₁₈ dendron and DNA without PA protein/C₀ dendron

Experiment 4 PLGA C₁₈ dendriplexes and PLGA C₀ dendriplexes
Animals: 15 BALB/c mice
Dose: 14 μg/20 μl
Route: I.M
Time points of booster vaccinations: 3, 6, 9 weeks
Control: PLGA-PA particles

Scheme 7.1: Scheme for experiments of in vivo study using dendriplexes and PLGA-dendriplexes incorporating PA DNA as antigen against anthrax.
Principally an analogous protocol was used for all four experiments, and contain slight variations as shown in scheme 7.2. For experiments 1 and 2 (low dose), procedure A was used, and for experiments 3 and 4 (high dose and PLGA particles) procedure B was used.

**Procedure A**

10 BALB/c mice and 10 A/J mice, each split into two groups of five animals.

**Group 1**- BALB/c mice: Intramuscular injection of 20 μl of PA-DNA-particle in right thigh of each animal.

**Group 2**- BALB/c mice: Intramuscular injection of 20 μl of control DNA particle in right thigh of each animal.

**Group 3**- A/J mice: Intramuscular injection of 20 μl of PA DNA-particle in right thigh of each animal.

**Group 4**- A/J mice: Intramuscular injection of 20 μl of control DNA particle in right thigh of each animal.

3 week test bleeds (1st boost injection)

6 week test bleeds (2nd boost injection)

9 week Terminal bleeds

**Procedure B**

15 BALB/c mice split into three groups of five animals

**Group 1**- Intramuscular injection of 20 μl of PA DNA-C₉ dendriplex particle in right thigh of each animal.

**Group 2**- Intramuscular injection of 20 μl of PA DNA-C₁₈ dendriplex particle in right thigh of each animal.

**Group 3**- Intramuscular injection of 20 μl of PA DNA in right thigh of each animal.

3 week test bleeds (1st boost injection)

6 week test bleeds (2nd boost injection)

9 week Terminal bleeds

**Scheme 7.2** The two procedures used A: low dose for experiment 1 and experiment 2 B: High dose dendriplexes, experiment 3 and related protocol for the PLGA study. Both procedures had control treatment groups containing control DNA complexed with dendron and naked PA DNA.

**7.2.7 Blood sampling**

Blood samples were taken from the tail vein of each animal. Approximately 100 μl of blood was collected into capillary tubes containing heparin. Once collected, samples were stored in Eppendorf tubes overnight at 4°C to allow the blood to clot. The samples were then centrifuged at 15000 rpm for 10 min and serum removed. This was then stored at -20°C in Eppendorf tubes until ready for use.
7.2.8 Anti-PA antibody levels in serum using an enzyme linked immunosorbent assay (ELISA)

Serum samples of individual mice were analysed by ELISA for specific antibody expression, in this case for anti-PA antibodies. All washing steps were carried out with PBS containing 0.05% Tween 20 (PBST). Each incubation step was carried out at room temperature, if not indicated otherwise. The 96 wells of high-binding protein microtiter plates (MaxiSorp™, Nunc) were each coated with 100 µl 5 µg/ml of recombinant PA in a 0.05 M carbonate buffer at pH 9.5 (at 4 °C overnight). The wells were washed and blocked with 200 µl/well of 2% Marvel (skimmed milk powder) in PBST for 2 h. Each serum sample was diluted starting with a 10-fold dilution, doubling across the plate and incubated for 2 h. The wells were washed extensively with PBS/Tween and anti-PA antibodies were detected by using goat anti-mouse IgG (Sigma) incubated for 1 h. The plates were developed by adding 100 µl of TMBBlue (Intergen) to each well and the reaction was stopped after 5 min. with 25 µl 2 M H₂SO₄. Optical densities were read at 450 nm. The serum collected from inoculated animals treated with each formulation was compared to the control.

**Fig. 7.3** An example of a 96 well plate with serial dilution across the wells, coated with antigen incubated with serum collected from the mice. The substrate enzyme (TMBBlue) was added for the colour development and enables optical density measurements using a spectrophotometer.

The basic concept of an enzyme linked immunosorbent assay (ELISA) is demonstrated in figure 7.4. It is a useful immunological technique as it can allow
antigens to become adsorbed onto solid surfaces, such as plastics. The 96 well microtitre plates are commercially available and are especially adapted for the ELISA procedure, allowing easy manipulation with the use of dispensing reagents. This permits the use of small volumes and gives ELISA the potential of dealing with a large number of samples. The final step of an ELISA is a colour reaction that is visible by the eye and can be quantified efficiently by using multichannel spectrophotometers.

**Figure 7.4** Schematic representation of the four steps involved in the ELISA technique. These include:

1) Antigen becomes bound to the plastic well surface

2) Serum is added to the well and if autoantibody to the antigen is present it will bind to the antigen well.

3) An enzyme-linked anti-mouse-Ig antibody is added to the well and will bind to any autoantibody to the antigen.

4) A colourless substrate is added (S) and this will be converted into a coloured product (C) once bound to the enzyme. The amount of coloured product produced can be measured spectrophotometrically. The amount of product is proportional to the amount of enzyme which in turn is proportional to the amount of autoantibody that is bound to the autoantigen.
The data from the ELISA was carefully analysed over the range of serum dilutions. The lowest serum dilutions resulted in the highest optical densities. The affinity of anti-PA antibody binding was dependent on the dilution factor of the serum, and as the dilution increased the affinity of the anti-PA antibody decreased, correlating to lower optical densities. Endpoint titres were defined as the reciprocal of the highest serum dilution that resulted in an absorbance greater than 5% standard deviation above the average of negative control serum samples at the same dilutions, with a minimum value of 0.03.

Fig. 7.5 Standard graph of log₁₀ sera dilution from mice inoculated with various formulations against the optical density (O.D.) measured from the ELISA plates. The graph shows a decrease in optical density as the serum dilution increases, and can be determined by the affinity of binding of anti-PA antibodies which was higher at lower serum dilutions, as illustrated by the diagrams above the graph. The black line represents the control with no detectable ELISA reactivity where the 95% confidence interval has been calculated (black dotted lines).

7.2.8.1 Determining IgG1 and IgG2a levels in serum using ELISA assay
This method involves the same procedure as described in section 7.2.8, but with differing methods for detection. The antigen-specific secondary antibodies, biotinylated anti-mouse IgG1 and IgG2a (Pharmingen) were used instead of the goat anti-mouse IgG. These conjugates were diluted with PBST and 0.1% Marvel
(skimmed powdered milk) and incubated for 1 h. The plates are washed with PBST and 100 µl of streptavidin-peroxidase (1:1000 in PBST and 0.1% Marvel) for 1h. The streptavidin binds to biotinylated antibodies and the peroxidase reacts with the substrate, therefore acting as a secondary antibody. The substrate TMBBlue (100µl) was added to each well and the reaction was stopped after 5 min. Using 25 µl 2 M H₂SO₄, and the optical densities for each plate was read at 450nm.

**Fig. 7.6** Indirect ELISA measures the level of antibodies IgG1 and IgG2a in the serum samples that specifically bind to the antigen coated on the plates. A secondary antibody streptavidin-peroxidase must bind to biotinylated antibodies (IgG1 and IgG2a), the peroxidase which reacts with the substrate to give a coloured product therefore the secondary antibody is a measure of detection for antigen specific IgG1 and IgG2a as shown in A. If streptavidin–peroxidase does not bind to the antibodies (IgG1 and IgG2a) and to the substrate, no colour product will be formed as depicted by illustration B.

### 7.2.9 *In vitro* toxin neutralisation assay (TNA)

Neutralizing assay antibody titres were determined by the ability of serum samples to protect the RAW 264.7 cells from the toxic effect of anthrax toxin, a cocktail of PA (protective antigen) and LF (lethal toxin) (LeTx). Cells were grown overnight in 96 well plate (Costar, Corning Incorporated, Corning, NY) and, were maintained in Dulbecco's Minimal Essential Medium (D-MEM) containing 5% heat-inactivated fetal bovine serum (FCS). The mouse serum samples were doubly diluted in cell culture medium and 5% FCS, starting with a serum dilution of 1:100. Serial two-fold dilutions of samples were preincubated with LeTx (0.08ng of rPA per ml and 0.1µg of LF per ml, final concentrations) in a humidified incubator set at 37 °C, 5% CO₂ for 1 h. Medium on the RAW 264.7 cells was removed from each well and any detached cells from the cell plate, replaced with preincubated serum LeTx. Each sample was done in duplicate. After incubating for 3h, 10µl WST-1 reagent (Roche) was added.
to each well and incubated for 4h at 37°C. The absorbance was then read at 450nm. Nine wells contained only medium and served as medium controls. Three LeTx wells contained only LeTx and served as blanks.

7.2.10 Statistical Analysis
ELISA titres of different treatment groups were compared, using a paired Student’s t-test when $t > 1.895$ depicted statically significant results. To test for associations between PA-ELISA titers over a 9 week period, we employed one-way ANOVA with pairwise comparisons, when $F > 3.39$, a significant difference was observed stated in both cases as $p < 0.05$. 
7.3 RESULTS AND DISCUSSION

7.3.1 Low dose naked DNA, C₁₈ dendriplexes and C₀ dendriplexes

Naked DNA at a dose of 1μg was administered by intramuscular injection to both A/J and BALB/c mice. The graphs in figure 7.7 indicate no increase in anti-PA antibodies over a period of 9 weeks, even at the lower dilutions for both strains of mice. The statistical analysis by one way ANOVA also illustrated no significant difference (p > 0.05) between the ELISA titres of mice immunised with naked DNA over the 9 week period. These results were corroborated by Williamson et al. (2002) in which mice primed only with plasmid DNA had low titres and were poorly protected when challenged with *Bacillus anthracis*. Furthermore, other groups such as Hahn et al. (2005) have observed similar findings when using a single PA plasmid, but when a combination of two plasmids pSecTag and pCMV/ER were administered, the antibody response was marginally elevated.

The complexation of DNA with dendron has several advantages, firstly by providing a level of protection from degradation, and secondly small peptides, in this case dendrons, potentially acting as adjuvants. Two dendrons were used Ala₃Lys₁₅(NH₂)₁₆ named (C₀) and (C₁₈)₃Lys₁₅(NH₂)₁₆ named (C₁₈). The characterisation of these particles can be found in chapter 6. The DNA content in both sets of dendriplexes was 1 μg/20 μl to match the dose of the naked DNA study. Two strains of mice were also used in the low dose dendriplex study to investigate any difference in antibody response and to allow comparison with the naked DNA antibody titres.

In figure 7.8, graphs A and B show five inoculations of BALB/c mice with each of the two dendrons, and the mean control, the DNA without PA. The C₁₈ dendriplexes on average have higher antibody titre values than the C₀ dendriplexes. Only one of the mice had a significantly higher antibody response compared to the control, while the other four showed results analogous to the C₀ dendriplexes. There was a statistically significant difference (p < 0.05) between the mean titres of C₁₈ dendriplexes and titres of BALB/c mouse 4 within the same treatment group (figure 7.8A). In the A/J strain, a more pronounced difference was observed between the C₁₈ dendriplexes and the C₀ dendriplexes. The titre profile of the A/J mice at 9 weeks for was relatively close to the control even at the lower dilutions. Hence, considerable
variation could be due to possible differences between the two mouse strains with regard to their humoral immune responses (Hahn et al., 2004).

Both dendriplexes for the BALB/c mice at week 9 (figure 7.9) produced antibody level that was approximately two fold higher relative to the naked DNA at its lowest dilution. Generally the dendriplexes induced higher anti-PA antibody responses compared to naked DNA. This becomes more obvious in the estimation of the dilution endpoint for both studies, the results of which are summarised in figure 7.10.

From the optical density versus serum dilution graphs (figure 7.7 to 7.9), the mean anti-PA antibody endpoint titres were calculated and are shown in figure 7.10. The C_{18} dendriplexes showed a large increase in antibody response in the BALB/c mice following the initial immunization not shown by the C_{0} dendriplexes. However after the first boost both levels of anti-PA antibody for both dendriplexes decreased to just over 500 and this was seen in both mouse strains. In contrast, the A/J strain had a lower anti-PA titre for both dendriplexes, compared to BALB/c at week 3, and by week 9 after the second boost the mean antibody titres increased, more so for the C_{18} dendriplexes. Both mouse strains illustrated a decrease in antibody titre following the primary boost. This could suggest that at week 3 the low dose dendriplexes induced an inflammatory response, increasing the initial antibody titre post primary immunisation. The secondary low dose boost was not sufficient to continue to induce antibody responses, hence two boosts of dendriplexes were required to sustain the longevity of the antibody response.
Figure 7.7: BALB/c mice were immunised with naked PA DNA at a dose of 1 μg/20 μl over a period of nine weeks. The graphs A, B, and C are ELISA titres measuring antibody response against serial dilution of serum collected. Naked DNA immunisations did not induce an increase of anti-PA antibodies over 9 weeks, showing non-significant difference as revealed by one-way ANOVA.
Figure 7.8: Five inoculations of BALB/c mice for each group at week 9. Groups A: PA-C_{18} dendriplexes and B: PA-C_{0} dendriplexes and a separate treatment group inoculated with control without PA insert into the plasmid construct. Each plot represents mean optical density (O.D.) of binding of serum antibodies at different serum dilutions. Both graphs show similar titres between the two dendriplexes at week 9, although the C_{18} dendriplex mouse 4 has statistically significantly higher titre than the mean antibody titre in the same treatment group depicted in graph A.
Figure 7.9: Five inoculations of A/J mice for each group at week 9. Groups A: PA-C₁₈ dendriplexes and B: PA-C₀ dendriplexes. Serum from control groups had no detectable ELISA reactivity. Each plot represents mean optical density of binding of serum antibodies at different serum dilutions collected from each A/J mouse. There was a statistically significant difference between the A/J and the BALB/c strains for both dendriplexes as calculated by one-way ANOVA.
Figure 7.10: ELISA titre of individual mice (Balb/c and A/J strains) serum samples over the course of 9 weeks. Serum samples were collected after each of the three vaccinations at week 3, week 6, and in week 9 the terminal bleeds. Each bar represents the mean titre of individual sera from a group (n = 5). The controls were mice inoculated with plasmid without PA. Two boosts were given with the same dose as the primary immunisation. BALB/c strain illustrates an initial increase in anti-PA antibody titre not shown in the A/J strain. After the primary boost both strains showed decreases in antibody level at week 6 which increased by week 9.
Overall the titres were low, but measurable in the low dose study. Consequently IgG1 and IgG2a ELISA assays were not conducted. Other groups also have shown low antibody titres which were increased by boosts of recombinant protein, therefore obviating the key advantages of pDNA vaccines (Galloway and Baillie, 2004). In the end this would increase the number of doses, which is one of the problems with the current AVA vaccine. Therefore in this work we focused on modifying the concentration of PA DNA in the dendriplexes. Flick-Smith et al. (2005) demonstrated anti-PA antibody production is dose dependent, by immunising A/J mice with varying concentrations from 0.25 to 12.5 µg rPA given by the I.M route. They determined that A/J mice produced antibodies to rPA in a dose-dependent manner, with antibody levels increasing as the immunising dose level increased. Williamson et al. (2002) also conducted a dose-response trial. The results from this trial indicated that a 50 µg dose of plasmid DNA should be selected for subsequent immunisations, as this dose induced a consistently high level of antibody response. This gave an indication of the levels of anti-PA antibodies produced for a low dose; the subsequent step was to increase the concentration of DNA. As other groups used 50 µg of DNA and achieved higher the antibody responses, this dose was used in our next in vivo study.

Evaluating the titre values from the low dose dendriplex study, the BALB/c strain had relatively higher titers by week 3 compared to the A/J strain. Strong immune responses are required within the first few weeks for a potentially viable vaccine against anthrax. Due to the prompt progression of the disease, rapid induction of anti-anthrax host responses is crucial in the scenario of bioterrorism (Kasuya et al., 2005). The BALB/c strain was thus selected as a potential model for the initial screening of the next high dose dendriplexes and the PLGA/dendriplex, as prospective vaccine candidates.

7.3.2 High dose naked DNA, C_{18} dendriplexes and C_{0} dendriplexes

Three sets of high dose DNA vaccines were used, C_{18} dendriplexes, C_{0} dendriplexes, and naked DNA, parallel to the low dose study. Again the naked DNA did not induce strong PA immune responses over the 9 weeks, even with a higher dose (P > 0.05). In figure 7.11 the titres obtained were analogous to the low dose study. The control titers were added as a comparison and to demonstrate the proximity of the antibody levels naked DNA to the control.
Both dendriplexes comprising 50µg of PA DNA at 3 weeks produced approximately double the titre values obtained in the low dose study, especially at the lower dilution ratios (i.e. 10:1) as shown in figure 7.12. This was not just the case for the week 3 time point; generally there was a higher level of anti-PA antibodies level for the 50 µg PA dendriplexes (both C₀ and C₁₈) relative to the 1 µg PA dendriplexes. The ELISA titre values at 3 weeks for the 50 µg C₁₈ dendriplexes started with approximately the same antibody level as C₀ dendriplexes.

At 6 weeks there was no significant difference between the two dendriplexes depicted by the ELISA endpoint titres (P < 0.05) in figure 7.13 and by the mean IgG anti-PA antibody responses in figure 7.15. In both studies a decrease in antibody titre was evident after the first boost at 6 weeks in comparison to the low dose, which had higher initial antibody levels. At week 9, the antibody levels for the C₁₈ dendriplexes increased by 9 fold, in contrast to the C₀ dendriplexes which did not change after week 6. After the second boost the C₁₈ dendriplex produced a significantly higher antibody response. The difference can be seen in figure 7.14, graph A, showing higher titre values with a longer endpoint compared to graph B. This suggests the longevity of antibody response is induced by boosting with two 50 µg doses of C₁₈ dendriplexes, which peaked at 9 weeks post primary immunisation. The dose of dendriplexes had a substantial effect on the level and long lasting antibody response as demonstrated by the different profiles shown in figures 7.13 and 7.14. This may imply that higher concentrations of DNA and dendron have an adjuvant effect by activating the inflammatory responses in the innate immune system, and therefore induce stronger humoral responses.

Previous studies have shown that humoral immune responses are not solely responsible for protection against anthrax (Ivins et al., 1992; Ivins et al., 1994; Turnbull, 1991). Despite this, by assessing the humoral responses linked to the cell mediated responses, we can predict the cascade of events leading to strong Th1 responses or activation of CD4⁺ T cells. To evaluate the humoral responses induced by the high dose PA DNA dendriplexes, we assayed individual mouse sera not only for IgG anti-PA antibodies but also for antigen-specific IgG1 and IgG2a by using ELISAs.
Figure 7.11: BALB/C mice immunised with 50 μg/20 μl dose of naked PA DNA over a period of 9 weeks. The graphs A, B and C represent mean optical density of binding of serum antibodies at different serum dilutions. Naked DNA even at the high dose did not elicit significant antibody responses over the 9 weeks shown by statistical analysis (ANOVA), a result similar to those shown in figure 7.7 for the low dose study.
Figure 7.12: BALB/c mice immunised with 50 μg/20 μl of PA dendriplexes after 3 weeks from initial inoculation, serum collect and examined by ELISA. Two sets of dendriplexes were used, graph B: represents C18 dendriplexes and C: C0 dendriplexes. Graph A correspond to the antibody titre reading for the low dose study at 3 weeks. The optical density of the various serial dilutions of serum collected from five mice was measured. Overall higher ELISA titres are evident for high dose dendriplexes (graph B and C) than for the lower doses (graph A) at 3 weeks.
Figure 7.13: Original ELISA titres measuring antibody response with serial dilutions of serum collected from individual BALB/c mice at 6 weeks (high dose study). These mice were immunised with two formulations both containing PA DNA. Graph A: C_{18} dendriplexes and graph B: C_{0} dendriplexes, on each graph the control titres (mice immunised with dendriplexes containing DNA without PA) were included to assess the antibody endpoint. Both graphs show no significant differences between the two dendriplexes calculated using paired Student’s t test.
Figure 7.14: Original ELISA titres measuring antibody response with serial dilutions of serum collected from individual BALB/c mice at 9 weeks for the high dose study. These mice were immunised with two formulations both containing PA DNA. Graph A: C_{18} dendriplexes and graph B: C_{0} dendriplexes, on each graph the control titres (mice immunised with dendriplexes containing DNA without PA) were included to assess the antibody endpoint. There was a statistically significant difference in antibody titres, C_{18} dendriplexes had higher primary titres at lower dilutions and a longer antibody endpoint compared to C_{0} dendriplexes calculated using paired Student’s t test.
Figure 7.15: A plot of the mean anti-PA IgG antibody levels calculated from the original ELISA endpoints shown in figure 7.11, 7.12, 7.13 and 7.14 versus the number of weeks post primary inoculation of BALB/c mice. Three sets of immunisations in the high dose study were recorded: naked PA DNA, C₀-PA dendriplexes, and C₁₈-PA dendriplexes each containing 50 μg of PA DNA. Two boosts were given, one after 3 weeks and the other after 6 weeks. At each time point; serum from BALB/c mice was collected. The terminal bleeds were collected after 9 weeks post primary immunisation and measured by ELISA. The naked PA DNA over the 9 weeks did not induce any significant antibody response, which was analogous to the results from the low dose study (statistical analysis using ANOVA). The C₀ dendriplexes elicited a constant level of anti-PA antibody over the specified period, whereas the C₁₈ dendriplexes produced a 9 fold increase at week 9 after the second boost was administered.
In the assessment of IgG1 and IgG2a, naked PA DNA was used as the control instead of the control plasmid (without PA). This was because the naked PA plasmid immunisations illustrated no significant antibody expression in the lower and higher dose studies. The C18 dendriplexes did not elicit IgG1 response at 3 weeks demonstrated by figure 7.16, but this was not the case by week 9 as the antibody expression increased, as shown by the higher titre levels found (figure 7.17).

The geometric mean titre for PA-specific IgG1 responses of mice immunised with C18 dendriplexes was higher, but not significantly different, than PA specific IgG1 responses of mice vaccinated with C0 dendriplexes. All sets of serum were tested for anti-PA IgG2a. Nevertheless no significant IgG2a responses were detected when directly compared to IgG1 responses. Both dendriplexes had predominantly higher IgG1 type responses than IgG2a shown in figure 7.18. These results are consistent with a Th2-type immune response (Hahn et al., 2005). Other groups such Gu et al. (1999) that observed IL-4 induces B cells to switch to IgG1 production explaining the bias noted in anti-PA serum from BALB/c mice. They used the Elispot assay to detect which cytokines were activated in spleen cells, and found that the cells were stimulated to produce the Th2 cytokine IL-4, which increased 4-fold following vaccination. Their findings corroborates the results obtained from the IgG1 ELISAs and gives us a further insight into the possible mechanism involved in the humoral system, lending to the production of specific antibodies. Despite the fact that there is a definitive role for the anti-toxin antibodies in the protection against anthrax, it is not clear what levels of antibodies are needed to protect humans against anthrax following vaccination and its equivalent in the mouse model (Brey, 2005). This was an important concern, as challenge studies cannot be performed in humans, therefore new vaccine systems are required to be evaluated in an appropriate animal model, especially if results are to be extrapolated and used to correlate with human clinical studies.

To our knowledge these were the first studies in which dendron and PA DNA (dendriplexes) were used as a possible vaccine against anthrax. Others such as Hermanson et al. (2004) have developed a cationic lipid-formulated plasmid DNA vaccine. The lipid used was vaxfectin at a final molar ratio of 4:1, DNA/cationic lipid in which they obtained geometric mean anti-PA titres of 10^5 in rabbits after two 1 mg dose of DNA via i.m injections. In our case, the C18 dendriplex obtained anti-
PA titres of $10^4$ in BALB/c mice after two boost injections with a 50 µg dose. Hence it was difficult to compare results as the two studies had differing animal models and doses of DNA. However it was shown that the rabbits after receiving two injections of Vaxfectin-formulated pDNA, were protected from spore challenge (Hermanson et al., 2004). The anti-PA titres were higher post spore challenge indicating sufficient spore generation to boost the pre-existing immune response in the vaccinated rabbits. In our study we demonstrated a level of high anti-PA antibodies for the dendriplexes, although this formulation needed to be further tested for its ability to protect against lethal toxin challenge in vivo.

7.3.2.1 In vitro toxin neutralisation assay (TNA)

Neutralising assay antibody titres were determined by the ability of serum samples from mice immunised with high dose PA dendriplexes, to protect the cells from anthrax toxin. Cell viability was measured to assess the degree of protection provided by anti-PA antibodies in the serum samples. Cell viability was less then 25% suggesting dendriplex formulations, even at higher doses, are likely to fail in protecting these animals against anthrax challenge (Figure 7.19). This failure of protection could be due to the low specificity of the antigen in the binding to the B cells. The role that antibodies play in vaccine induced resistance for protection against anthrax infection has not been well defined (Little et al., 1997). Hahn et al. (2005) obtained similar results in their sheep vaccination study using PA plasmid complexed with vaxfectin. In their study they also observed low TNA titres but significantly higher anti-PA antibodies. The same group also conducted a study of plasmid PA administered by I.M. vaccination to mice and they obtained corresponding results to their sheep study. Other groups such as Ivins at el. (1994) have demonstrated that there was no correlation between the titre of anti-PA antibodies and the survival from bacterial infection. This therefore suggested that the cell mediated immune response may contribute to the clearing of anthrax infections.

The route of administration of the anthrax vaccine may be an essential factor in inducing protection when challenged with spores. Boyaka et al. (2003) has revealed that neutralising antibody was induced in external secretions of mice mucosally immunised with rPA. The mucosal site is the most common route of entry of the anthrax pathogen, therefore is an alternative to the traditional systemic compartment
that is now currently achieved by the AVA. Hence consideration of this as an alternative route of inoculation is a possibility to further induce protection against the anthrax pathogen and to develop these dendriplex particles as an anthrax vaccine.

Recently rhesus macaques were used as a model as they are considered to be the model most comparable to humans (Ivins et al., 1998). When rhesus macaques were inoculated with two doses of rPA (50 or 5 μg per dose) formulated with Alhydrogel at 0 and 4 weeks, 90-100% protection was observed against an aerosol challenge at 3 months. Hence for our dendriplex vaccine investigation into larger animals such as rabbits or sheep needs to be undertaken, as higher neutralising antibodies may be obtained. The next step would be the inoculation of sheep with 1 mg of PA DNA complexed with C18 dendron, which might then provide a better understanding of the humoral response (specific antibody production) and also the cell mediated response (testing cytokine production from spleen cells).
Figure 7.16: Original ELISA titres of antigen specific IgG1 of serum collected from individual BALB/c mice vaccinated with two formulations of dendriplexes at week 3 post primary immunisation. Graph A: C_{18} dendriplexes; graph B: C_{0} dendriplexes. The C_{18} dendriplex vaccine did not elicit IgG1 response in mice (n=2) at week 3, as shown by the titre values which were lower than the control (naked PA DNA).
Figure 7.17: Original ELISA IgG1 and IgG2a titres of BALB/c mice immunised with C₁₈ dendriplex vaccine at week 9 post primary vaccination. Graph A: IgG1 and graph B: IgG2a response. There was no significant change in IgG2a response especially when compared to the IgG1 titres, this was not only the case for week 9, but also after the first boost.
Figure 7.18 A graph of the geometric mean anti-PA titers specific for IgG1 and IgG2a of serum collected from BALB/c mice at three timepoints post primary vaccination. Two sets of dendriplex vaccines (C_{18} dendriplexes and C_{0} dendriplexes) were used, each at 50 μg dose. The original ELISA antibody titres were used to estimate the dilution endpoint. The geometric mean titre for PA-specific IgG1 responses of mice immunised with C_{18} dendriplexes was higher, but not significantly different, compared to IgG1 responses of mice vaccinated with C_{0} dendriplexes. Both dendriplexes produced predominantly higher IgG1 type responses than IgG2a responses.
Figure 7.19: Levels of neutralising anti-PA antibodies in serum from BALB/c mice i.m immunised with high dose dendriplexes. Serum samples serial diluted of each sample added to J774A.1 (mouse macrophage cell line) culture in the presence of anthrax toxin (cocktail of PA and LF). Results shown are % cell viability and are expressed as reciprocal dilution titres.
7.3.3 PLGA encapsulated naked DNA, C_{18} dendriplexes and C_{0} dendriplexes

The immunogenicity of dendriplexes encapsulated in PLGA particles was assessed by I.M. vaccination in BALB/c mice. The characterisation of these PLGA/dendriplex particles was discussed in chapter 6. The particles used in the study were approximately 500 nm in diameter. The relationship between structural properties of these PLGA formulations and the immunological outcome provides an insight into the mechanism of action to stimulate immune responses. It has been shown that PLGA particles greater than 30 μm induce no greater antibody titer than DNA alone (Singh et al., 2001).

In this thesis, we have shown that the formulation of PLGA and dendriplexes requires optimisation to obtain the properties such as size and charge to elicit a strong antibody response. The PLGA/naked PA DNA treatment group were unable to generate significant humoral response for this reason, it was used as the control in this study. At first glance both sets of PLGA/dendriplex particles were noted to have higher antibody expression, particularly post primary boost compared to the low dose dendriplex study. Although the PLGA particles had a higher PA DNA content, approximately 12 times more than the dendriplex particle alone (low dose study). Therefore considering this factor there was no significant difference between the studies. Within the PLGA treatment group, the C_{18} dendriplexes had a more marked elevation in Ig anti-PA antibodies after the second boost at week 9, whereas at weeks 3 and 6 this was not the case. IgG subtype analysis demonstrated that the IgG response elicited consisted predominantly of IgG1 rather than IgG2a-type antibodies for the PLGA/C_{0} dendriplexes and the PLGA/C_{18} dendriplex particles. Other studies by Jilek et al. (2004) found that IgG1 and IgG2a production was dependent on the surface charge of the microparticles. Comparison of anionic microparticles with cationic particles showed that the anionic particles resulted in reduced titres and therefore confirming the importance of particle design before conducting in vivo studies.

The Ig anti-PA antibodies and the subtype IgG1 both consistently have demonstrated higher titres for PLGA/C_{18} dendriplex particles. As demonstrated in figure 7.20, titres for Ig, IgG1 and IgG2a were observed to increase with the number of immunisations, consistent with a Th1 predominant response.
A gradual increase in antibody response was observed for both PLGA formulations over the 9 week period in comparison to the low dose and high dose dendriplex treatment groups. This suggested that the PLGA particle group may have provided a sustained release of the DNA, and transfection of muscle cells. This may imply that a controlled production of antigen and activation of antigen-presenting cells may be required to elicit an immune response. Gupta et al. (1998) suggests that PLG particles used in their vaccine formulations provide a depot of vaccine with controlled release properties. Mize et al. (2003) has used fluorescence-labelled DNA and PLG particles to assess cell trafficking after I.M. administration. They have observed the presence of a depot of PLG-DNA particles at the injection site after 7 days post-injection. Gene expression of the target antigen was reported in the draining lymph nodes following intramuscular injection. Mize et al. (2003) has proposed that the persistence of plasmid enables the recruitment of mononuclear cells, and that those become active and acquire a gene-encoding antigen to incite the humoral and cell-mediated responses.

7.3.3.1 In vitro toxin neutralisation assay (TNA)

The toxin neutralisation titres depicted by figure 7.21 for the PLGA particulate treatment groups of BALB/c vaccinated with C18 PLGA, C0 PLGA, and PA DNA PLGA, elicited no protection when challenged with lethal toxin with in vitro cell viability assessment. The in vivo spore challenge was attempted by other groups, Xie et al. (2005) used mice immunised with PLG/AVA particles. In the preliminary results, these PLG particles yielded intermediate protection (50% survival, P < 0.01) against spore challenge. We did not attempt in vivo studies to measure protection, although these results would supply additional information to enable comparison with the TNA in vitro study.

PLGA nanoparticles can be designed in such a way that encapsulated DNA can be released in a well-defined and long lasting manner. These nanoparticulate systems offer the opportunity of increasing the DNA payload to induce Th1 immune responses. In further studies by increasing the DNA content as shown in the high dose dendriplex study we may also enhance the level of anti-PA antibodies and thus provide protection against anthrax challenge. PLGA particles may be useful as
adjuvants for a broad range of novel vaccines, and evidence of its progress has allowed a number of human clinical trials to be performed (Sheets et al., 2003).

Figure 7.20: Mean anti-PA IgG antibody levels with the specific subgroups IgG1 and IgG2a calculated from the original ELISA endpoints. Three sets of immunisation in the PLGA treatment group: C₁₈ PLGA, C₀ PLGA and PA PLGA (used as the control). Two boost were given, one after 3 weeks and the second after 6 weeks. The serum of each BALB/c mouse was collected and measured as specified in the methodology section. The terminal bleeds were collected at 9 weeks post primary inoculation. The previous low dose dendriplex antibody titres were added to the graph above for comparison. At first glance the results illustrate that the PLGA elicited higher antibody responses than the low dose dendriplexes, but the DNA content was considerably different. Hence the great advantage of using PLGA particles is increasing the payload of DNA. As in the high dose dendriplex trail, the IgG1 was predominantly higher than IgG2a for both dendriplexes over the course of 9 weeks.
Figure 7.21 Toxin Neutralisation Assay (TNA) titres were determined similarly as the previous treatment groups. BALB/c mice were immunised with PLGA particulates (C₁₆ PLGA, C₀ PLGA and PA DNA PLGA). The serum collected from each individual mouse was used to assess the ability of these serum samples to protect the mouse macrophage cell line J774A.1 from the toxic effect of anthrax toxin composed of a cocktail of PA (protective antigen) and LF (lethal toxin) depicted as (LeTx). All three groups fail to protect the BALB/c mice vaccinated with the PLGA formulations (noted above) against anthrax challenge.
7.4 CONCLUSIONS

Many attempts have been made to improve the immunogenicity of PA vaccine through the use of immunological adjuvants. As both particulate systems and small peptides have been reported to possess adjuvant properties, dendriplexes and encapsulated dendriplexes in PLGA were investigated as potential genetic DNA vaccines. Naked PA immunisation with multiple dosing did not induce sufficient antibody response even after secondary boosting post primary intramuscular immunisation. However, both dendriplexes and PLGA particles produced strong anti-PA antibody responses. These responses were dose dependent in both "low" and "high" dose dendriplex treatment groups. Throughout the in vivo study, both dendriplexes and PLGA formulations improved immunomodulatory efficiency over the naked PA DNA. However, they did not provide protection against lethal toxin challenge in the in vitro studies. In vivo spore challenge trials need to be performed to correlate data with the present toxin neutralisation results. This was not the first animal trial in which mice were poorly protected even though they demonstrated a serological response to PA as evidenced by their high ELISA titers (Hahn et al., 2005). Guinea pigs with the same inoculation as the mice developed varying degrees of partial protection. As a result, selection of the animal model has become a critical factor, larger animals must be investigated as they have a close correlation to human immune responses.

To our knowledge, this was the first in vivo study utilising dendriplexes and encapsulated dendriplexes as a vaccine against anthrax. Further work needs to be conducted to investigate what levels of antibodies are needed to protect humans against anthrax after vaccination (Little et al., 1997). The role that antibodies play to induce resistance against this biological weapon needs to be defined (Brey, 2005).
CHAPTER EIGHT

CONCLUSION AND FUTURE PERSPECTIVES

In this thesis a wide range of disciplines were applied from the chemistry of lysine-based dendron synthesis, to the formulation aspects of the dendron and DNA complexes named “dendriplexes” together with encapsulation in PLGA particles, to the final stage of biological assessment of both dendriplexes and PLGA-dendriplexes particles as non-viral vectors for gene delivery.

An assembly of dendrons were synthesised all containing the same number of periphery groups, but varying alkyl chain lengths of C_{10} and C_{18} including numbers of lipidic core chains from 0 to the maximum of 3 in the dendron molecule. These partial dendrimers were selected instead of the fully branched parent molecule, mainly due to reduced toxicity and the ability to condensed DNA into nano-sized stable structures as it is well known that nanoparticles are taken up by cells more efficiently than large-sized microparticles (Panyam et al., 2003).

The preliminary results illustrate that lipophilicity of the dendron when complexed with DNA enhanced the uptake across cell membranes tracked by radiolabelling the DNA. The rate of uptake was dependent on molar charge ratio (N/P ratio) of the dendriplexes. The N/P ratio above 5 produced compact spherical particles less than 80 nm in size and the in vitro studies demonstrated higher transfection efficiency and gene expression.
The colloidal properties including size morphology, charge was accomplished by standard dynamic light scattering and electron microscopy. The DNA utilised was pDsRed1-N1 encoding a red fluorescent protein and once complexed with dendron transfection studies were visualised by confocal microscopy. The results indicated a possible link between the physical properties of the dendriplexes and effective gene delivery.

An extensive study of the biophysical characterisation was conducted for both formulations. A change in morphology was observed for the 10:1 dendriplexes when incubated in Opti-MEM, depicted by transmission electron micrograph and verified by PCS. The results confirm instability to electrolyte challenge which is sensitive to charge ratio particularly at the neutralisation point between DNA and dendron at stoichiometric ratio above 2:1, linking the aggregation to changes in the electrical double layer leading to reduced charge repulsion between particles. These findings correspond to the particle size data, confirming higher molar charge ratio must be utilised to minimise the aggregation and form compact complexes. The release of active DNA from these complexes was evaluated using gel electrophoresis, 58% of DNA was released when 0.2 units of heparin was added to the dendriplex which did not significantly increase when the heparin concentration was elevated from 0.2 to 0.8 units. Spectroflurometric EtBr exclusion and anionic displacement data together with the gel electrophoresis supported the hypothesis that the stability of the (C_{18})_3 dendron and DNA complexes are distinctive from the other dendriplexes.

The cytotoxicity of the dendrons alone and as dendriplexes including PLGA-dendriplexes were studied. It is well documented that dendrimers toxicity increases with increasing generation due to the number of termini amino groups. In our case partial dendrimers, dendrons were used containing only 8 periphery amino groups per molecule, which have reduced cytotoxicity in comparison to their parent dendrimer. However the results showed a relationship between the number of lipidic chains of the dendrons synthesised and their toxicity shown by the IC_{50} values obtained from their toxicity results and ranked as follows: (C_{10})_1(Lys)_7(NH_2)_8 = (C_8)(Lys)_7(NH_2)_8 > (C_{18})_1(Lys)_7(NH_2)_8 > (C_{18})_2(Lys)_7(NH_2)_8 > (C_{18})_3(Lys)_7(NH_2)_8.

The dendriplexes had a more pronounced increase in cell viability compared to the dendrons alone.

To our knowledge this is the first study incorporating lipidic dendron and DNA complexes in PLGA matrix by using double emulsion w/o/w technique producing
nano-sized particle with an approximate size of 500 nm. An optimised double emulsion method was employed adding the dendriplexes to 3% w/v PVA solution, which was homogenised with PLGA and DCM. This primary emulsion was further homogenised in a PVA solution (1.25% w/v) forming w/o/w emulsion. Nanoparticles were characterised by PCS and electron microscopy. These PLGA-dendriplex particles have shown to be advantageous in controlling DNA release and providing additional protection against DNA degradation. The encapsulation rate and the release of these particles depend on the primary formulation of the dendriplexes depicting the 10:1 as the optimal ratio. A series of SEM photomicrographs suggested that not only does the surface area link to the particle size effect DNA release but, other factors, such as the dendron PLGA interaction may cause “unravelling” of the particle matrix. The release is comparable to a burst effect at the initial 24h with continuous controlled release of dendriplexes over a 2 week period. The stability of the encapsulated dendriplexes was confirmed when aliquots taken to measure the release were additionally incubated with Caco-2 cells and illustrated maximal expression at 24 h therefore corroborates the previous release results.

A therapeutic agent in this case PA DNA was utilised, in order to provide immunogenicity against *Bacillus anthracis*. Both PA dendriplexes and encapsulated PA complexes were used as novel anthrax vaccines providing mass protection for humans particularly when utilised as a warfare agent. The virulence of anthrax bacilli is due to the production of three-component protein exotoxin including PA (protective antigen), LF (lethal factor), and EF (edema factor). PA is recognised as the antigen required to induce antibodies against LF providing immunogenicity against anthrax. Plasmid DNA encoding PA83 and a control plasmid with the PA gene in the DNA construct was complexed with two dendron assemblies. A comparative study of anti-PA antibody production of mice immunised with two selected dendrons which were \((C_{18})_3(Lys)_7(NH_2)_8\) and \((C)_0(Lys)_7(NH_2)_8\) complexed with DNA and a secondary formulation of PLGA-dendriplex particles. Two different mouse strains were used in this study; BALB/c and A/J, 10 of each strain I.M administered approximately 1ug in 20ul PBS per mouse. An enhanced antibody response was observed for the dendriplexes when compared to naked DNA and control plasmid in both sets of mice for the low dose study but no significant difference between the two sets of dendriplexes containing structurally diverse dendrons. Naked PA immunisation with multiple dosing did not induce sufficient
antibody response even after secondary boosting post primary I.M immunisation. The dose of DNA complexed with each dendron increased to 50µg in 20µl of PBS per mouse using only BALB/c mice which illustrated higher antibody responses in the previous low dose study. The response was dose dependent with a significant increase after the second boost vaccination primarily for the C_{18} dendriplexes. To evaluate the humoral responses induced by the high dose PA DNA dendriplexes, we assayed individual mouse sera for antigen-specific IgG1 and IgG2a by using ELISAs. Both dendriplexes had predominantly higher IgG1 type responses than IgG2a coherent with a Th2-type immune response (Hahn et al., 2005). Both dendriplexes and PLGA-dendriplex particles did improve their immunomodulatory efficiency in comparison to the naked PA DNA, although they did not provide protection against lethal toxin challenge in the in vitro studies.

Further work needs to be conducted to evaluate the specific level of antibodies needed to protect humans against anthrax after vaccination. Therefore immunising larger animals would be the next step to develop this work in order to relate the antibody results discovered for mice and to correlate them to a larger animal model with a comparative immune system to humans.

As concluded this DNA vaccine is merely at the initial stages of development, further work is required as mentioned in order to provide a viable alternative to the present available vaccine. Anthrax Vaccine Absorbed (AVA) is the current UK licensed human vaccine. Recent reports have shown a variety of unexplained illnesses which have been speculated to be linked to AVA vaccine, administered to US troops during the gulf war (Pittman et al., 2006). These side effects include severe systemic and local reactions, reported to develop after administration of AVA vaccine (Mahan et al., 2003). Hence this vaccine has been at the centre of controversy, composed of a cell-free filtrate, a mix of dead bacteria to stimulate the appropriate immune response. As previously stated our vaccine is based on recombinant protein antigen (rPA), which was complexed with the dendrons synthesised and applied in vivo. The animal study conducted illustrated after two boost injections, in contrast to the AVA which requires four intramuscular injections, the PA-dendriplex (50 µg) vaccine provided high anti-PA antibody production.
Challenge studies however were not as positive, but have shown to be an essential part of evaluating the efficiency of anthrax vaccines, as this can not be conducted in humans (Brey, 2004). Generally performed in higher primates like rabbit models that correlate well with humans, to assure that protection can be obtained and that the vaccine is safe and doesn’t stimulate undue responses. There would, however, be no real certainty of the vaccine being protective in humans. Matyas et al. (2004) did note, though, that there are many ways to obtain a sense of the protective capability of a vaccine including conducting a series of in vivo spore challenge. Protection against anthrax is believed to depend upon the production of an effective humoral immune response (serum antibodies), against the protective antigen produced by the infecting microorganism (Pitt et al., 2001; Fellow et al., 2001; McBride et al., 1998). These reports hypothesize that additional factors may also contribute to development of total immunity against this pathogen, but remain unclear to the exact mechanism that is required for full protection.

It has been stated that DNA vaccinations represent the next generation in the development of vaccines (Donnelly et al., 1997). Although naked DNA vaccine strategies have continued to suffer one main limitation. Principally, inefficient gene transfer when using direct plasmid injection makes it difficult to provide the antigen presenting cells with sufficient antigen to produce long lived immune responses and this appears to be the principle factor in poor antibody levels in primates. By using dendrons as carrier systems we are able to protect the DNA and further aid the delivery to antigen presenting cells, which may explain the disparity between the dendriplexes and encapsulated dendriplex particles elevated antibody levels and low titers for the naked DNA immunisations.

Designs of delivery system together with improvements in vaccine efficacy are both fields that should begin to collaborate to deal with the key problems with current vaccine technology that need to be addressed. These include the mechanism by which infection induces long-lived immunity and how genetic immunisation can induce the necessary memory CTL and Th cells to achieve a similar outcome? Improvements in vaccination strategies will develop particularly with our expanding knowledge of host immune system and specific targeting in order to provide sufficient protection from various pathogens.
Resin tests are used to monitor the completion of reactions such as coupling or deprotection. The most widely used qualitative test for the presence or absence of free amino groups was devised by Kasier. The following three solutions were prepared;

**Solution 1**: 19 g of liqueffied phenol (99.5%) was dissolved, with the aid of heat, in 6 ml absolute ethanol.

**Solution 2**: 0.5 ml aqueous solution of potassium cyanide (0.001 M) was added to 24.5 ml pyridine (99%).

**Solution 3**: 1.25 g ninhydin (99%) was dissolved in 25 ml ethanol (95%).

Following deprodection of amines with TFA and washing with DMF, a small amount of the peptide-resin sample was washed five times with DCM/MeOH (50:50) ratios and transferred to a small glass tube. Two drops of solution 1, four drops of solution 2 and two drops of solution 3 were added to the sample and tube was placed in an oil bath at 100 °C for 5 min. Next 5 ml of 60% ethanol is added to the contents of the tube and a dark blue coloured solution indicated the presence of free primary amino groups.
### APPENDIX II

**List of chemicals used for synthesis of dendrons and their sources**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-(1H-Benzotriazole-1-yl-1,1,3,3-Tetramethyluronium hexafluorophosphate (HBTU))</td>
<td>Severn Biotech Ltd Worcester, UK</td>
</tr>
<tr>
<td>2-methyl-2-propanol</td>
<td>Aldrich Chemical Co., Dorset,</td>
</tr>
<tr>
<td>Boc-Lys(Boc)-OH</td>
<td>Calbiochem AG, Laufelfingen, Switzerland</td>
</tr>
<tr>
<td>1-Bromododecane</td>
<td>Lancaster, England</td>
</tr>
<tr>
<td>1-Bromodohexane</td>
<td>Lancaster, England</td>
</tr>
<tr>
<td>Chloroform</td>
<td>BDH laboratories</td>
</tr>
<tr>
<td>Citric acid</td>
<td>Lancaster, Morecambe, UK</td>
</tr>
<tr>
<td>Dichloromethane (DCM)</td>
<td>BDH Laboratories, Poole, UK</td>
</tr>
<tr>
<td>Ethanol</td>
<td>BDH Laboratories, Poole, UK</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>BDH Laboratories, Poole, UK</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>BDH Laboratories, Poole, UK</td>
</tr>
<tr>
<td>HCl</td>
<td>BDH Laboratories, Poole, UK</td>
</tr>
<tr>
<td>Hydrogen fluoride (HF)</td>
<td>Air Products PLC, Cheshire, UK</td>
</tr>
<tr>
<td>Magnesium sulphate dried</td>
<td>BDH Laboratories, Poole, UK</td>
</tr>
<tr>
<td>N,N-diisopropylethylamine (DIEA)</td>
<td>Avocado Research Chem. Ltd, Lancashire, UK</td>
</tr>
<tr>
<td>N, N-dimethylformamide (DMF)</td>
<td>Rathburn Chemicals Ltd, Walkerburn, UK</td>
</tr>
<tr>
<td>NaOH</td>
<td>BDH Laboratories, Poole, UK</td>
</tr>
<tr>
<td>Ninhydrin</td>
<td>Avocado Research Chem. Ltd, Lancashire, UK</td>
</tr>
<tr>
<td>p-cresol</td>
<td>Avocado Research Chem. Ltd, Lancashire, UK</td>
</tr>
<tr>
<td>Phenol</td>
<td>Avocado Research Chem. Ltd, Lancashire, UK</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>KMZ Chemicals, Surrey, UK</td>
</tr>
</tbody>
</table>
APPENDIX III

\[(C_{18})_3(Lys)\gamma(NH_2)_8\]
\((C_{18})_2\text{Lys}_7\text{NH}_2_8\)
(C_{18})(Lys)_{7}(NH_{2})_{8}
(C₁₀)₁₁(Lys)₇(NH₂)₈
(C)₆(Lys)-(NH₂)₈
REFERENCES


Gibbons, W. A. (1988) Racemic and optically active fatty amino acids, their homo-and hetero-oligomers and conjugates, the process of their production, their pharmaceutical composition and activity. [GB2217319A].


transfer from packaged DNA enveloped in a dendrimeric photosensitizer. Nat.Mater., 4, 934-941.


