DEVELOPMENT OF PLASMID DNA FORMULATIONS FOR APPLICATION AS MICROSPHERIC DNA VACCINES

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June 2001

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THESIS SUMMARY

The advent of DNA vaccines has heralded a new technology allowing the design and elicitation of immune responses more adequate for a wider range of pathogens. The formulation of these vaccines into the desired dosage forms extends their capability in terms of stability, routes of administration and efficacy.

This thesis describes an investigation into the fabrication of plasmid DNA, the active principle of DNA vaccines, into microspheres, based on the tenet of an increased cellular uptake of microparticulate matter by phagocytic cells.

The formulation of plasmid DNA into microspheres using two methods, is presented. Formulation of microspheric plasmid DNA using the double emulsion solvent evaporation method and a spray-drying method was explored. The former approach involves formation of a double emulsion, by homogenisation. This method produced microspheres of uniform size and smooth morphology, but had a detrimental effect on the formulated DNA. The spray-drying method resulted in microspheres with an improved preservation of DNA stability.

The use of polyethylenimine (PEI) and stearylamine (SA) as agents in the microspheric formulation of plasmid DNA is a novel approach to DNA vaccine design. Using these molecules as model positively-charged agents, their influence on the characteristics of the microspheric formulations was investigated. PEI improved the entrapment efficiency of the plasmid DNA in microspheres, and had minimal effect on either the surface charge, morphology or size distribution of the formulations. Stearylamine effected an increase in the entrapment efficiency and stability of the plasmid DNA and its effect on the micropshere morphology was dependent on the method of preparation. The differences in the effects of the two molecules on microsphere formulations may be attributable to their dissimilar physico-chemical properties. PEI is water-soluble and highly-branched, while SA is hydrophobic and amphipathic. The positive charge of both molecules is imparted by amine functional groups.

Preliminary data on the *in vivo* application of formulated DNA vaccine, using hepatitis B plasmid, showed superior humoral responses to the formulated antigen, compared with free (unformulated) antigen.

Keywords: DNA vaccine delivery; Microspheres; Stearylamine; Polyethylenimine; Spray-Drying; Double Emulsion Solvent Evaporation.

To Mummy & Daddy, Kofi, Akwasi, and Abena

Acknowledgements

To Prof. Oya H. Alpar, I extend my sincerest gratitude, for her incessant support, guidance and encouragement throughout my undergraduate and postgraduate studies. Through her supervision I have gained a lot more than scientific development, and I shall always be grateful.

I am also grateful to Prof. Hans P. Merkle of the Swiss Federal Institute of Technology, Zürich, for the collaborative research undertaken in his laboratories, and Dr. Elke Walter, under whose direct supervision the cell culture and spray-drying experiments were performed. My time in Zürich was a fruitful and enjoyable one, made more so by the wonderful people I met and worked with; I would like to express special a "thank you" to Elke, Lars Thiele, Martina and Herbert Meinecke, and Christiane "Lola" Schmidt, and the other members of the Department of Applied Biosciences in whom I found valuable support, and friendship.

I thank the James Watt Memorial Foundation for awarding me a Prize Travel Fellowship.

My heartfelt appreciation is proffered to Dr. Jowan Bowen, Dr. Pete Lambert, Dr. Anna Hine, and Dr. Steve Smith for providing assistance and training where required, especially in molecular biology and microbiology.

I must also acknowledge the technical assistance and training received from Dr. Sheila Handley, Mel Gamble, Steve and Bryan, Chris Bache, Roy Tilling, Rita, Roy McKenzie, and Jitendra; and to Julie Simpson, Jan, Emma and Alison Hunt for administrative support.

To Dr. Jim Eyles, Dr. Barbara Conway and Dr. Ian Spiers, thanks for the directions out of dire straits, on countless occasions! I would also like to thank Manjinder Kang, Satyaranayana Somavarapu, Khalid Shah, Dr. Marcus Hughes and all others to whom I will always be indebted for your support and friendship over the years. May we always remain the friends that we have come to be.

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List of Abbreviations

[α-^{35}S]dCTP	Sulphur-35 labelled deoxycytosine triphosphate
AAV	Adeno-associated virus
ABTS	2,2'-Azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid
ADD	Adenosine deaminase deficiency
APC	Anitgen presenting cell
BSA	Bovine serum albumin
CMI	Cell mediated immunity
CMV	Cytomegalovirus
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cell
DCM	Dichloromethane
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmid reticulum
EtBr	Ethidium bromide
FCS	Foetal calf serum
FDC	Follicular dendritic cell
GALT	Gut- associated lymphoid tissue
GFP	Green fluorescence protein
GI	Gastrointestinal
GIT	Gastrointestinal tract
GM-CSF	Granulocyte macrophage cell stimulating factor
GMP	Good manufacturing practice
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HIV	Human immunodeficiency virus
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
IBB	Importin-β domain
IEL	Intraepithelial Lymphocyte
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
kD	Kilo dalton
LB	Luria-Bertani
LC	Langerhan cell
MLN	Mesenteric lymph node
mRNA	Messenger ribonucleic acid
NALT	Nasal-associated lymphoid tissue

NLS	Nuclear localisation sequence
NP	Nucleoprotein
NPC	Nuclear Pore complex
OVA	Ovalbumin
PAA	Poly (dl-aspartic acid)
PBS	Phosphate buffered saline
PCLN	Posterior cervical lymph node
PEI	Polyethylenimine
PLA	Poly lactic acid
PLGA	Poly (lactide-co-glycolide)
PMN	Polymorphonucleocytes
PP	Peyer's patch
PVA	Poly vinyl alcohol
RES	Reticuloendothelial system
RNA	Ribonucleic acid
RSV	Rous sarcoma virus
SA	Stearylamine
SC	Subcutaneous
SCLN	Superior cervical lymph node
SEM	Scanning electron microscopy
SF	Serum-free
SFM	Serum-free medium
ssDNA	Single-stranded DNA
SV40	Simian virus 40
TAE	Tris-acetate EDTA
TAP	Transfer-associated protein
TB	Tuberculosis
TCR	T-cell receptor
TE	Tris-EDTA
UV	Ultra violet

1. Introduction

1.1 Vaccines

The official parchment certifying the global eradication of smallpox, dated December 9, 1979 reads "*We the members of the global commission of smallpox eradication, certify that smallpox has been eradicated from the world*" and bears the signatures of the representatives of the 20 member states (Mackett M and Williamson JD, 1995). This remarkable achievement was predicted by Dr. Edward Jenner (1749 - 1822), and greatly strengthened the perception of vaccination as a highly effective public health measure. Vaccination has come to be regarded as a cost-effective measure against infectious diseases, and the recent advent of nucleic acid vaccines will only aid in the total eradication of totally preventable diseases.

1.1.1 Historical Background

The procedure of variolation is thought to have originated in India and China where it was a commercially important procedure to traders transporting young maidens from Circasia to the Seroglios of the Turkish Sultan and the Persian Sophy (Plotkin SL and Plotkin SA, 1994). During the second half of the 18th century it was observed in several European countries that milkmaids were rarely pockmarked, and it was thought that a pre-infection acquired from cows protected them from smallpox. In 1774 a farmer called Benjamin Betsy who considered himself immune to smallpox inoculated his wife and children with material from pocks on cows. The family remained free from smallpox infection for 15 years. Dr. Edward Jenner's inoculation of the young Mr. James Phipps with cowpox was followed with a deliberate inoculation of smallpox. It is by this critical experiment that credit is given to the Jenner, for he proved that infection with cowpox produced a much less severe disease than variolation, and vaccination with cowpox rapidly became the accepted practice.

In 1958, delegates from the Soviet Union proposed to the 11th World Health Assembly that smallpox should be eradicated globally, and the proposal was approved the following year. The voluntary nature of the program, combined with the innate scepticism of many senior persons within and outside WHO, following the difficulties of a malaria eradication program, hampered the progress of the proposal, and many

developing countries continued to show undiminished rates of incidences of the disease. During the 19th World Health Assembly in 1966 it was decided that special funds should be allocated for the mounting of an intensified campaign to eradicate smallpox. The goal was formally achieved in 1979 at an estimated cost of $300 million (Mackett M and Williamson JD, 1995).

The second half of the 19th century saw the acceptance of the concept that infectious disease was caused by 'germs', and specific agents were identified in many cases. Louis Pasteur found that a culture of chicken cholera bacillus left exposed to the air for some weeks failed to produce a disease when inoculated into chickens, but these chickens were protected against a later challenge with the virulent organisms. Pasteur proposed that the procedure of inoculation of attenuated microbes should be called 'vaccination' and that the product be called a vaccine. In pursuance of proof of this theory, Pasteur in 1881, showed that animals immunised with his vaccine could survive lethal challenges with the virulent organism, although all the non-immunised animals died. He repeated these experiments in humans using an attenuated strain of rabies with similar success (Ada G and Ramsay A, 1997).

In 1881 Roux and Yersin showed that immunisation with a bacterium-free filtrate of a diphtheria culture would induce a protective response. Von Berhring and Kitasato (1890) showed that such immunisation resulted in the formation of antibodies that neutralised the activity of the bacterial toxin or antigen produced by the bacteria. The discovery of antibodies led to two important findings: the first was that sera from immunised animals could produce rapid cures in infected children, particularly if administered in the early stages of the disease. The second was that the toxicity of bacterial toxins could be modified if they were mixed with an appropriate amount of the antibody and the floccule was carefully washed and injected. This approach was replaced by a chemical procedure, treatment with formalin, which detoxified the toxin, converting it to a toxoid. By the early 20th century, activated bacterial vaccines against typhoid fever, plague and cholera made from killed or inactivated infectious agents had been developed. This period saw the progress of basic approaches to vaccine design such as, live, attenuated organisms, inactivated organisms, and subunit vaccines.

Thus, immunisation may be defined as the process of administering antigen to a live host with the purpose of inducing an immune response for academic or public health purposes. Vaccination is used mainly for the latter reason, but the terms are partly interchangeable. It is defined as a process of antigen administration once or a few times before the host expects to be confronted by a challenge.

1.1.2 Need for vaccines

Although the final cost of small pox eradication by an expanded program of immunisation was about £300m (Ada G and Ramsay A, 1997), the effect was calculated to save the USA the total of all its contributions to the program every 26 days (Mackett M and Williamson JD, 1995). Vaccination programs in developing countries have reduced the incidence of measles in children under 5-year olds from 2.5 million in 1980 to just over 1 million in 1992; with similar successes in whooping cough and neonatal tetanus. In 1994, the Pan-American health authorities announced the eradication of poliomyelitis from the North, Central and South America. Despite these successes there are numerous diseases for which effective vaccines are required. Figure 1.1 shows the incidence of mortality caused by vaccine-preventable diseases, with acute respiratory tract infection causing more than 4 million deaths *per* year.

In 1993, a report of the World Bank concluded unambiguously that vaccination is the single most cost-effective public health measure (Whalen RG, 1996).

1.1.2.1 Tuberculosis

Protective immunity against *M. tuberculosis* (TB) is mediated mainly by cellular rather than humoral immunity. Gastrointestinal tract (GIT) infections are also associated with a very wide range of pathogens including, viruses, bacteria, protozoa and worm infections. This antigenic diversity of pathogens present an immense challenge in the field of vaccine development. Many *E. coli* are associated with diarrhoeal disease, and there are more than 2000 members of the genus *Salmonella*. Rotaviruses are an important cause of nonbacterial gastritis, a condition which kills several million infants

a year. These viruses are noted for the difficulty in their *in vivo*. Thus, one approach to a rotavirus vaccine has been to use bovine or monkey strains of the virus as heterologous vaccines to immunise infants. Tuberculosis, a disease once thought to be under control,

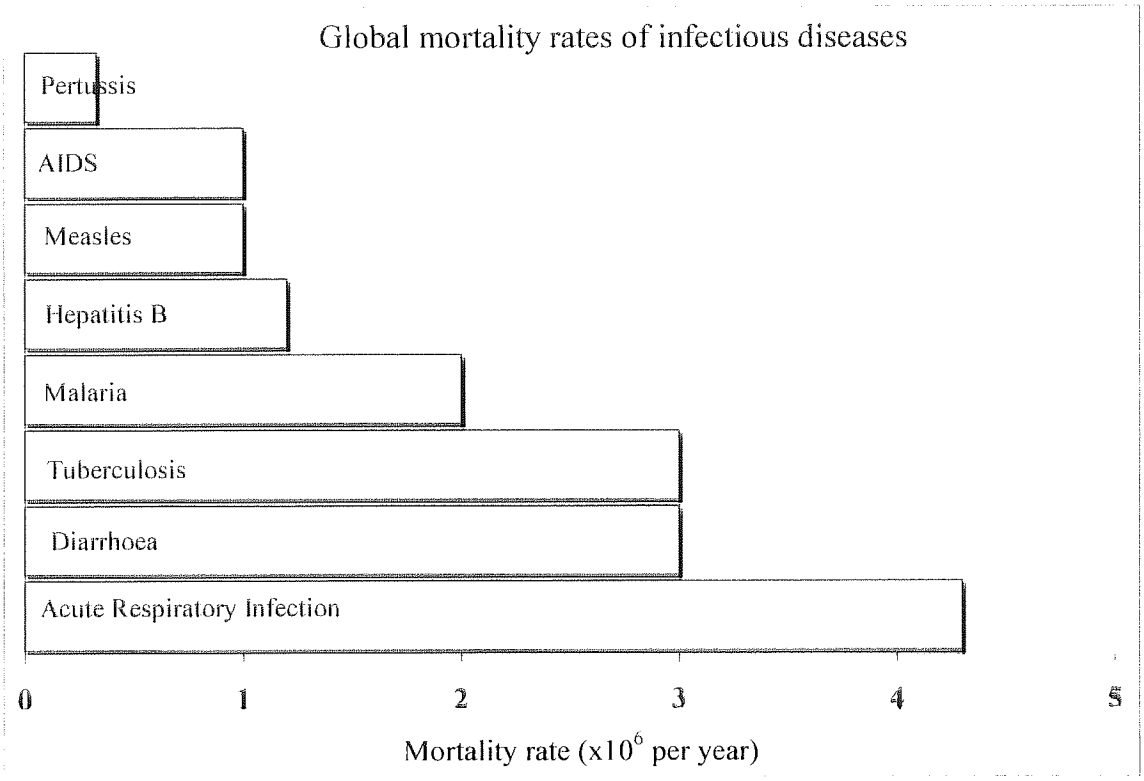


Figure 1.1 Major causes of death in the world, adapted from Robinson *et. al.*, 1997 (Robinson HL *et. al.*, 1997)

has an annual adult fatality rate of 1.5 million, with half a million more in combination with HIV/AIDS (Heyman DL, 2000). Nearly two billion people - one-third of the world's population - are estimated to have latent TB infection. Together, they constitute a huge potential reservoir for the disease; infection with HIV weakens the immune system and can activate latent TB infection. About one-third of all AIDS deaths today are caused by TB (Ada G and Ramsay A, 1997).

1.1.2.2 Acquired immune deficiency syndrome

A vaccine for AIDS is yet to be realised about two decades after the emergence of the HIV. Much attention has been directed towards the surface glycoproteins of the virion, particularly gp 120 or its precursor, gp 160. Part of this molecule, the V3 loop, contains epitopes which stimulate neutralising antibodies, however this region is hypervariable, and neutralisation-escape mutants readily appear. An alternative approach is to elicit

responses to internal antigens such as the p24 core antigen. This approach is based on the induction of CTL by the virion's internal nucleoprotein (NP) antigen. Cellular responses to its NP have been shown to aid recovery from influenza virus infection (Bagarazzi ML *et. al.*, 1998). However, there is evidence from cytokine profiles that there is a shift towards the predominance of a Th-2 like helper cell population as the HIV infection progresses. This may be related to the decline of the ability of CTLs to inhibit HIV replication as the disease progresses. This could be exacerbated by the progressive depletion of dendritic cells and the concomitant loss of their antigen-presenting function, together with the decline of CD4+ Th cells (Ada G and Ramsay A, 1997). Although an attenuated vaccine to protect against simian AIDS has been developed, progress towards attenuated or inactivated HIV vaccines is severely limited by safety issues. It is envisaged that future developments in subunit or DNA vaccines will aid overcome these barriers.

1.1.2.3 Malaria

Another disease of global significance is malaria, deaths from which are mainly in infants below 5 years of age. The challenge of a malaria vaccine is to elicit humoral responses to attack each of the extracellular stages; sporozoites, merozoites, gametocytes and gametes. There is considerable antigenic variation during the blood stage of the parasite's life cycle, and there is also a need for a cell mediated response to combat the intracellular stage. Subunit vaccines are favoured because the epitopes responsible for the B and T cell responses may be identified on the protein, and those giving rise to non-protective or spurious immune respones may be eliminated. A promising approach has been based on a chimeric molecule SPf(66)n, which is a chemically synthesised 45 amino acid peptide derived from four different proteins of the sporozoite (Heyman DL, 2000).

1.1.2.4 Acute Respiratory infections

Acute respiratory infections (ARIs) are responsible for about 4.2 million deaths each year. Pneumonia, the deadliest ARI, kills more children than any other infectious disease. Most of these deaths (99%) occur in developing countries. The influenza virus is another cause of lung infection that can lead to pneumonia. There is very little

information available on the number of influenza deaths in developing countries. Nevertheless, in the United States alone, the disease kills 10,000-40,000 people in an average influenza season.

1.1.2.5 Other infectious diseases

Diarrhoeal diseases claim nearly two million lives a year among children under five. These diseases impose a heavy burden on developing countries - accounting for 1.5 billion bouts of illness a year in children under five. Other major diarrhoeal diseases include typhoid fever and rotavirus which is the main cause of severe dehydrating diarrhoea among children.

Measles is the most contagious disease known; it is a major childhood killer in developing countries - accounting for about 900,000 deaths a year. The measles virus may ultimately be responsible for more child deaths than any other single microorganism - due to complications from pneumonia, diarrhoea and malnutrition.

1.1.3 Modern implications of infectious diseases

In the Middle Ages deadly plagues were shipped from one continent to another - carried by flea-infested rats. Today, 1.4 billion passengers travel internationally by airline every year, potentially transporting deadly airborne diseases such as pneumonic plague, influenza and TB, which can easily spread in crowded airport lounges, jumbo jets or by passengers after their return home. Infectious diseases can also be carried across borders by their animal or insect hosts. In the United States in 1977, over 70% of the passengers on board an airliner grounded for several hours were infected with influenza by a fellow passenger. In 1994, a person with active TB is believed to have infected six fellow passengers on a flight from Chicago to Honolulu. Published reports show that the majority of multi drug-resistant typhoid cases in the United States originated in six developing countries. In 1978 and again in 1992, poliovirus was imported into Canada by people travelling from western Europe. All the eleven people who were affected by polio paralysis in the first outbreak had refused immunisation. Also in Canada, health care authorities traced two outbreaks of methicillin-resistant *Staphylococcus aureus*

(MRSA) to a small village in Northern India (Heyman DL, 2000).

A recent report by the United States national intelligence agency stated '*that infectious diseases are likely to continue to account for more military admissions than battlefield injuries... at the highest risk will be U.S. military forces deployed in support of humanitarian and peacekeeping operations in developing countries.*' (Heyman DL, 2000). The probability of a bio-terrorist attack against civilian and military personnel overseas or at home is likely to grow as more states and groups develop a biological warfare capability.

Vaccines therefore represent an effective healthcare measure in terms of disease prevention, and reduction of human morbidity and mortality. In fact, in 1993, a report by the World Bank concluded unambiguously that vaccination is the single most cost-effective public health measure (Whalen RG, 1996).

1.1.4 Types of vaccines available today and their limitations

Today the types of vaccines vary from live attenuated whole pathogen to the most recent genetically engineered sub-unit proteins.

1.1.4.1 Live attenuated pathogen

The pathogenic organism (e.g. Rubella, measles virus or BCG bacteria) is rendered non-virulent (attenuated) by serial passage of the virus in cell culture of non-human origin (e.g. chicken embryo). A successful attenuation results in the loss of virulence of the pathogen while retaining its antigenic capability, and thus stimulating protective immunity. This was first successfully achieved by Calmette and Guérin with a bovine strain (*M. bovis*) of *Mycobacterim tuberculosis* which during 13 years of culture *in vitro* changed to the much less virulent form now known as BCG (bacille Calmette-Guérin)(Goodyer L, 2000). Subsequent attenuation of viral strains of the yellow fever virus (17D) by passage in mice and chicken embryos and similar approaches with polio (Sabin), measles, mumps and rubella (MMR vaccine) viruses have yielded successful immunonological protection(Mackett M and Williamson JD, 1995). Live vaccines are

easier and less costly to produce, do not require extensive purification, and elicit long-lasting immunity without the need for adjuvants. The major disadvantage with this type of vaccine lies in its potential to revert to the virulent form, as observed with the Type 2 and 3 polio vaccines (Roitt I *et. al.*, 1998).

1.1.4.2 Inactivated pathogen

The rabies and Salk polio vaccines are examples of killed pathogens which retain immunological qualities. The pathogen is inactivated by heat, or chemically (usually with formaldehyde).

1.1.4.3 Passive immunity -immunoglobulins

Immunity with immediate protection against infective organisms can be obtained by injecting preparations made from the plasma of immune individuals with adequate levels of antibody to that particular pathogen. This passive immunity however, lasts only a few weeks.

1.1.4.4 Purified component vaccines

Developed in the early 20th century, this type of vaccine comprises purified components from a pathogen. Examples of this include the tetanus and diphtheria bacteria, whose toxins are purified and inactivated (toxoid) and used as a vaccine (Mehta DK, 2000).

1.1.4.5 Genetically engineered vaccines

1.1.4.5.1 Sub-unit vaccines

This is a technique which uses the proteins produced by recombinant DNA technology. The antigenic protein is cultured in a suitable vector and harvested. Hepatitis B virus vaccine is a recombinant subunit protein which was previously purified from the blood of infected carriers, it is now cultured in a eucaryotic expression system (*Saccharomyces cerevisiae*), and is currently the only recombinant vaccine to be licensed by the FDA for use in humans (Medaglini D *et. al.*, 1995).

1.1.4.5.2 Live attenuated vaccines

The advent of recombinant DNA technology has allowed live vaccines to be engineered to contain highly defined mutations that minimise the risk of virulence. An alternative approach is the use of genetically engineered commensal bacteria. These are inherently safer than the attenuated pathogens, but may not elicit the best immunity. The oral commensal *Streptococcus gordonii* has been used to this effect , using the M6 protein of *S. pyogenes*; a mucosal inoculation with this vaccine in mice produced secretory immunoglobulin A (sIgA) and serum immunoglobulin G (IgG) responses to M6 and to the bacterium, which lasted for up to 11 weeks (Medaglini D *et. al.*, 1995).

1.2 Gene therapy

Experiments performed in the 1950s showed that foreign genetic material can be expressed by host cells. The table1.2 (Chattergoon M *et. al.*, 1997) shows a brief history of these experiments.

1.2.1 Mechanism of the therapeutic action of gene therapy

The aim of gene therapy is to cure an inherited disease by providing the patient with a correct copy of the defective gene. There are two basic approaches to gene therapy: germline therapy, in which the new gene is injected into the fertilised egg, resulting in its expression in all cells of the individual and somatic cell therapy, where the relevant gene is inserted into somatic cells. Obvious ethical implications of the applications of this technology have limited it to somatic therapy.

Gene therapy has been used for the treatment of a range of diseases including cystic fibrosis, muscular dystrophy and adenosine deaminase deficiency. More recently, clinical trials have focused on the use of this technology for the treatment of cancer and HIV infection (Chattergoon M *et. al.*, 1997). The one major obstacle to gene therapy, however, is the effective delivery and deposition of functional genetic material into the cell.

1.2.2 Types of vectors used in gene delivery

The main obstacle to successful gene therapy is the delivery of the required gene to its site of action. There are several main methods of vector design applied to circumvent these barriers, and some of these will be briefly addressed.

Table 1.1: Currently available vaccines in the United Kingdom adapted from (Goodyer L, 2000; Mehta DK, 2000).

Name of vaccine	Type of vaccine
BCG[1]	Live attenuated strain derived form *M. bovis*
Hepatitis A[1]	Formaldehyde-inactivated hepatitis A adsorbed onto aluminium hydroxide
Hepatitis B[1]	Surface antigen made using recombinant DNA technology
Japanese *encephalitis*[1]	Lyophilised formaldehyde-inactivated virus
Meningitis A[1] and C[1]	Meningococcal polysaccharide
Polio (oral)[1]	Live attenuated trivalent
Rabies[1,5]	Lyophilised inactivated Wistar rabies strain cultivated in human diploid cells
Tetanus[1]	Toxoid
Typhoid (oral)[1]	Live attenuated *Salmonella typhi*
Typhoid (parenteral)[1]	Vi capsular polysaccharide
Tick-borne *encephalitis*[1]	Killed virus
Yellow fever[1]	Live attenuated
Diphtheria[1]	Prepared from diphtheria formol toxoid adsorbed on a mineral carrier
Combined Diphtheria and Tetanus Vaccine[2]	Prepared from diphtheria formol toxoid and tetanus formol toxoid adsorbed onto a mineral
Adsorbed Diphtheria, Tetanus and Pertussis Vaccine[2]	Prepared from diphtheria formol toxoid, tetanus formol toxoid and pertussis vaccine
Haemophilus Influenza type b[1]	Protein- conjugated capsular polysaccharide of pathogen
Measles Mumps and *Rubella* (MMR) [1,4]	Live measles, mumps and rubella vaccine
Acellular Pertussis[3]	Single-antigen acellular pertussis vaccine

Notes:

1) Administered as part of childhood immunisation schedule in the UK.

2) Administered mainly as a travel vaccine in the UK.

3) Available as an unlicensed vaccine by the department of health under Crown immunity, only for completion of immunisation against *pertussis* in those children whose course of *pertussis* vaccine was not given or completed as part of their triple vaccine course.

4) The safety of the MMR vaccine had been confirmed by the Department of Health, in response to reports by Wakefield *et. al.*, proposing a link between MMR and autism and Inflammatory Bowel Disease (Breckenridge A, 2001).

5) A second rabies vaccine cultivated from purified chick embryo cells is now available in the UK (Haynes H, 2001).

1.2.2.1 Viral vectors

Viral-mediated gene therapy involves the use of attenuated or defective viruses designed to transfer therapeutic genes into a patient's cells without causing viral disease. Viruses have developed and optimised, over years of evolution, mechanisms to infect mammalian cells, and effect a transfer and expression of their genetic material. This inherent efficiency of gene transfer provides a rationale for their use as gene delivery vectors. Some of these viruses, like retroviruses, adenoviruses, adeno-associated viruses, and Herpes viruses have been investigated for *in vivo* gene delivery.

Table 1.2: Brief history of genetic immunisation techniques (Chattergoon M *et. al.*, 1997)

Year	Form of DNA	Delivery Route	*In vivo* function
1955	Chromatin	s.c.	Tumour formation
1958	Phenol extract	s.c.	Tumour formation and antibody
1962	Purified DNA	s.c.	Tumour formation and antibody
1964	Purified DNA	s.c.	Tumour formation and antibody
1979	Phage	i.p.	Tumour formation and antibody
1984	Non-replicating Plasmid	i.p.	Viral production
1985	Liposomal plasmid	i.v.	Insulin production
1985	Plasmid	Liver, Spleen	Viral gene
1985	Ca^{2+} Plasmid	i.p.	Viral gene
1990	Plasmid	i.m.	Reporter gene
1992	Gold-adsorbed plasmid	i.m.	Human growth hormone
1993	Gold-adsorbed plasmid	i.m., mucosal, s.c.	Immune response to Influenza A
1993	Plasmid	i.m.	Immune response to Influenza A
1993	Bupivacaine with plasmid	i.m.	Immune response to HIV-1

1.2.2.1.1 Adenoviruses as gene delivery vectors

Adenoviruses are known to efficiently infect dividing and non-dividing cells such as airway epithelial cells, endothelial cells, hepatocytes, and cancer cells. Genes introduced into the cells using adenoviruses are maintained for a finite period of time in the nucleus as episomes. The main drawback to the use of this virus as a delivery system is the cytopathic and immunologic responses demonstrated in preclinical and clinical studies.

1.2.2.1.2 Adeno-associated viral vectors (AAV) as gene delivery vectors

The AAVs are DNA viruses capable of permanently inserting their genome into the chromosomes of the host cells. A DNA virus may be less susceptible to recombination than a retrovirus, and has been shown to infect both dividing and non-dividing cells. The ability to engineer these viruses to be completely defective, yet retain their gene integration properties has made these a promising viral gene delivery vector. The main limitation to the use of AAV to date has been the difficulty in developing packaging cell lines to produce sufficient titres of the virus for clinical use (Flotte TR *et. al.*, 1995).

1.2.2.1.3 Retroviral vectors

Retroviruses are RNA viruses that can introduce genes permanently into dividing somatic cells by integration into the host chromosomal DNA. The clinical consequence of this effect is to prevent any necessary modification or termination of the therapy in the event of any adverse side effects or cure of the disease. Additionally, the random insertion of viral sequences into host chromosomes may result in either the activation of oncogenes or inactivation of tumour suppresser genes. Another danger in their use lies in the potential for the combination of viral and cellular RNA to produce replication-competent viruses of unknown characteristics.These drawbacks have led to efforts in the developments of artificial methods of gene transfer.

1.2.2.2 Artificial (non-viral) methods for gene transfection

1.2.2.2.1 Liposomes and lipidic systems

Liposomes have been used to successfully deliver exogenous DNA into cells since 1978 (Mukherjee AB *et. al.*, 1978). Liposomes were one of the first systems to be investigated for the delivery of genes, and formulations approved for commercialisation are a testimony to their safety. Liposomal formulations have been shown to be non-toxic and nonimmunogenic at therapeutic doses (Nicolau C and Cudd A, 1989). Properties which make them attractive as gene delivery systems are several; (1) the constituents are usually natural products like phosphatidylcholine, reducing toxicity and inherent immunogenicity; (2) the formulation aspects permit versatility in the choice of physico-chemical properties required; i.e. a large variety of lipids, lipoproteins, or

lipopolysaccharides can be modified greatly to achieve the desired surface charge, hydrophobicity or steric properties, for example. (3) through the method of preparation diameters from 30 nm to 10 μm, may be achieved, and the liposomes may be uni or multi- lamellar (Nicolau C and Cudd A, 1989). (4) they can be prepared in bulk, sterile, and in most cases the method of entrapment results in little or no damage to the DNA. The encapsulated DNA has been shown to be protected from enzymatic degradation. There are however, disadvantages to the use of liposomes as gene delivery agents: low encapsulation efficiencies and *in vitro* transfection efficiencies. The major obstacle to the use of liposomes *in vivo* is rapid clearance by cells of the morphonuclear phagocyte system (MPS), with about 62% of the dose accumulating in the liver following i.v. delivery. However, there have been advances in liposome formulation to evade this rapid clearance; these techniques include the addition of cholesterol to the liposome, and attachment of hydrophilic moieties to the surface of the liposome (Wivel N, 1995).

1.2.2.2.2 Polycationic systems (polymer-plasmid complexes)

These systems are composed primarily of positively charged polymers. The neutralisation of the negative charge by the cationic molecule results in a collapse of the extended structure of the plasmid molecule. (Anwer K *et. al.*, 2000), (Pouton CW and Seymour LW, 1998). The efficiency of cellular transfection achieved with these polymers *in vitro* is dependent on the charge ratio, the size and morphology of the particles formed. Generally, cationic polymers have been shown to be capable of forming homogeneously small and stable complexes. Two important examples of cationic polymer-plasmid complexes for gene delivery are the use of poly-L-lysine (PLL) and polyethylenimine (PEI).

1.2.2.2.2.1 PLL-plasmid complexes for gene delivery

Henner *et. al.*, (Henner WD *et. al.*, 1973) showed the use of PLL as a tranfection agent at a time when DNA condensation was not viewed as a useful gene formulation. The advent of gene therapy and the need for small stable complexes kindled efforts in the investigation of PLL-DNA as gene targeting delivery agents. Methods of targeting complexes to specific cell types or organs include the attachment of antibodies. The

high affinity for targets, broad range of tissue selectivity, and efficiency of receptor-mediated internalisation makes antibody targeting an attractive delivery strategy. Trubetskoy *et. al.*, attached a thiol-derivitized antithrombomodulin antibody to a 3,000 Mw PLL and showed specific binding to thrombomodulin-positive cells *in vitro* and *in vivo* (Trubetskoy VS *et. al.*, 1992). However, the expression of chloramphenicol acetyl transferase (CAT) in the complex was found to be low. The addition of chloroquine increased transgene expression (Foster BJ and Kern JA, 1997), suggesting that endosomal escape is a major block to efficient transfection. The low *in vivo* transfection efficiency of PLL-DNA complexes has prevented the clinical application of this stable complex to date. However, methods of increasing lysosomal escape and alternative targeting technologies, like fibroblast growth factor (Sosnowski BA *et. al.*, 1996), asialoglycoproteins (Wu GY and Wu CH, 1988), galactose (Hashida M *et. al.*, 1998), and mannose (Erbacher P *et. al.*, 1996) make PLL-DNA conjugates worth further exploration.

1.2.2.2.2.2 Polyethylenimine complexes for gene delivery

Polyethylenimine (PEI) is an organic molecule with a high cationic-charge density. Every third atom is an amino acid nitrogen that can be protonated. Boussif *et. al.*, demonstrated its use as a transfection agent *in vivo* and *in vitro* (Boussif O *et. al.*, 1995). Luciferase-encoding plasmid was complexed with various amounts of PEI and injected into the brains of new-born mice, resulting in transfection levels as high as that obtained with complexes *in vitro*. The authors attributed the high transfection capability of PEI-DNA complexes not only to the buffering capacity of the vector, but also to a perturbation of the lysosomal trafficking. Suh *et. al.*, have shown that PEI contains primary, secondary and tertiary amine groups, in the ratio 1:2:1 (Suh J *et. al.*, 1994). The protonation pattern of PEI indicates clearly the influence of multiple nitrogen atoms in close proximity; at pH 7.5 only 11% of the nitrogen atoms are protonated, compared with 67% at a pH of 4. Experiments designed to evaluate the effects of PEI on the stability of the lysosomal membrane, endocytosis and intracellular degradation led Klemm *et. al.*, (Klemm AR *et. al.*, 1998) to propose that PEI in the culture medium is carried into cells by adsorptive endocytosis, subsequently increasing the permeability of the membrane and affording DNA conjugated to the PEI an otherwise unavailable mode

of entry into the cytoplasm. However, the 'proton sponge' hypothesis by Boussif *et. al.*, is the most plausible mode of action by which PEI buffers the lysosome. More recently, direct evidence of inhibition of lysosomal fusion to the phagosome has been shown with confocal imaging *in vitro* (Godbey WT *et. al.*, 2000). Other experiments have focused on improving the PEI association (Boussif O *et. al.*, 1996), (Ogris M *et. al.*, 1998), (Godbey WT *et. al.*, 1999) and targeting (Bandyopadhyay P *et. al.*, 1998) of PEI-DNA complexes *in vitro*. Complexes of luciferase plasmid with PEI reductively coupled to lactose gave high transfection efficiencies in HepG2 (Zanta MA *et. al.*, 1997). Incubation with excess asialoferritin to block the asiaglyocoprotein receptor reduced transfection to levels below those of untargeted complexes. The potential of polycationic-DNA complexes as gene delivery systems is yet to be realised. However, the high specificity and reduced toxicity as well as relative ease of preparation will continue to stimulate research into their further development.

So the potential of genes delivery to mammalian cells for the purpose of curing disease or alleviation of symptoms can be appreciated. The design of safe vectors to effect transfer of the genetic material is an important aspect of current research efforts. Genetic vaccination is a relatively new concept, but faces drawbacks similar to that outlined for gene therapy.

1.3 DNA vaccines

DNA vaccines fall under the general umbrella of nucleic acid vaccines, a term adopted by the scientific community during a WHO meeting in Geneva, 1994. Nucleic acid vaccination refers to the induction of an immune response to a protein expressed *in vivo*, subsequent to the introduction of its encoding gene, (Davis HL and Whalen RG, 1995), be it the deoxyribonucleic or the ribonucleic acid (DNA or RNA).

Wolff *et. al.*, in 1990 reported the expression of the protein encoded by a plasmid injected into murine muscle *in vivo* (Wolff JA *et. al.*, 1990). This direct transfection of cells by naked DNA in normal saline was followed by a report by Ulmer *et. al.*, in 1993 (Ulmer JB *et. al.*, 1993) that intramuscular injection of a DNA expression vector encoding a conserved, internal protein of influenza A resulted in the generation

protective levels of nucleoprotein (NP)-specific antibodies and primary CTLs, allowing survival against subsequent viral challenge.

1.3.1 Cellular uptake of plasmid DNA

DNA vaccines provide protective immunity by the expression of a foreign protein within the host cells. The cells take-up the DNA in the form of a double stranded plasmid, express the encoded protein antigen and present it to the cells of the immune system. Thus, this method of vaccination is unique in that the antigen is synthesised *de novo*, without the use of a live vector. This mechanism of presentation of the antigen effectively mimics a natural infection, while employing the benefits of a subunit vaccine, precluding the introduction of the live pathogen to the immunised subjects. Figure 1.2 is a schematic representation of the mechanism by which plasmid DNA is thought to be internalised and intracellularly processed and presented on the surface of APCs. Stages 1 to 7 depicted in figure 1.2 are discussed below.

Phagocytosis involves attachment, internalisation and digestion of particulate matter. It is thought that initial adsorption to or association with the cell surface is the first stage of cellular internalisation, and this is achieved mainly through formulation into complexes with cationic liposomes or polymers. The particles are engulfed within phagocytic vacuoles or phagosomes close to the plasma membrane (stage 1) and initiate the oxidative burst. Fusion of the phagosomes with lysosomes deeper within the cytosol forms phagolysosomes, into which the lysosomal enzymes are discharged, and the enclosed material including any DNA or protein, is effectively degraded by hydrolytic enzymes. Peptide fragments of degraded proteins are processed and presented *via* MHC class II molecules to T cells bearing the CD4 ligand.

For the effective elicitation of an immune response to encoded antigen, the plasmid DNA taken-up must be intact and enter the cell nucleus for subsequent transcription and translation of encoded antigen. Hence the DNA must escape the endosomal compartment before the lysosomic stage, or be protected from the harsh environment induced by the lysosomes (stage 2). Methods of achieving this include the use of pH-sensitive liposomes, whose conformation in a low-pH environment disrupts the vesicle

membrane, allowing the DNA to escape into the cytosol. Weakly basic molecules like ammonium chloride and chloroquine which naturally sequester within the acidic lyososomal compartments have been shown to increase the efficiency of transfection *in vitro*, and polyethylenimine has a lysosomolytic effect by a proposed 'proton sponge' mechanism, in which the hydrogen ions of the lysosomes are progressively reduced the amine groups of the PEI molecules, increasing the internal pH and osmotic pressure. The higher pH either denatures or inactivates the hydrolytic enzymes and water drawn into the vesicles by osmosis raptures the phagolysosomal membrane, releasing the DNA into the cytosol.

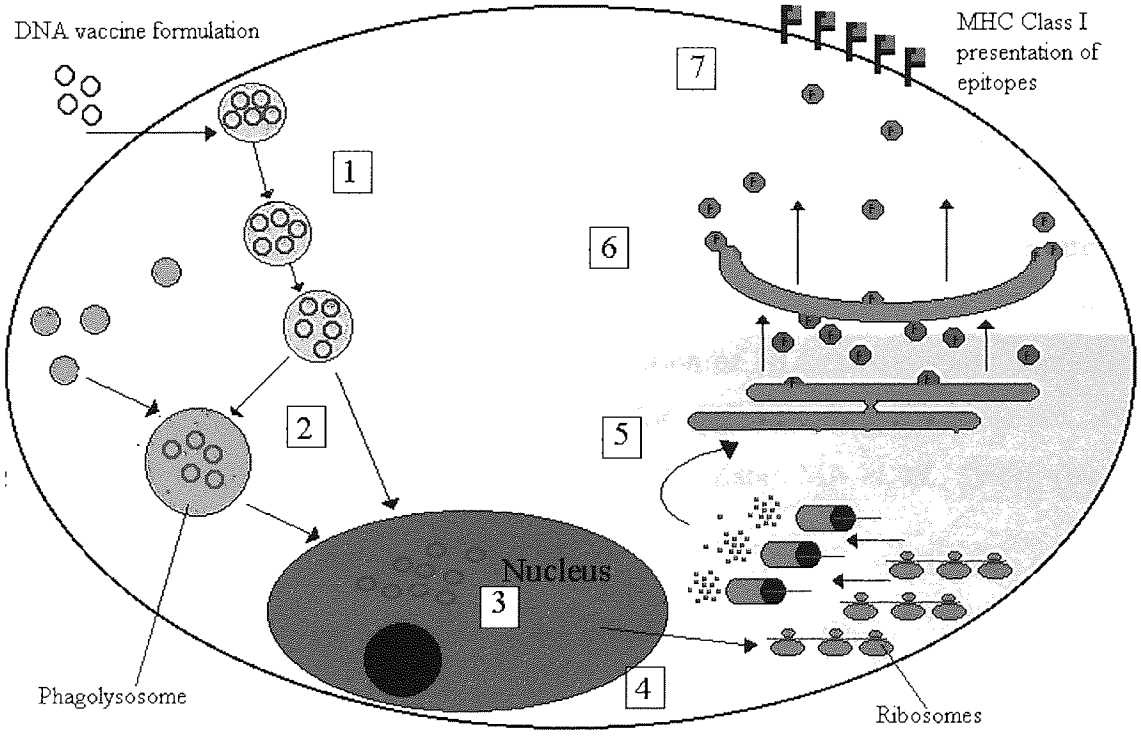


Figure 1.2: Schematic representation of the proposed mechanism of entry of plasmid DNA into mammalian cells, and subsequent expression of encoded protein antigen. See text for discussion of numbered stages.

Once in the cytosol, the DNA has to gain access to the interior of the nucleus, and this presents one of the main barriers to transgene expression.

1.3.1.1 The nucleus as barrier of gene delivery

One of the major challenges to gene delivery is the accessibility of the gene-vector system to the cell nucleus. It is within this intracellular organelle that the genetic

material is transcribed by RNA polymerases. The nucleus is enclosed by a bi-lipid membrane that is continuous with the ER. This membrane separates the nucleoplasm from cytoplasmic plasma, and the passage of the plasmid DNA into the nucleus is dependent on breaching this barrier. The sites on the nuclear membrane where macromolecules are exchanged are the nuclear pore complexes (NPCs), which have a mass of about 125 MDa and consists of about 100 different polypeptides (Gorlich D and Mattaj IW, 1996). The pore has a diameter of about 9 nm, allowing the passive diffusion of small proteins (around 13 kDa). With the nuclear pores making-up only about 10 % of the membrane surface, the nuclear membrane is a formidable barrier to gene delivery (Zanta MA *et. al.*, 1999). The passage of larger proteins like ovalbumin (43 kDa) is delayed, and that of BSA (66kDa) is virtually prevented. Such size excluded proteins may enter the nucleus by a carrier mediated active process, accommodating particles up to about 25 nm. The transport of proteins across the NPCs is generally selective and signal-dependent. Existence of a nuclear localisation sequence (NLS) was demonstated in the large T antigen of SV40, and is a 7-amino acid sequence. NLS are short sequences that have been identified as generally one or two clusters of four or more basic amino acids (lysine or arginine). Conjugation of NLSs to synthetic peptides and DNA have enhanced their nuclear accumulation (Gorlich D and Mattaj IW, 1996), (Morris MC *et. al.*, 2000), (Neves C *et. al.*, 1999), (Zanta MA *et. al.*, 1999), (Neves C *et. al.*, 1999).

1.3.1.1.1 Mechanism of import

The import of proteins into the nucleus involves 2 major steps; binding of the protein to the nuclear pore complexes (NPCs), and an energy-dependent translocation through the nuclear pore. Four soluble factors are currently known to be required for nuclear protein import. The importins (or karyopherins) α (also called NLS receptor), and β (also called p97 or PTAC97), the guanosine triphosphatase (GTPase) Ran, and pp15 (also called p10 or NTF2) (Gorlich D and Mattaj IW, 1996). The importin subunits (α and β) consist largely of repeated domains, or arm repeats, that are found in several funtionally unreleated porteins. A diagrammatic representation of the mechanistic steps involved in the nuclear protein import cycle is given in Figure 1.3.

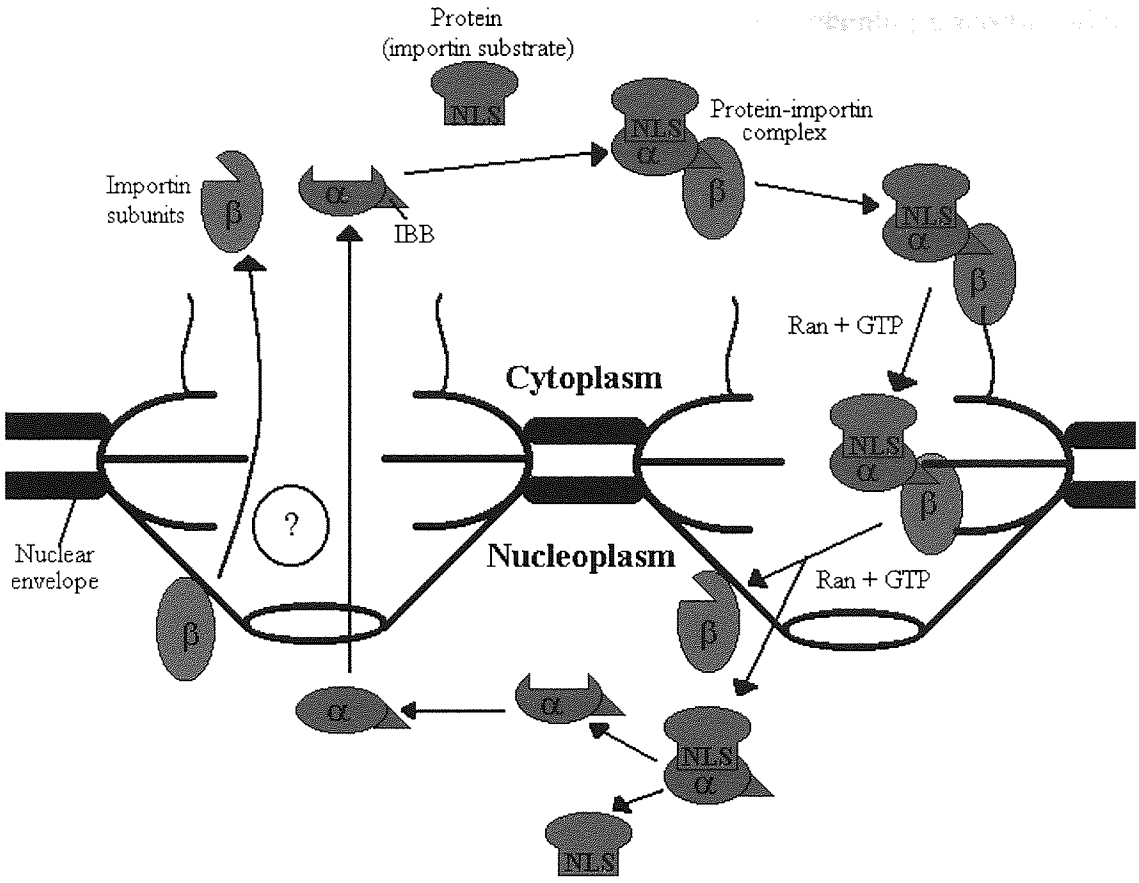


Figure 1.3: Proposed mechanism of the nuclear protein import cycle. NLS-containing proteins bind to the importin heterodimer in the cytosol. The NLS interacts primarily with the α subunit; the IBB domain of the α unit mediates the heterodimerisation. NLS binding to a may precede α–β interaction. The β subunit mediates docking of the complex at the NPC. Translocation involves GTP hydrolysis by Ran and is probably a multi-step process. The α–β heterodimer dissociates, and α enters the nucleoplasm with the substrate. Dissociation of α from the nuclear protein must then occur. The subunits of importin are returned to the cytoplasm, possibly separately (Gorlich D and Mattaj IW, 1996).

The import substrate binds *via* its NLS directly to the arm repeat region of the α subunit of the importin α–β heterodimer in the cytoplasm. This NLS protein-importin complex docks to the nuclear pore complex *via* the importin-β binding domain (IBB) of the importin-β complex, and is subsequently translocated through the pore by an energy-dependent mechanism that requires Ran and pp15, though the precise role of pp15 in this process is unknown. Ran is a GTPase that hydrolyses GTP and exchange the formed guanosine diphosphate (GDP) for GTP, liberating the bond energy which then drives the translocation. The constituents of the NLS recognition sequence become separated as a consequence of this process. The import substrate and importin-α reach the nucleoplasm, whereas importin-β accumulates at the nuclear envelope. In the

nucleus, the importin-α has to dissociate from the NLS-containing importin substrate probably by conversion of the importin-α to a form with a low affinity for NLS. The different rates of re-export of the α and β subunits indicate that they return to the cytoplasm separately, possibly by different routes.

It is not known how many NLSs can bind simultaneously to a single importin-α monomer, and whether all NLS-containing proteins interact similarly with importin-α. The IBB appears to have all the features of an NLS, being characterised by clusters of basic amino acids. When fused to a heterologous protein, the IBB domain confers not only binding to importin-b but also transit into the nucleus, bypassing the requirement for impotin-α. This suggests that the interaction with the NPC and the mechanism that drives nuclear translocation must target importin-β. Thus, the IBB domain may be considered as the archetypal nuclear targeting signal in the sense that it is sufficient to target a protein to which it is attached to the nucleus, and its structural similarity to an NLS suggests that the two may be evolutionarily related (Gorlich D and Mattaj IW, 1996).

1.3.1.1.2 Targeting to the nucleus

With some knowledge of the process of nuclear import, it is possible to exploit the mechanism for nuclear localisation of gene vectors. Neves *et. al.*, (Neves C *et. al.*, 1999) developed a method to covalently associate a cationic NLS peptide with plasmid DNA. The NLS of the SV40 large T antigen was covalently linked to a 19 bp oligonucleotide. This oligonucleotide-NLS conjugate was covalently associated with a β-galactosidase-expressing plasmid. The functionality of β-galactosidase plasmid, as assessed by tranfection of NIH 3T3 cells, was not significantly different to the that of the NLS-coupled plasmid, in spite of increased association with importin-α.

Zanta *et. al.*, (Zanta MA *et. al.*, 1999) proposed that plasmid condensation with NLS-coupled cationic lipids or polymers generally led to products larger than the size-exclusion limit for signal-mediated nuclear import, and previous means of coupling NLS or karyophilic signals led to intracellularly unstable conjugates. Based on the

premise that a free DNA double helix is thin enough (3nm) to enter the nuclear pore, this group prepared a dsDNA fragment (encoding the reporter protein, luciferase) coupled to an oligonucleotide-NLS conjugate. The product was effectively a linear, double-stranded capped luciferase gene linked to a single NLS peptide. It was found that this modification led to a 1000-fold reduction in the amount of DNA required for the same level of luciferase expression *in vitro*, and this enhancement was attributed to an increase in the nuclear import of the luciferase gene fragment, directed by importin-mediated nuclear translocation (Zanta MA *et. al.*, 1999).

Using a different approach, Langle-Rouault *et. al.*, (Langle-Rouault F *et. al.*, 1998) reported a 100-fold increase in luciferase expression after *in vitro* transfection of a 293 cell line with a luciferase-expressing plasmid. The cell line was stably transfected with Epstein Barr virus nuclear antigen 1 (EBVNA1), which has a NLS located within its C-terminal region. EBVNA1 also has a binding site for *oriP*, the origin of replication of the virus genome. The luciferase gene was inserted into a plasmid possessing the *oriP* sequence. It was found that luciferase expression was increased 100-fold in the EBNA1-positive 293 cells transfected with the $oriP^{+}$ plasmid construct, compared with that in EBNA1-negative cells transfected with the $oriP^{-}$ construct. The authors proposed the *oriP*+ plasmid constructs become tightly associated with the EBVNA1 molecules in the cell cytoplasm, with a high enough affinity, forming a stable plasmid-EBVNA1 complex, which were transported through the NPCs into the nucleus, thereby increasing the intranuclear presence of luciferase plasmid.

These two differing approaches to nuclear targeting of gene vectors highlight the effectiveness of NLSs, and though the precise mechanisms by which gene-NLSs are imported into the nucleus are not defined, the use of karyophilic moities for increasing gene expression has been proved. What remains is the development of delivery systems to effectively exploit this phenomenon.

1.3.2 Expression and MHC class I presentation of antigen

Bacterial plasmid DNA is susceptible to transcription by mammalian polymerases in the nucleus, DNA. Following the natural progression of post-transcriptional modification,

the mRNA is translocated into the cytoplasm, and translated (stage 4). The *de novo* synthesis of the antigenic peptides or proteins mimics a natural viral infection, and the post-translational processing of the antigen by APCs results in MHC class I presentation of the epitope. The newly synthesised peptide fragments must pass through into the lumen of the ER (stage 5) before they can bind to the MHC class I molecules. A set of genes homologous to the ATP-binding cassette (ABC) family of protein transporters, called 'transorter in antigen processing' or TAP are located within the MHC locus (York IA and Rock KL, 1996). The TAP proteins comprise of two subunits which are non-covalently associated, and located in the ER and Golgi apparatus. The function of these TAP proteins is that of transportation of peptides from the cytosol to the ER. The binding of the peptide to the TAP complex is independent of ATP, while the peptide translocation into the ER lumen requires ATP. It is thought that peptide-MHC class I association probably occurs in the ER; MHC class I molecules bound to an adenoviral protein, E3bp19k (which binds MHC class I complexes and causes their retention in the ER), is observed to be loaded with peptides *in vivo* (York IA and Rock KL, 1996). To be efficiently transported to the cell surface and displayed to the immune system, the heavy chain (HC) and β2 microglubulin (β2-m) subunits of the MHC I molecule, as well as the peptide must assemble together. The HC associates with the calnexin, which maintains partial folding of the MHC class I molecule in the ER, and also prevents degradation. This dimer is then transferred to TAP for association of the peptide, which itself enhances the stability of the trimeric complex (Cruse JM and Lewis RE, 1999). After assembly in the ER, the MHC-bound peptide is transported to the golgi (stage 6) where N-linked carbohydrates are modified. They are then translocated to the cell surface *via* the exocytic pathway (stage 7).

1.3.3 Plasmid features required for an effective DNA vaccine

Plasmids are essentially replicons which are stably inherited in an extrachromosomal state. They have a constant monomeric unit size and the ability to replicate independently of the host chromosome. They may be maintained *per* cell as multiple copies (relaxed plasmids) or as a limited number of copies (stringent plasmids). They are widely distributed throughout the prokaryotes, and vary in size from less than 1 megadalton to more than 200 megadaltons (Old RW and Primrose SB, 1994). The

phenotypic traits carried by natural plasmids include antibiotic and heavy metal resistance, production of enterotoxins, haemolysin and antibiotics, and sugar fermentation (Old RW and Primrose SB, 1994). Plasmids are naturally transmitted to new hosts by a process known as bacterial conjugation. This ability is conferred by a set of transfer or *tra* genes. Some plasmids without the *tra* gene may be mobilised by a conjugative plasmid in the presence of a mobility protein that nicks the plasmid at a site (*nic*) close to the *cis*-acting element (Sambrook J *et. al.*, 1989). Plasmids for use in gene therapy need to be non-mobilisable to preserve global antibiotic sensitivity of microbes by ensuring that the antibiotic resistance gene is not transferred to other bacterial species during cloning and amplification of the gene. Non-mobilisable plasmids also prevent any cross-species transfer of potentially harmful geneproducts. Figure 1.4 illustrates the essential features of a luciferase plasmid (pGL3-Control, Promega (UK) Ltd.).

1.3.3.1 Copy number

The number of molecules of a plasmid contained in a single bacterial cell is important for high yields during the cloning and amplification stage of production. This property of plasmids is termed the copy number, and ranges from less than 15 *per* cell (e.g. pACYC and ColE1) to more than 700 (pUC) (Birnboim HC and Doly J, 1979). The copy number is determined primarily by the origin of replication. The pUC vectors have a replicon which is a mutant of the pMB1, and relies on long-lived enzymes supplied by the host for replication (Sambrook J *et. al.*, 1989). It can therefore continue to replicate in the bacterial host cell after inhibition of protein synthesis by chloramphenicol, enabling copy numbers as high as two to three thousand to be attained by the plasmid. Thus, the choice of plasmid vector with respect to the copy number can influence the efficiency of its production.

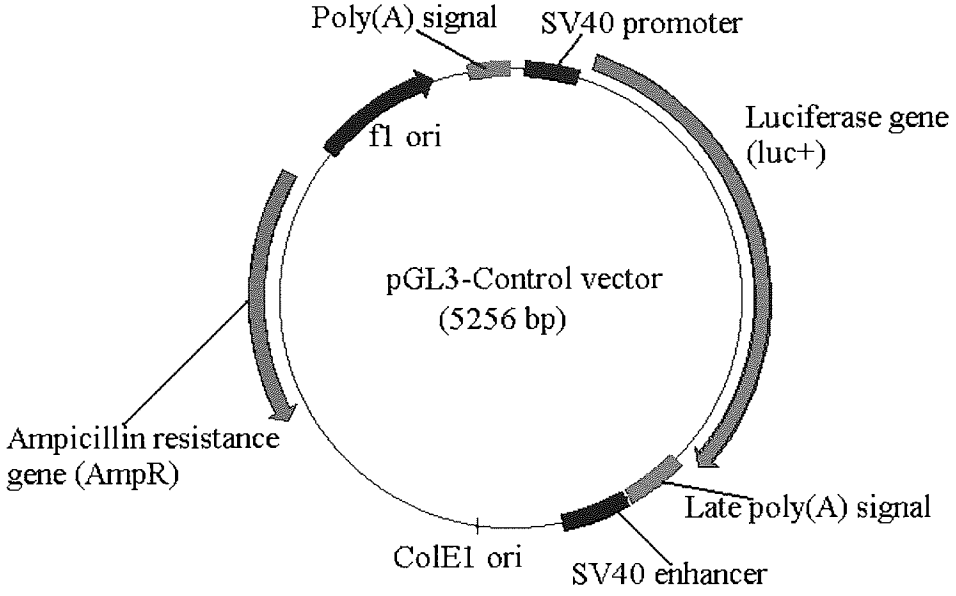


Figure 1.4: Luciferase plasmid (pGL3-Control) showing the essential features required for a high efficiency of transfection [Beckler, 1996 #313].

1.3.3.2 Promoter

The type of promoter used to initiate the transcription can influence the level of expression of the gene of interest; Lee *et. al.,* (Lee RJ and Huang L, 1997) showed that the cytomegalovirus (CMV) immediate early promoter was stronger than the Rous sarcoma virus (RSV), murine leukemia virus (SL3-3) and simian virus 40 (SV40) early promoters in expressing chloramphenicol acetyl transferase (CAT) *in vitro* (Lee AH *et. al.*, 1997). Similar results were observed by Norman *et. al.,* 1997 (Norman JA *et. al.*, 1997).

1.3.3.3 Intron sequence

Introns are portions of DNA between coding regions in a gene which is transcribed, but which is excised from the mRNA. Palmiter *et. al.*, demonstrated that these regions of DNA increased the expression transgenes in mice (Palmiter RD *et. al.*, 1991).

1.3.3.4 Poly A

Most eukaryotic mRNAs have a poly (A) tail added onto their 3' ends as part of the maturation of the primary transcript. The polyadenylation of the mRNA confers stability and translatability to the RNA transcript, it also acts to signal the RNA polymerase II to terminate transcription of the corresponding gene. (Proudfoot N, 1991). Most plasmid vectors have poly (A) signals incorporated into the backbone to increase the efficiency of expression of the gene of interest by stabilising the transcribed mRNA and terminating any spurious transcription initiated in the vector backbone.

1.3.3.5 Adjuvant (immunostimulatory) DNA sequences

Bacterial DNA has direct immunostimulatory effects *in vitro* due to the presence of unmethylated cytosine-guanine (CpG) dinucleotides. It is likely that a rapid immune reaction in response to these motifs may have evolved as a defense mechanisms against microbial pathogens. CpG DNA can trigger B cell proliferation, polyclonal immunoglobulin, IL-6 and IL-12 secretion and protect B cells from apoptosis (McCluskie MJ and Davis HL, 2000), (Krieg AM *et. al.*, 1995). Klinman *et. al.*, 1997 reported that unmethylated CpG motifs in plasmid backbones act as internal adjuvants, promting the development of IgG responses against the DNA vaccine-encoded antigen (Klinman DM *et. al.*, 1997).

1.3.4 Advantages of DNA vaccination

DNA vaccine technology is based on the expression of only one, or a subset of proteins, from the pathogen and has a number of advantages over more classical vaccines; DNA vaccines mimic the action of live attenuated vaccines in the *de novo* synthesis of the antigen. However, DNA vaccines lack the propensity to carry out reversion to pathogenesis. Classical subunit vaccines are produced in fermenters or cell cultures (Dertzbaugh MT, 1998), and the desired antigen(s) purified and used for subsequent inoculation. Recombinant virus vectors revolutionised this process by producing the subunit vaccine in the host. DNA vaccines take this one step further by eliminating the need for a virus vector. Thus, the production of subunit vaccines in hosts by DNA vaccine technology has a number of positive consequences for the success of

vaccination

1.3.4.1 Native form of protein antigen is presented to the immune system

Many viral proteins have folded structures amenable to disruption during the purification process of conventional vaccines. Antibodies that fail to recognise the native form of a protein frequently prove ineffective at containing an invading micro-organism. The ability of DNA to produce the immunising protein(s) in host cells yields a vaccinating protein in its native form. This ensures an effective antibody-antigen reaction on challenge with the pathogen (Robinson HL *et. al.*, 1997).

1.3.4.2 Cytotoxic T lymphocyte (CTL) and long-lived responses are elicited

The production of the immunogen in host cells supports processing and presentation by both Class I and Class II MHC molecules. By contrast, killed, whole or protein subunit vaccines generally undergo presentation by Class II MHC molecules. These differences in presentation result in DNA vaccines raising both cytolytic T-cells and antibody (Yoon SJ *et. al.*, 1999), (Ashok MS and Rangarajan PN, 1999), (Xin KQ *et. al.*, 1999), whereas more classical subunit vaccination elicits a predominantly humoral-based response (Denis O *et. al.*, 1998). A cytolytic T-cell response is important for infections like HIV and TB, where the pathogen is intracellular. The use of DNA, a nonliving agent, to raise cytolytic T-cells represents a milestone in vaccinology (Robinson HL *et. al.*, 1997).

1.3.4.3 Incorporation of multiple antigens into a single plasmid

In the United States, the full course of childhood immunisations currently requires 18 visits to a physician or clinic (Heyman DL, 2000). Giving several vaccines at once could reduce this number of visits, but differences in the formulations of the different vaccines limits their use in combination. DNA vaccines, by having the same formulation, might eliminate this problem. The ability to use DNA for immunization against multiple antigens against the same or different pathogens represents a particular strength of the DNA vaccine approach (Hoffman SL *et. al.*, 1997). For example, Rogers *et. al.*, 1999 (Rogers WO *et. al.*, 1999) constructed eight nucleic acid vaccines against

P. vivax based on four antigens, the circumsporozoite protein (PvCSP) and sporozoite surface protein 2 (PvSSP2) from the pre-erythrocytic stage, and apical membrane antigen 1 (PvAMA1) and merozoite surface protein 1 (PvMSP1) from the erythrocytic stage. Targeting of *Mycobacterium tuberculosis* can be made more effective by immunising against several known antigens, a technique not too unlike multidrug therapy. Grifantini *et. al.*, also found that multi-plasmid vaccination increased the immunogenicity of a poorly immunogenic antigen (Grifantini R *et. al.*, 1998).

1.3.4.4 Good stability at high and low temperatures

In contrast to live virus and protein sub-unit vaccines, DNA vaccines remain stable at both high (below boiling) and low temperatures. Proteins may be irreversibly unfolded or denatured under relatively mild conditions, but plasmid DNA readily and quickly renatures under the appropriate conditions with little or no loss of activity (Middaugh CR *et. al.*, 1998). Hence, plasmid DNA requires chemical, rather than mere structural modification to effect biological loss. This physical stability allows the storage of DNA vaccines in dry or aqueous solution (Evans RK *et. al.*, 2000), which in turn facilitates distribution, administration, and eliminates the need for cold chain storage; the series of refrigerators required to maintain the viability of a vaccine during its distribution. Currently, maintaining the cold chain represents 80% of the cost of vaccinating individuals in developing nations (Ada G and Ramsay A, 1997), (Robinson HL *et. al.*, 1997).

1.3.4.5 Ease and reproducibility of production

Process development for manufacturing plasmid DNA involves initial construction and selection of expression vectors and cloning microorganisms (upstream processing), followed by the selection and optimisation of the fermentaion conditions. The desired plasmid is then isolated and purified (downstream processing) from the cultured cells (Ferreira GNM *et. al.*, 2000). It can be seen that all DNA vaccines, regardless of the properties of the required antigen, can be produced using similar fermentation, purification, and validation techniques, once the conditions for upstream processing have been optimised. This ability to use generic production and verification techniques

reduces the cost and complexity of vaccine development and production.

1.3.4.6 Potential for rapid identification of the antigenic unit of pathogens

DNA vaccinology allows potential antigenic epitopes or units of a pathogen to be rapidly screened, using expression library immunization (Ulmer JB and Liu MA, 1996), (Manoutcharian K *et. al.*, 1999). In cases where the immunologically relevant genes remain unknown, DNA libraries can be used to screen the entire genome of a micro-organism for sequences that raise protective immune responses. This technique has been used to identify antigenic units of *Trypanosoma cruzi* (Alberti E *et. al.*, 1998).

1.3.4.7 Support the recovery of candidate vaccine from infected tissue

Using the polymerase chain reaction (PCR) supports the recovery and amplification of specific DNA sequences present at low levels from biological material. Candidate vaccines can be directly recovered from infected tissue, eliminating or reducing the upstream processing time. The ability to make a vaccine directly from the tissue of an infected animal also allows construction of vaccines for microorganisms that fail to grow in cultures, like hepatitis B and C viruses and papilloma viruses.

1.3.4.8 Ability to prime for Th1 or Th2-biased responses

DNA vaccines can be used to bias an immune response towards one of two different types of T-cell help. These two types of T-cells, called T-helper 1 and T-helper 2, affect the types of antibody raised and the nature of the inflammatory cells mobilized to fight an infection. The type of T-helper cell and its associated inflammatory responses are important in determining how effectively the body clears an infection. The ability of DNA immunizations to bias the type of T-cell help may also support the use of this new technology for the control of autoimmune disease (an inappropriate T-helper 1 response) and allergy (an inappropriate T-helper 2 response).

1.3.4.9 Current limitations of DNA vaccination

DNA vaccination raises immune responses against the protein components of

pathogens, thus precluding its use against polysaccharide antigenic units of pathogens like p*neumococous*, where sub-unit vaccines are more applicable. Another limitation of the DNA vaccine technique is the lack of post-translational modification of some bacterial protein antigens, such as glycosylation. Despite this limitation, early work with DNA vaccines has provided impressive results against bacterial and parasitic agents.

1.4 Microspheric formulation of vaccines

The formulation of vaccines into delivery systems has become a major field in vaccinology and pharmaceutical sciences alike, the various types and advantages will now be discussed.

1.4.1 Advantages and disadvantages of formulating vaccines

Current vaccines mostly require repeated administration to induce protective antibody titres. Frequently, three applications are necessary to increase or boost the antibody titres or cellular responses sufficiently for protection (Heyman DL, 2000). Such immunisation schemes are generally not conducive to patient compliance, especially in regions where access to health care systems is poor, and this has been found to be notoriously low. A vaccine which induces protective responses after a single administration is desirable in terms of both patient compliance and potential cost-savings.

Recent advances in biotechnology have led to the production of purified antigens which, while improving on purity, require adjuvants for potentiation of the immune response due to their reduced immunogenicity. Adjuvancy can be achieved by the formulation as well as more traditional means like the addition of a variety of chemical compounds like Fruend's complete adjuvant, which consists of a water-in-oil emulsion of mineral oil, lanolin, and killed mycobacteria (Kissel T *et. al.*, 1995). Additional benefits of formulation are the increased stability of the antigen, possibly avoiding cold chain storage and prolonged shelf life of the vaccine. A reduced dosage and enhanced local tolerability might also contribute to better compliance and a reduced financial healthcare burden.

1.4.2 Microspheric formulations

1.4.2.1.1 Methods of microsphere preparation

Microencapsulation may be defined as the incorporation of the solid or liquid phase of a substance into spherical particles. This may be achieved with a variety of polymers, both biodegradable and non-biodegradable, using any number of the 2000+ patented methods (Kissel T *et. al.*, 1995). This section will be limited to the discussion of the double emulsion solvent evaporation and spray-drying.

1.4.2.1.1.1 Double emulsion solvent evaporation method of microsphere formulation

The double emulsion technique consists of a two-step process in which microspheres are generated after the formation of a water-in-oil-in-water (w/o/w). The process begins with the emulsification of the aqueous antigen solution and the polymer solution. Biodegradable, biocompatible polymers such poly (dl-lactic-co-glycolic acid) (PLGA) or poly (lactic acid) (PLA) are dissolved in chlorinated organic solvents like dichloromethane (DCM) or chloroform ($CHCl_3$), though other polymers and solvents non-miscible with water may also be used. Emulsification is achieved by homogenisation, ultrasonication or vortex mixing, resulting in a primary water-in-oil emulsion. In the second step, the primary emulsion is gradually added to a larger aqueous phase containing an emulsifier like poly(vinyl alcohol) (e.g. PVA) and the resulting double emulsion is stirred gently until the organic solvent has evaporated. The double emulsion method was first described by Ogawa *et. al.* (Ogawa Y *et. al.*, 1988) for the entrapment of leuprolide acetate, a water-soluble peptide.

The production of microspheres by the double emulsion is influenced by various parameters which determine the final characteristics of the product. Ogawa *et. al.*, (Ogawa Y *et. al.*, 1988) regarded the viscosity of the internal aqueous phase as essential to increasing entrapment efficiencies, and to this end included gelatin in this phase. Maa and Hsu (Maa Y-F and Hsu CC, 1997) studied the effect of the primary emulsion and found that the homogenisation speed and viscosity of this phase greatly affected the size and encapsulation efficiency of the microspheres. The authors observed that the

formation of large aqueous droplets in the primary w/o emulsion resulted in smaller microspheres with a reduced encapsulation efficiency, a phenomenon explained by fragmentation of the large aqueous droplets, which exposes the drug during secondary emulsification, causing leakage of the protein into the external phase. They also confirmed Ogawa's observations that increasing the viscosity of the disperse phase improves the drug-loading. Alex and Bodmeier (Alex R and Bodmeier R, 1990) also found that a concentrated protein solution resulted in the a higher loading. Jeffery *et. al.*, 1993 (Jeffery H *et. al.*, 1993), observed that the actual loading of ovalbumin (OVA) in PLGA microspheres increased as the ratio of the OVA to the polymer increased; however, at high ratios (1:2 and above) there was some disruption to the microsphere surface morphology, as observed by scanning electron microscopy (SEM). The effect of various formulation parameters on the particle sizes is shown in table 1.3 below. The concentration of $PVA_{13\text{-}23kDa}$ in the external aqueous phase had the most effect in reducing the particle size. The authors also reported that an increase in the external phase volume (10 mL to 100 mL) increased the entrapment efficiencies (42.6 to 98.4 %, respectively).

Table 1.3: Effect of various formulation parameters on the size of microspheres prepared by the double emulsion solvent evaporation method, adapted from Jeffery *et. al.*(Jeffery H *et. al.*, 1993).

Formulation parameter	Range			Respective change in particle size (μm)		
OVA/PLGA ratio	1:10	→	1:1	1.8	→	3.9
Concentration of $PVA_{13\text{-}23kDa}$ in external phase	1	→	10 %	8.3	→	3.7
Volume of external phase	10	→	100 mL	2.8	→	6.5
Volume of internal phase	0.5	→	2 mL	2.5	→	4.1

The effect of the primary emulsion stability was investigated by Nihant *et. al.*, 1994 (Nihant N *et. al.*, 1994), who showed that the addition of small amounts of BSA (0.25 $\%^{m}/_{m}$) improved the stability of the primary water-in-oil emulsion; but keeping the aqueous BSA concentration constant and increasing the Pluronic™ F68 (a polyoxypropylene-polyoxyethylene-polypropylene triblock polymer surfactant with 80 % polyoxyethylene *per* molecule) concentration in the oil phase had a detrimental effect on the emulsion stability. The effect of the emulsion stability on the encapsulation efficiency was not discussed, but the stabilising effect of the protein could explain the increase in encapsulation seen by Jeffery *et. al.*, 1993.(Jeffery H *et. al.*, 1993). The

importance of solvent selection has been reported by Bodmeier and McGinity, 1988 (Bodmeier R and McGinity JW, 1988). It was found that of the wide range of PLGA solvents, those with high water miscibility like DMSO and acetone were not conducive to microsphere formation. They also showed that rapid polymer precipitation at the outer surface reduced drug diffusion into the external phase, increasing the entrapment efficiency. It can thus be envisaged that a water miscible organic solvent will result in mal-formed microspheres due to a poor dispersion of discrete water-in-oil droplets in the primary emulsion, but can increase the encapsulation efficiency by encouraging rapid loss of the solvent from the surface of the microspheres during the drying phase of the secondary double emulsion. The importance of solvent is illustrated in this dichotomy.

1.4.2.1.1.2 Spray-drying method of DNA vaccine formulation

Spray-drying is in the family of suspended-particle processing systems together with fluid-bed drying, flash drying, spray granulation, spray agglomeration, spray reaction, spray cooling and spray absorption. This family of methods is characterised by the drying being accomplished while particles are suspended in air. By definition, spray-drying is the transformation of feed from a fluid state into a dried particulate form by spraying the feed into a hot drying medium (Masters K, 1991).

The first description of this method is credited to a Mr. Samuel Percy in 1872, who first described in detail, the "*...principle of atomising and desiccating simultaneously by dried or heated air forced forward, and thus throwing substances into a state of atoms, and drying them while in this state of division.*" However, it was not until after 1907 that the first successful spray-dryer was marketed in the USA (Masters K, 1991).

This method may be divided into four stages: the atomisation of the liquid feed into a fine spray; contact of the feed with the heated gaseous stream; drying of the feed in the gaseous stream; and separation and collection of the solid product (spray-dried particles).

The atomisation of the feed is achieved by a number of systems classifiable by their

nozzle design, of which there are several, like the rotary atomisation, pressure atomisation or pneumatic (two-fluid) atomisation. The rotary atomiser introduces the feed into the drying chamber through a rotating wheel, which creates a spray of droplets whose shape and size are dependent on the shape, size and rotating speed of the wheel. With the pressure atomisation, the feed is forced through the nozzle under a high enough pressure to disperse the fluid into droplets as it leaves the nozzle; there is no supply of air to create the spray. The two-fluid nozzle atomisation involves the separate delivery of the feed and air into the nozzle, where they mix and create a spray of droplets as they leave the nozzle. This nozzle is commonly seen on laboratory scale spray-dryers like the the SD-03 (LabPlant, Huddersfield, UK), and Buechi Mini Spray-Drier (Buechi Laboratotiums- Technik AG ,Flawil, Switzerland). The spray may be introduced into the drying chamber counter current to, or co-current with, the direction of drying-airflow. In both cases the final stage of the process is the separation of the solid product from the air stream (Kissel T *et. al.*, 1995). This is achieved by the creation of a cyclone in the cyclone chamber, which deposits the particulate product into the product collector (see figure 6.1).

There are several parameters which govern the formation of microspheres by the spray-drying method, the ratio of encapsulated drug to encapsulating polymer, the viscosity of the feed, the rate of spray of the feed, and the outlet temperature (Conte U **and** Giunchedi P, 1995).

Conte *et. al.*, 1994 (Conte U *et. al.*, 1994) studied the effect of the concentration of the organic polymer solution (poly-,dl-lactide in a $CHCl_3$:DCM mixture), the inlet air temperature and spray rate of feed on the characteristics of microspheres. SEM performed on the microspheres showed that a variation of the inlet air temperature (from 44°C to 63°C) and feed spray rate(2 to 7 mL/min) with a corresponding variation of outlet air temperature from 37°C to 51°C had no effect on the microsphere morphology, which was spherical with smooth surfaces. However, varying the concentration of the polymer solution from 1.25 $\%^{m}/_{v}$ to 5 $\%^{m}/_{v}$ produced noticeable differences in the particle morphologies. It was reported that a 3 $\%^{m}/_{v}$ concentration produced the best microsphere shape. Solutions at 1.25 %m/v resulted in incompletely-

formed particles, while microspheres formed at a 5 $\%^{m}/_{v}$ solution were spherical but very aggregated. The increase in the temperature was shown to reduce the microsphere size. The effect of the rate of feed spray was generally temperature-dependent; at high temperatures, increasing the rate of spray (from 53°C to 63°C) slightly reduced particle size, and increased recovery or yield of product; while an increase in particle size and a reduction in yield was seen when the spray rate was increased at a lower temperature. Wagenaar and Mueller however, reported that polymer concentrations above $2\%^{m}/_{v}$ resulted in fibres instead of microspheres (Wagenaar BW and Mueller BM, 1993). This was ascribed to large intermolecular forces which exists in linear molecules which requires large forces to break-up into droplets, an explanation proposed by Bodmeier and Chen (Bodmeier R and Chen H, 1988). This apparent conflict in observations is perhaps a reflection of the need for precise control of the discussed parameters.

The advantages of preparing microspheres from biodegradable polymers using the spray-drying method are several; microparticles are produced from the start emulsion or suspension a single step. The method also allows the encapsulation of both water soluble and water insoluble drugs into hydrophilic or hydrophobic polymers with relatively high encapsulation efficiencies. The process allows tight control of process parameters, leading to high reproducibility, and a good potential for scale-up.

1.5 Microspheric formulation of DNA vaccine

The formulation of DNA vaccine into microspheric formulation presents all the advantages of formulated subunit vaccines, coupled with the added advantage of the inherent stability of the polynucleic acid. To this end, several groups have investigated and reported different aspects of particulate DNA vaccine formulations. Table 1.4 lists some publications of DNA vaccines formulated into biodegradable microspheres.

Alpar *et. al.* reported the potential of mucosally administered particulate DNA vaccines (Alpar HO *et. al.*, 1996); (Alpar HO *et. al.*, 1997) OVA plasmid was adsorbed onto cationic and anionic surfaces of latex particles, and a correlation was observed between the hydrophobicity of the particles and the serum IgG levels. Jones *et. al.*, (Jones DH *et. al.*, 1997) showed that orally administered luciferase plasmid encapsulated in PLGA

microspheres was capable of eliciting systemic and mucosal responses to the enzyme. It was demonstrated by Singh *et. al.*, (Singh M *et. al.*, 2000) showed immune responses to HIV-1 DNA vaccine adsorbed unto the surface of cationic microparticles; in this study, the effect of the particle size was shown to be crucial to the strength of the immune response, with 300 nm particles showing twice the serum IgG levels of 1μm particles. Serum IgG levels were undetectable in animals immunised with 30 μm particles, and this was thought to be due to lack of uptake by APCs. It was also shown in the same study that the conferment of a positive charge onto the particles and the subsequent adsorption of the DNA vaccine was responsible for the increase in immune response. Particles with no positive charge administered with the DNA vaccine displayed a 100-fold decrease in serum IgG levels. These formulations also induced CTL responses higher than naked DNA alone.

Formulation and process parameters have been investigated by Hsu *et. al.*, 1999 (Hsu Y-Y *et. al.*, 1999). The authors investigated the effect of using either sonication or homogenisation to create the primary emulsion. The effect of the water/oil ratio and surfactant concentration on particle size, DNA integrity and release kinetics, and biological activity of the encapsulated plasmid were investigated. Sonication resulted in a finer primary emulsion, but DNA integrity, encapsulation efficiencies and release profiles were comparable. A reduction in the water/oil ratio (increasing the volume of organic polymer solution) of the primary emulsion lead to a reduction in the particle diameter and an increase in the encapsulation efficiency. This effect might be a polymer-concentration rather than a polymer solution volume effect, as evidenced by the reduction in release rate. This parameter did not affect the DNA integrity. There was an inverse correlation between the PVA13-23kD concentration and size, and the smaller microspheres formed displayed increased encapsulation efficiencies. Increasing the PVA concentration beyond 1% increased the release rate of DNA. There was no effect on the DNA integrity by varying the concentration of PVA.

Tinsley-Brown *et. al.*, 2000 (Tinsley-Bown AM *et. al.*, 2000), optimised the process of plasmid DNA encapsulation in microspheres. PLGA 50:50 polymers of inherent viscosities 0.2 and 0.4 dL/g were used on their own or as blended mixtures, resulting in

a range of release characteristics. It was observed that increasing the proportion of the more viscous polymer increased the encapsulation efficiency, a trend seen also with higher total polymer concentrations, and reported by Hsu *et. al.*, 1999 (Hsu Y-Y *et. al.*, 1999). The high encapsulation efficiencies were attributed to the use of ethyl acetate instead of DCM, and optimisation of temperature and volume ratios. The reported number size distributions showed that 90% of the microspheres were below 10 μm, with a mode sizes between 2.9 and 3.2 μm. The method of size measurement did not allow volume distributions to be determined. The use of Waring blending was claimed to reduce the loss of the supercoiled conformation of the plasmid to 30 - 40%, as opposed to around 10% when using the Silverson homogeniser. The released DNA retained a significant proportion of the supercoiled conformation after 6 weeks

Walter *et. al.*(Walter E *et. al.*, 1999) explored the stability issues surrounding microencapsulation of plasmid DNA by spray-drying, using PLGA polymer. It was reported that exposure to low pH (3.5) causes reversion of the plasmid to the open circular form, whereas a pH of 8 conserved the plasmid supercoiled (SC) form. In contrast to the observation by Tinsley-Brown *et. al.*, (Tinsley-Bown AM *et. al.*, 2000), sonication of the plasmid in water dramatically reduced the transfection activity of plasmid, but Walter *et. al.*, found that physiological concentrations of PBS or 0.1 M $NaHCO_3$ prevented the degradation of DNA by sonication, and preserved the transfection activity. To investigate the quality of DNA released from the microspheres, the authors selectively assayed for double-stranded DNA (dsDNA), as well as total nucleic acid. It was found that the release of the total DNA was bi-phasic, showing an initial burst release followed by a second release, possibly due to the polymer degradation. The assay technique revealed that significantly lower amounts of dsDNA were released during the second release phase, and the transfection activity of the DNA released after 16 days confirmed the almost complete loss of biological activity. The spray-drying method however showed that encapsulation efficiencies of up to 76% could be reached by the incorporation of $NaHCO_3$.

Other methods of increasing the encapsulation efficiency and preserving the integrity of plasmid DNA in microspheres have been reported. Capan *et. al.* (Capan Y *et. al.*, 1999)

complexed DNA with poly-l-lysine (PLL) and showed that compared with the free DNA, DNA-PLL complexes showed increased preservation of the supercoiled conformation and stability to DNase I degradation, after microencapsulation. Ando *et. al.*, (Ando S *et. al.*, 1999) reported that freezing the DNA solution prior to homogenisation protected the DNA from the shear forces of the process, preserving the structural integrity of the plasmid.

Table1.4: Current publications of DNA vaccines formulated with biodegradable microspheres

Reference	Polymer	Formulation method	Antigen encoded	Investigation	Results / major finding
(Alpar HO *et. al.*, 1997)	PLA	O/w (surface- adsorbed plasmid)	Ovalbumin	Immune response after i.n. and i.m. administration	Increase in serum IgG with increasing hydrophobicity of particles.
(Jones DH *et. al.*, 1997)	PLGA	W/o/w	Luciferase	Immune response after i.p., and oral administration	Oral route results in high IgA and IgM compared with i.p., which elicits stronger IgM.
(Jones DH and Farrar GH, 1998)	PLGA	W/o/w	Luciferase	Immune response after i.p., and oral administration	Oral route results in high IgA and IgM compared with i.p., which elicits stronger IgM.
(Walter E *et. al.*, 1999)	PLGA 50:50	Spray-drying	[4]GFP	Release profiles of DNA of different Mw and stability to formulation parameters.	Low pH (<7) and sonication causes DNA degradation. $NaHCO_3$ increases encapsulation efficiency. Tri-phasic release profile
(Ando S *et. al.*, 1999)	PLGA 50:50	W/o/w, frozen internal aq. phase	β-gal	Preservation of SC state of plasmid DNA, encapsulation efficiency	Up to 95 % SC retention by cryoencapsulation method, and increased encapsulation.
(Capan Y *et. al.*, 1999)	PLGA 50:50	W/o/w, plasmid/PLL complex encapsulated	Not reported	Effect of PLL on encapsulation efficiency, release profile and protection from serum enzymes	Complexation with PLL increased resistance to serum enzymes. No effect of PLL on loading or release profile
(Hsu YY *et. al.*, 1999)	PLGA 50:50	W/o/w	Luciferase	Effect of encapsulation process parameters on microsphere characteristics	Size, plasmid release profile and loading affected by surfactant and water/oil ratio
(Wang D *et. al.*, 1999)	PLGA 50:50	W/o/w	β-gal	Preservation of plasmid biological activity, *in vitro* microsphere uptake, loading and release profiles	Optimal loading efficiency (~50 %) at 30 to 60 kDa, with reduced release rate. *in vitro* rate of microsphere uptake increased by addition of [3]MPLA.
(Tinsley-Bown AM *et. al.*, 2000)	PLGA 50:50	W/o/w	Not reported	Effect of blending polymers of different Mw on loading, morphology and plasmid release.	Optimal loading efficiency (54%) with RG503. Increasing Mw reduces plasmid release rate. SEM shows hollow microspheres
(Singh M *et. al.*, 2000)	PLGA 50:50	W/o/w, Surface adsorbed DNA	HIV-1 p55 gag	Immune response after i.m. administration of naked and formulated DNA.	Adsorbed DNA shows enhanced antibody response. Reducing microsphere size increases serum IgG titre.
(Kaneko H *et. al.*, 2000)	PLGA 50:50	W/o/w	HIV env Glycoprotein	Immune response after oral and i.m. administration	100-fold reduction in virus titres after oral administration of PLGA encapsulated DNA vaccine.
(Lunsford L *et. al.*, 2000)	PLGA 50:50	W/o/w	HPV antigen	Distribution of DNA after i.m. s.c. and i.v. delivery of DNA-loaded microspheres in mice.	I.m. and s.c. leads to persistence at site of injection and draining LNs. I.v. leads to persistence in organs rich in APCs and lymphoid cells, e.g. spleen and liver
(Mittal KS *et. al.*, 2001)	Sodium alginate	Cross-linked alginate emulsion	[1]β-gal [2]BAd3	Immune response after oral, i.n., i.m., s.c., and i.p.	Mucosal route results in higher IgA titres, sytemic routes results in stronger systemic IgG.

1) β-galactosidase; 2) Bovine adenovirus type III; 3) MPLA 4) Green fluorescent protein

1.6 Cells and organs of the immune system

The lymphoid system is a complex network mainly of lymphatic vessels, lymphoid nodules, lymph nodes, tonsils and the spleen. The cellular components are the lymphocytes, macrophages and polymorphonuclear (PMN) leukocytes. Figure 1.5 is an illustration of these cells, displaying their respective precursors (Sell S, 1987).

1.6.1 Neutrophils, eosinophils and basophils

Briefly, PMNs are subdivided into neutrophils, eosinophils and basophils. Neutrophils make up 50 to 90% of the total circulating PMN population and have a diffuse presence in many tissues. Rapidly migrating and phagocytic, neutrophils appear in areas of infection or tissue damage. The phagocytic function of these cells is aided by the extensive distribution of primary lysosomes. With a half-life of 6 to 10 hours, and about 10 billion produced daily, neutrophils are the first line of active defence against bacterial and fungal infections (Ada G and Ramsay A, 1997).

Eosinophils make up 1 to 3% of the total circulating leukocyte population. They have lysosomic granules similar to those found in neutrophils, but have a high peroxidase content. Found in high numbers around antigen- antibody complexes, eosinophils appear to limit or modulate inflammation.

Basophils located in solid tissue are called mast cells and are found in loose aureolar connective tissue. Blood basophils make-up 1% of the total circulating PMN population. The granules found in basophils contain heparin, histamine, 5-hydroxytryptamine and a range of hydrolytic enzymes. The release of these agents by mast cells is the mechanism for the unleashing of anaphylactic or atopic allergic reactions (Cruse JM and Lewis RE, 1999).

1.6.2 Lymphocytes

The lymphocyte is a small round cell found in the peripheral blood, lymph nodes, spleen, thymus, tonsils, and appendix and scattered throughout the many other tissues. It is responsible for the primary recognition of antigen and is an immunologically specific effector cell. Lymphocytes produce cell surface molecules that serve as receptor

sites for reaction with the antigen. The lymphocyte is the carrier of immunologically specific information. They are broadly classified as T and B cells.

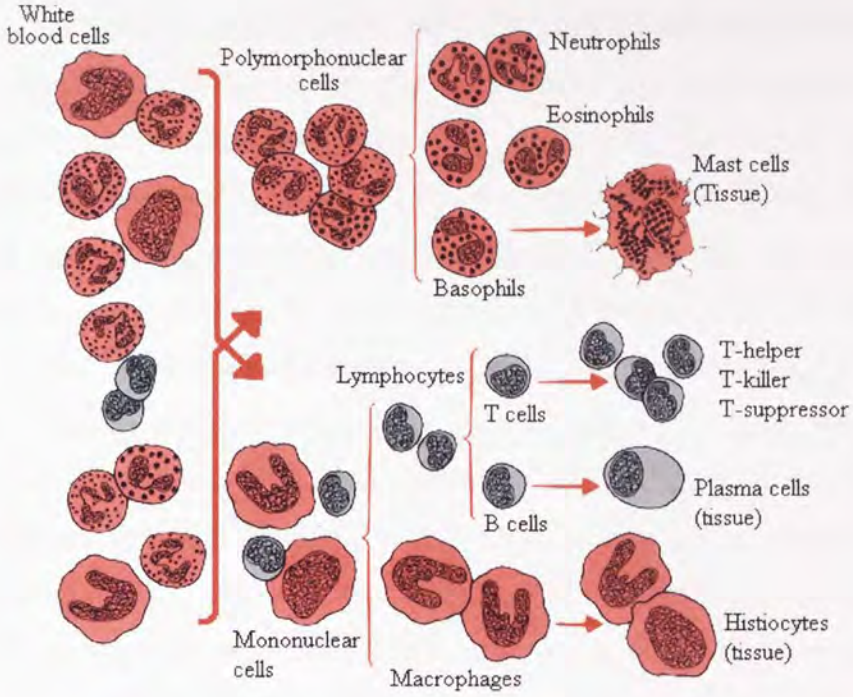


Figure 1.5: A diagrammatic representation of the cells of the immune system, showing their origins and precursors (Sell S, 1987).

Thymus-derived lymphocytes (or T cells) originate in the thymus from pro-thymocytes (or T cell precursors). They are re-released into the circulation and subsequently localise in thymus-dependent areas of the other lymphoid organs. Approximately 65 to 85% of lymph node cells and 30 to 50% of spleen cells are T cells. A further subdivision of these cells identifies T helper (T_h), T cytotoxic (CTL), T delayed hypersensitivity and T suppresser cells (T_s).

The B cells arise from precursors in the bone marrow and are the precursors of the cells that synthesise immunoglobulins. It is found that 10 to 20% of lymph node cells and 20 to 35% of spleen cells contain surface immunoglobulin (Ig) markers indicative of B cells. They develop from stem cells that originate in the foetal bone marrow or liver, the gastrointestinal lymphoid tissue, or the peripheral lymph nodes. On antigenic stimulation, B cells differentiate into antibody-secreting plasma cells.

1.6.3 The lymph nodes

In humans, the lymph node is a relatively small (0.5cm) secondary lymphoid organ that is a major site of immune reactivity. It is surrounded by a capsule and contains lymphocyte, macrophages and dendtritic cells (DC) in a loose reticulum. (see figure 1.6). Lymph enters this organ from afferent lymphatics at the periphery, percolates through the node until it reaches the efferent lymphatics, where it exits at the *hilus* and circulates to central lymph nodes, and finally to the thoracic duct. The lymph node is divided into a cortex and medulla. The superficial cortex contains B lymphocytes in follicles, and the deep cortex is comprised of T lymphocytes. Differentiation of the specific cells continues in these areas and is driven by antigen, and thymic hormones. Conversion of B cells into plasma cells occurs chiefly in the medullary region where enclosed lymphatics are protected from undesirable influences by a macrophage sleeve. Macrophages and follicular dendritic cells (FDCs, located in the paracortex) interact with antigen molecules that are transported to the lymph node in the lymph. T lymphocytes percolate through the lymph nodes; they enter from the blood at the post capillary venules of the deep cortex then enter the medullary sinuses, and pass out of the node through the efferent lymphatics. T cells that interact with antigens are

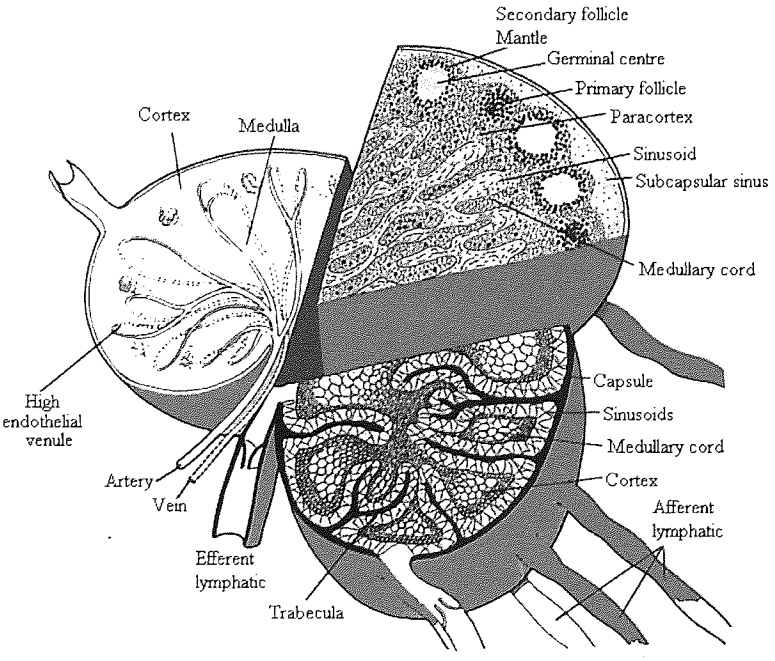


Figure 1.6: The normal lymph node showing the cortex, medulla and lymph vessels (Sell S, 1987).

detained in the lymph node which may be a site of major immunologic reactivity. The lymph node is divided into B and T lymphocytes regions. The lymph node acts as a filter and may be an important site for phagocytosis and for the initiation of immune responses (Austyn JM and Wood KJ, 1993).

Killer cells (K, or null cells) have lymphocyte-like morphology, but their functional characteristics differ from that of B and T cells. They are involved in the antibody-dependent cellular cytotoxicity, killing target cells coated with IgG antibodies. An IgG antibody molecule binds through an Fc region to the K cell's Fc receptor. Following contact with a target cell bearing antigenic determinants on its surface, the lymphocyte-like K cell releases lymphokines that destroy the target. This represents a type of immune effector function in which cells and antibody participate. Besides K cells other cells that mediate antibody-dependent cell-mediated cytotoxicity include natural killer (NK) cytotoxic T cells, neutrophils and macrophages.

Natural killer (NK) cells attack and destroy virus-infected cells. They constitute an important part of the natural immune system, do not require prior contact with antigen, and are not MHC restricted by the major histocompatibilty complex (MHC) antigens. NK cells and killer cells induce lysis through the action of antibody. Immunological memory is not involved as previous contact with antigen is not necessary for NK cell activity. In addition to the ability to kill selected tumour cells and some virus-infected cells, they also participate in antibody-dependent cell mediated cytotoxicity by anchoring antibody to the cell surface through an Fc gamma receptor. Thus, they are able to destroy antibody coated nucleated cells. NK cells are believed to represent a significant part of the natural immune defence against spontaneously developing neoplastic cells and against infection by viruses (Austyn JM and Wood KJ, 1993).

Macrophages are the primary phagocytic cells, and the largest of the lymphoid cells, ranging form 12 to 15 μm. The cytoplasm of the macrophage contains a great variety of organelles including endoplasmic reticulum (ER), a Golgi complex, mitochondria, free and aggregated ribosomes and various membrane-limited phagocytic vacuoles. Tissue-located macrophages are slightly larger (15 to 18 μm), and may contain many more

cytoplasmic vacuoles than monocytes (Cruse JM and Lewis RE, 1999).

1.7 The immune system

Infectious agents, for the most part, gain entry into the host either by direct access into the circulation *via* a breached skin site or by infection at a mucosal surface. The mammalian response to pathogenic invasion has two main components; the humoral and cell mediated immune responses.

1.7.1 The humoral immune system

Humoral immunity is mediated mainly by B cells and immunoglobulins. There are 5 isotypes of the immunoglubulins, namely, IgA, IgD, IgE, IgG and IgM. The structures of these immunoglobulins or antibodies are such that each is composed of an antigen-recognizing (Fab), and a readily crystallisable constant fragment (Fc). The amino acid sequences in the Fab fragment are variable, and within the variable regions are hypervariable regions that bind with antigens and are responsible therefore, for immunologic specificity. The Fc segment determines the biological properties of the particular class of antibody.

B cells have two classes of immunoglobulin receptors, IgM and IgD receptors. Both are dimeric molecules with two antigen binding sites. Generally, the affinity of each site of each dimer on antigen-naïve cells for the epitopes of antigens is generally low. If both binding sites are occupied, the total avidity of binding is substantially higher. B cells secrete IgM molecules after activation in the form of a pentameric molecule. In contrast, IgD molecules are not secreted by activated cells (Sell S, 1987).

Antigens that react with B cells are of two types. The type that are pre-processed by APCs and result in activation of T cells, which may aid the process of B cell replication and differentiation. The proliferation and differentiation of B cells by such antigens is referred to as a T cell-dependent process. The second type of antigen are those that are not processed by APCs, most notably poly- or oligo-saccharides. The activation and differentiation of the B cells are thus T cell-independent.

After presentation of a T cell dependent antigen to B cells, primary blast cells are formed. Some of these cells rapidly differentiate to form relatively short-lived plasma cells, producing low-affinity antibodies. B cells that respond to a T-independent antigen such as a polysaccharide become IgM - secreting cells. Antibody-antigen complexes thus formed together with associated complement components become attached by Fc receptors to the surface of follicular dendritic cells (FDCs), which occur in a diffuse pattern in primary follicles. Under the influence of the FDC associated antigen, in the primary follicle, B-cell blasts undergo further clonal expansion to form centrocytes. It is during this process that germinal centres in which antigen-bearing FDCs form a cap encapsulating a rapidly replicating cell, and antibody isotype switching occurs. Somatic hypermutation of the B cell receptors takes place, and centrocytes with mutated receptors move towards the antigen depot. Those with receptors of highest affinity for the antigen are selected by the antigen to further differentiate into either plasma cells or memory B cells. The cells that fail to receive a positive signal from antigen die by apoptosis and are consumed by macrophages. Plasma cells migrate to the bone marrow or Peyer's patches, and the memory B cells recirculate. When antigens in the germinal centres come into contact with re-circulating memory B cells, they are processed and presented to specific T cells within the germinal centre, and differentiate into antibody-secreting plasma cells. This continuing cycle of plasma cell and memory B cell formation maintains a reservoir of high affinity antibodies (Ada G and Ramsay A, 1997).

1.7.2 The cell mediated immune response

The efficient presentation of antigen to lymphocytes requires the expression of MHC class I or MHC class II by the APCs, as well as the co-expression of co-stimulatory molecules, B7.1 or B7.2. The lymphocytes express CD28, the receptors for these ligands. Expression of these molecules is enhanced by cytokines like IFN-γ and IL-7. A virus may infect an APC by receptor-mediated endocytosis or by fusion with the plasma membrane, and liberate RNA or DNA into the cytoplasm. During transcription and translation of this nucleic acid into the new viral protein, degradation of the protein occurs by enzymes that form part of the proteasome. The resulting peptides are moved into the lumen of the endoplasmic reticulum by a 'transporter of antigenic peptides' or

TAP. They combine with MHC class I molecules and are translocated to the cell surface. This mechanism of MHC class I presentation of endogenous peptides, called the endogenous pathway, leads to recognition of this complex by T lymphocytes (CD8+). Most or all newly synthesised proteins, including self proteins contribute to peptides through this pathway (York IA and Rock KL, 1996).

Non-infectious preparations may be internalised into endosomes of APCs, which then fuse with lysosomes to form an endolysosome. Proteolysis in the prevailing mild conditions generates peptides. MHC class II molecules move to the trans-golgi network, where they are targeted to the endosome. In this mildldy acidic environment they bind the antigenic peptides and the peptide-MHC complex is transported to the cell surface, where it is recognised by a CD4+ T cell. This is the exogenous antigen pathway of antigen processing.

1.7.3 The mucosal immune system

Besredka observed and reported in 1919 that rabbits were protected from fatal dysentery after oral immunisation with the killed *Shigella bacillus*, regardless of the serum antibody titres. It was found in 1963 that fluids surrounding the mucosal surfaces contained high levels of an immunoglobulin, designated IgA, and contrasted with the low levels found in the serum. The IgA was found to also contain a secretory component and a J chain that linked two IgA monomers together (Ada G and Ramsay A, 1997)

The production of a s-IgA on the mucosal surface is a result of the local exposure of antigens to the mucosal-associated lymphoid tissue (MALT), especially those in the upper respiratory tract (NALT), and the GIT (GALT). Anatomically, the GALT consists of the Peyer's patches (PP), the appendix, and solitary lymph nodes (LNs) in the GIT, and the NALT contains the palatine and pharyngeal tonsils (Austyn JM and Wood KJ, 1993). The epithelial surfaces of both the NALT and GALT contain specialised antigen sampling cells known as M cells. These cells can transport antigens from the mucosal surfaces into the underlying lymphoid tissues. GALT describes lymphoid tissue in the gastrointestinal mucosa and submucosa. It consists of radially arranged and closely

packed lymphoid follicles termed Peyer's patches, which impinge upon the intestinal epithelium, forming dome-like structures. Specialised epithelial cells overlie the lymphoid follicles, forming a membrane between the lymphoid cells and the lumen. These are the 'M' cells, or the follicle-associated epithelial cells (FAE), and have several features which enrich them for the function of antigen sampling; they have short microvilli, small ctyoplasmic vesicles and few lysosomes and are adept at uptake and transport of lumenal antigens including proteins and particulates such as viruses, bacteria and even small parasites. Antigen uptake by M cells results in intact delivery into the underlying lymphoid tissue (Cruse JM and Lewis RE, 1999).

The *lamina propria* of the GIT is a thin connective tissue layer that supports the epithelium of the GI, respiratory and genitourinary tracts. It is a major mucosal effector site, and the main cell types found are lymphocytes, including B cells (20 to 40%), and T cells (40 to 60%). Other cell types include macrophages, oesinophils and mucosal mast cells. Macrophages found in significant numbers in the *lamina propria* may be involved in antigen processing and presentation at this site (Cruse JM and Lewis RE, 1999).

The MALTs can be divided into two functionally distinct compartments or sites: the inductive site, which is a defined lymphoid microcompartment, and are located in the various anatomical sites primarily the bronchus (BALT), the gut (GALT) and other sites including the breast, cervix, and skin. The effector sites, which contains diffuse accumulations of large numbers of lymphoid cells, do not associate into apparently organised structures. Antigens are first encountered in the inductive sites, and initial responses are induced. In the effector sites IgA plasma cells are found and the production of s-IgA antibodies results in local and distal mucosal protection (Partidos CD, 2000). Figure 1.7 is a diagrammatic representation of a typical response in MALT, after introduction of an antigen or pathogen. The lymphoid cells of the MALT are either found as diffuse aggregates or are organised into germinal centres. Lymphocytes found in this system mainly re-circulate within the MALT and do not localise to other areas of the lymphatic system. Following stimulation by antigen, these cells migrate *via* the local lymphatics to the systemic circulation from which they re-circulate to other mucosal

surfaces. These leucocytes are responsible for mediating mucosal immunity, an important role, as the majority of pathogens enter the body by these surfaces

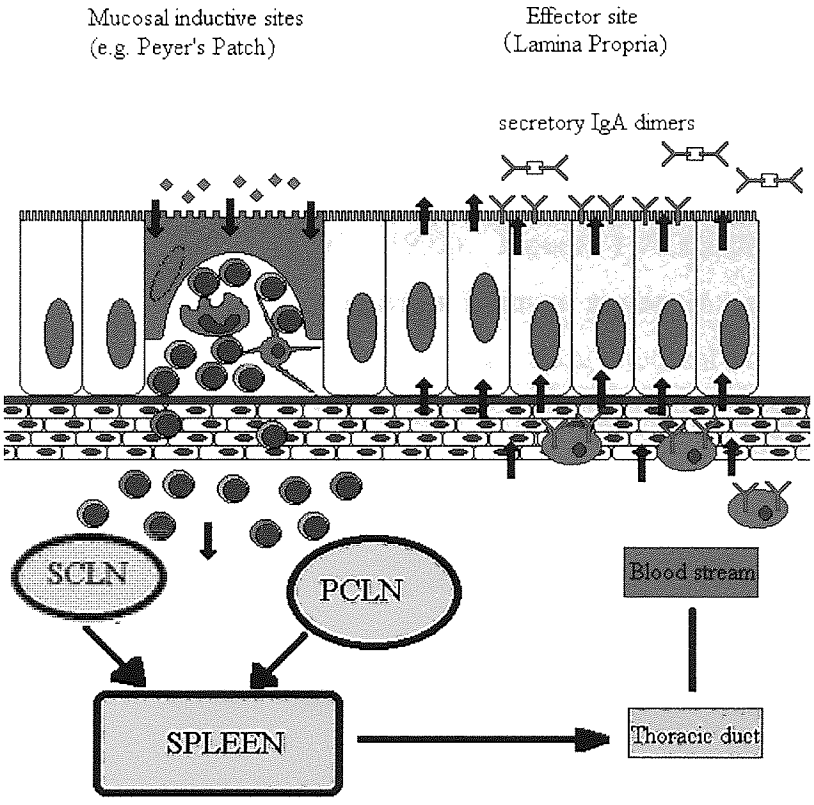


Figure 1.7: Diagrammatic representation of the mucosal immune response, adapted from Roitt *et. al.*, (Roitt I *et. al.*, 1998) (SCLN: Superior cervical lymph node; PCLN: Posterior cervical lymph node)

Distinct follicles or B cell zones occur beneath the dome region of the PP. The follicles contain germinal centres, where significant B cell division occurs. These germinal centres are considered to be sites where frequent B cell switches to IgA and affinity maturation occur, and they also contain the majority of surface IgA+ B cells. Adjacent to follicles are the T cell dependent areas which contain all major T cell subsets. The T cells present in the PP are mature; they contain a T cell receptor CD3 complex, and more than 95% of these T cells use the α/β form of the T cell receptor (TCR) (Ada G and Ramsay A, 1997).

Upon entry into the MALT, antigens are rapidly internalised and processed by APCs

such as subepithelial DC and macrophages, and B cells proliferate and switch to s-IgA-committed cells. The s-IgA molecules are produced by plasma cells in the *lamina propria*, and are subsequently transported across the epithelial cells with the aid of the polyimmunoglobulin receptor. T lymphocytes are found in the *lamina propria* of the mucosae as well as the epithelial layer. The functions of these T cells are the induction and regulation of responses by the antigen-specific IgA B cells as well as effector T cells. These B cells migrate through the systemic circulation to other mucosal sites, including the initial site of induction for terminal differentiation to s-IgA producing plasma cells (McGhee JR *et. al.*, 1992). Figure 1.7 illustrates this process diagrammatically. Stimulation of the mucosal immune system at one mucosal site can lead to s-IgA production in the local as well as distal mucosal surfaces. This inter-connected mucosal system of s-IgA induction and production is termed the common mucosal immune system (CMIS) (McGhee JR *et. al.*, 1992). There is some evidence of compartmentalisation within the CMIS; the upper aero-digestive tract is primarily supplied by IgA-committed B cells form the NALT, while the genito-urinary tract preferentially receives these from the GALT (Chen H, 2000), however, i.n. administration of DNA vaccines result in genito-urinary tract IgA responses (Klavinskis LS *et. al.*, 1999).

The induction of effective immunity to pathogenic viruses and bacteria must consider the activation of two well-defined classes of T cells. These are the CD3+, CD4+, and CD8- T-helper cells which bind to epitopes presented on MHC class II molecules by APCs. In mucosal inductive sites these APCs include B cells, dendritic cells and macrophages. Activation of antigen-specific CD4+ Th cells can lead to secretion of appropriate cytokines (e.g. IL-4,IL-5, IL-6) for B cell responses and Ig synthesis. In addition, antigen-activated CD4+ Th cells can also up-regulate the function of CTLs which are major effectors for the elimination of virus-infected cells. CTLs normally express CD8 molecules and respond to viral epitopes presented together with MHC class I molecules (Ada G and Ramsay A, 1997). T cells therefore play a crucial role in the induction of mucosal responses.

The mucosal immue system thus provides a large capacity for the induction of immune

responses, and is a useful route of vaccine administration. Increased knowledge of the CMIS will maximise the exploitation of this system for vaccine delivery.

1.7.4 The skin immune system

The superstructure of the skin comprises of the epidermis, the dermis and the hypodermis. The hypodermis is the lowest layer of the skin comprising fibroblasts and adipocytes. Above this is the dermis, which is 2 to 3 mm thick and composed of mostly fibroblasts (see Figure 1.8 for a diagrammatic representation of the skin superstructure). The basal *lamina* lies between the dermis and the epidermis, and serves as a basement membrane for the cells of the epidermis, the outermost layer of the skin. It is within the viable epidermis, composed of 90% keratinocytes that Langerhan cells (LC) are found. The LCs are immune competent dendritic cells with extensive protrusions which form a an effective immunological barrier. In spite of being only 1% of the total cell population in the epidermis, they cover 20% of the surface area. When the skin becomes damaged, keratinocytes synthesise immune-modulating cytokines and can express intracellular cell adhesion molecules (ICAM). Thus, keratinocytes are immunologically active cells, as well as providing a physical protective. barrier LC show an increase in phagocytic activity and migrate into draining lymph nodes where they encounter foreign antigens and initiate immune responses. The skin serves as the first line of defence against external pathogen attack, as well as providing protection against water loss and ultraviolet radiation (Babuick S *et. al.*, 2000).

Within the epidermis are also melanocytes, epidermotropic lymphocytes and Merkel cells. The epidermis can be further separated into the basal, spinuous, granular, clear and horny layers, each representing a differentiation stage of the keratinocytes. The basal layer consists of undifferentiated stem cells above the basal *lamina*. The cells above the basal layer contain many desmosomes, giving them a spinuous appearance. The transition zone above the granular zone consists of both living and dead cells, above this is the stratum corneum (SC). The SC is 10 - 20 μm thick and consists of terminally differentiated keratinocytes called corneocytes. These are flattened cells which contain intracellular cross-linked macrofibrillar bundles of keratin, giving the cell a rigid structure. Desmosomes connect corneocytes into the ordered superstructure of

the SC. Within the SC are multilamellar arrays of lipids found extracellularly between the corneocytes. These arrays are composed of cholesterol, free fatty acids and ceramides. A chemical or physical breach of the skin's barrier induces the synthesis of fatty acids, cholesterol and free fatty acids, and pre-formed lamellar bodies are secreted to normalise the barrier.

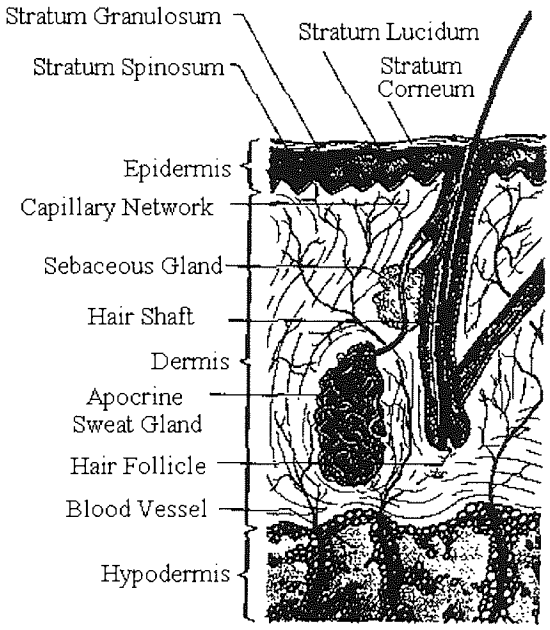


Figure 1.8: Superstructure of the skin

The LC are immune competent, skin-located DC which form a second line of defence, initiating specific immune responses by processing and presenting antigens to other cells of the immune system. The involvement of DC in the induction of immunity to foreign antigens encountered in the skin is evidenced by experiments which showed that removing the vaccination site after cutaneous DNA vaccination still resulted in specific immune responses to the DNA vaccine. It was illustrated that DNA was being rapidly transported from the site of administration. Condon (Condon C *et. al.*, 1996) showed that LC, following gene gun administration of plasmid encoding green fluorescence protein (GFP) migrated to local lymph nodes and expressed GFP. Other studies have demonstrated the role of B cells (Chen Y *et. al.*, 1998) and CD4+ helper T cells (Maecker HT *et. al.*, 1998).

1.8 Immunological aspects of DNA vaccines

The immune response to DNA vaccination is dose-dependent, long-lasting and can be characterised as strongly Th-1, driving a more prominent cell mediated response (Shroff KE *et. al.*, 1999). This cell-mediated aspect of the response is important for protection against intracellular pathogens like Tuberculosis (TB) and human immunodificiency virus (HIV). The initial experiment by Ulmer *et. al.* showed that heterologous protection against influenza by DNA vaccination, using a plasmid that encoded the conserved viral protein influenza A nucleoprotein (NP) was not mediated by the NP-specific antibodies, but was most likely due to the virus nucleoprotein NP-specific cellular immunity, as evidenced by significant amounts of primary CTLs directed against the antigen (Ulmer JB *et. al.*, 1993).

1.8.1 The humoral response to DNA vaccines

The proposed mechanisms for priming humoral and cellular immune responses by DNA vaccines have reflected the distinct pathways of MHC class I and class II antigen processing as defined *in vitro*. Antibody responses have been proposed to occur when antigens encoded by the transduced myocyte are released into the circulation either *via* secretion or cell death. These antigens are then taken-up by macrophages and B cells, thereby initiating a T helper-dependent antibody response (Boehm W *et. al.*, 1998).

1.8.2 The cell mediated response to DNA vaccines

Alternatively, CTL priming has been proposed to occur *via* endogenous proteosome-dependent processing and presentation of antigens within the transfected myocyte, followed by transfer associated protein (TAP)-dependent presentation of the myocyte MHC class I molecule. Pardoll *et. al.*, proposed that the stability of the DNA sequences would produce an essentially continuous supply of antigens to drive the immune responses indefinitely (Pardoll DM and Beckerleg AM, 1995)., This theory is consistent with the long term persistence of immune responses seen with DNA vaccination, as observed by Wolff *et. al.*, (Wolff JA *et. al.*, 1992).

1.8.3 The role of professional antigen presenting cells

APCs have been shown to be critical to the priming of humoral and cell mediated responses. Co-injection of naked DNA encoding granulocyte-macrophage colony stimulating factor (GM-CSF) with that for a rabies glycoprotein enhanced both humoral and cell mediated responses, with protection against rabies virus challenge. The proposed mechanism of this protection was the induction and differentiation of haematopoetic precursors into professional APCs. GM-CSF has been shown to induce the differentiation and maintenance of DCs and up-regulate their expression of B7-1 and B7-2 molecules (Larsen CP *et. al.*, 1994).

DCs have been aptly described as 'mobile sentinels' which are central to T cell induction. Infected cells frequently lack the co-stimulatory molecules that drive T cell clonal expansion, production of cytokines and development into killer T lymphocytes. DCs capture and process antigens, migrate to the spleen and the lymph nodes where they activate antigen-specific T cells. Either terminally differentiated or mature DCs readily prime T cells, which complete the immune response by interacting with other cells such as B cells for antibody formation, and macrophages for cytokine release. DCs can stimulate the vigorous proliferation of CD8+ T cells and turn CD4+ T cells into Th 1 cells which produce IFN-γ. This cytokine activates the antimicrobial activity of macrophages and promotes the differentiation of T cells into killer T cells. In the presence of IL-4, DCs induce T cells to differentiate into Th-2 cells which secrete IL-5 and IL-4. These cytokines promote the humoral arm of the immune response.

The immature DCs have several features which make them good APCs; they are highly phagocytic, they form large pinocytotic vesicles in which extracellular fluid and solutes are sampled, and they express receptors that mediate adsorptive endocytosis. It is this combination of macropinocytosis and receptor-mediated endocytosis that make antigen presentation so efficient as to require low antigenic concentrations for activation. Once the immature DC has captured an antigen, its phagocytic capabilities rapidly decline as it enters the mature stage. To generate CTLs, antigenic peptides have to be complexed to CD8+ expressing T cells. Catabolism of the antigen by the cell proteosome turns over the bulk of cellular proteins and provides the class I pathway with one source of

peptides for presentation. A peptide transporter, TAP, transfers the peptides from the cytosol to empty class I molecules in the endoplasmic reticulum. After assembly, the MHC class I -peptide complexes are transported by the exocytic pathway to the plasma membrane (York IA and Rock KL, 1996). It is not clear how DCs process and present antigens with no access to the cytosol in an MHC class I-restricted manner.

Follicular DCs (FDCs) directly sustain the viability, growth and differentiation of activated B cells. They also organise the primary B cell follicles. These FDCs are not bone marrow derived, are located in the paracortices of the lymph nodes and bear surface receptors for complement and Fc. They capture antibody-antigen complexes and display the whole complex at their surface for long periods in the germinal centres of the lymph nodes, where they direct antigen specificity of plasma cells. Proliferating B cells undergo somatic mutation and stop dividing until triggered by an immune complex on the FDCs. The B cells that recognise an immune complex with high affinity process the antigen and present it as a peptide-MHC complex to antigen-specific T cells. The T-B cell interaction ensures the survival of these high-affinity B cells, while the low-affinity B cells are not stimulated, and apoptose to be cleared by macrophages. A second type of DC found in the germinal centres of these inductive sites, the CD11c+ DC also carry the immune complexes, but are much more powerful stimulators of T cells than the B cells. It is thought that these bring the antigens to the germinal centre and display processed antigens (Ada G and Ramsay A, 1997). Thus, it can be seen that DCs are likely to play a crucial role in immune responses by DNA vaccines; this has been highlighted by several studies (Casares S *et. al.*, 1997; Ni K and O'Neill HC, 1997; Iwasaki A *et. al.*, 1997; Akbari O *et. al.*, 1999).

The immunobiology of the muscle reveals a sparse presence of professional APCs like DCs (Hohfeld R and Engel AG, 1994), though they may be recruited to this tissue by local irritation. This makes the direct transfection of APC by i.m. injection unlikely. The question then remains as to how this route of immunisation gives rise to the CTL response.

It has been shown that transfected fibroblasts (non-professional APCs) are able to

induce an antigen-specific MHC class I-restricted response if they are physically relocated to secondary lymphoid tissue, which is effectively a reservoir of professional APCs (Kundig TM *et. al.*, 1995). This credits the possibility that CTLs could receive a peptide-MHC class I-restricted complex expressed by the directly-transfected myocyte, and a second signal from haemopoietic cells recruited by a local inflammatory response to the site of injection. Corr *et. al.*, 1996 (Corr M *et. al.*, 1996) using murine parent →F1 chimeras showed that muscle cells at the site of plasmid DNA injection did not present the encoded NP peptide to the immune system, but that this presentation occurred at the surface of bone-marrow-derived APCs. However, the paucity of the APCs at the injection site favoured the hypothesis that the transfected myocytes transferred the NP to APCs. In a further study by the same group, it was noted that plasmid DNA with a macrophage-specific promoter elicited very weak humoral and CTL responses, even when co-injected with GM-CSF to stimulate antigen presentation by macrophages. They also showed that the immunogenic protein responsible for the CTL response in mice was expressed predominantly by nonlymphoid tissue, and not by APCs (Corr M *et. al.*, 1999). Thus, the existence of an alternative pathway by which non-endogenously produced antigen is processed and presented by APCs is suggested. Evidence of this is provided by several studies (Kavocsovics-Bankowski M and Rock KL, 1995; Kovacsovics-Bankowski M and Rock KL, 1994; Reis e Sousa C and Germain RN, 1995). Endogenously produced antigen is degraded by cytosolic proteosomes and shunted into the endoplasmic reticulum by TAP peptide transporters for stable association with MHC class I molecules, with subsequent translocation to the cell surface (York IA and Rock KL, 1996; Kavocsovics-Bankowski M and Rock KL, 1995). Kovacsovics-Bankowski and Rock (Kavocsovics-Bankowski M and Rock KL, 1995) demonstrated a pathway whereby proteins can traffic from vesicular compartments into the cytosol. Selective blockade of the proteolytic activity of the proteosome resulted in a marked decrease in the supply of peptides to newly synthesised class I molecules. It was also observed that chloroquine, an inhibitor of lysosomal degradation, did not affect the presentation of exogenous OVA with MHC class I molecules. Thus, antigens taken-up by APCs may be shunted to the cytosol for processing and presentation to CD8+ cells by MHC class I molecules.

The proof of the existence of several pathways and mechanisms by which DNA vaccination results in cellular and humoral responses is currently unavailable, but the central role of APCs like DCs cannot by refuted, and further work to uncover the precise pathways will probably show a mixture of the proposed mechanisms.

1.8.4 The role of co-stimulatory molecules

The proposed role of co-stimulatory molecules in addition to the MHC-peptide complex is now well established (Linsley PS and Ledbetter JA, 1993). The most potent known co-stimulatory signals are the B7 family of molecules, including B7-1 and B7-2 (designated cluster differentiation numbers CD80 and CD86 respectively). Binding of these molecules to CD28 augment proliferation, cytokine production and differentiation of T lymphocytes, and it has been shown that CD28-deficient mice have a decreased T cell function (Santra S *et. al.*, 2000). Santra *et. al.*, have shown clearly that B7-2 is a critical requirement for the elicitation of both humoral and cellular immune responses to intracellularly-synthesised antigen. In contrast, B7-1 was not required for T cell priming, though it augmented the level of CTL activity. This study also revealed that co-administration of plasmid with B7-1, but not B7-2 amplified DNA vaccine-elicited CTL responses. B7-1 is expressed in activated B cells, macrophages, dendritic cells (DCs), and T cells, whereas B7-2 is constitutively expressed at moderate levels on resting and activated B cells, monocytes and DCs (Santra S *et. al.*, 2000). This further proved that the level of B7-1 expression affects the level of CTL activity.

It is apparent that antigen processing and presentation alone cannot fully explain the observed induction of immunity. The evidence for the critical role of co-stimulatory molecules implies that direct presentation of endogenously synthesised antigens by MHC class I molecules of transfected myocytes, is not sufficient to prime CTL precursors to evoke the observed strong cell-mediated immune activity. The role of APCs expressing co-stimulatory molecules is important.

1.9 Common routes of administration of DNA vaccines

There are a many reported routes of administration of DNA vaccines, most notably the intra-muscular injection (i.m.), but the subcutaneous (s.c.), intradermal (i.d.), oral,

intranasal (i.n.), intratracheal (i.t), intravenous (i.v.) and intraperitoneal (i.p.) have also been investigated. Among these routes, the i.m., mucosal, s.c. and i.d. routes are most successful and will be discussed in brief detail.

1.9.1 Intramuscular administration of DNA vaccines

Skeletal muscle has been shown to be an effective site for the administration of DNA vaccines for eliciting systemic humoral and cell mediated responses (Donnelly JJ *et. al.*, 1995; Davis HL *et. al.*, 1993; Raz E *et. al.*, 1994; Fu TM *et. al.*, 1997; Whalen RG and Davis HL, 1995; Shiver JW *et. al.*, 1996), the proof of the concept was first demonstrated by Wolff *et. al.*, 1990 (Wolff JA *et. al.*, 1990). Although muscle cells are not APCs, they have been shown to maintain the expression of injected plasmid for the effective lifetime of the mice (Wolff JA *et. al.*, 1992). This long term expression acts as a reservoir of antigen for the sustained stimulation of immune responses to the antigen and may be envisaged as a 'depot' vaccine. Intramuscular administration of plasmid generally results in low transfection efficiencies and high variabilities of gene expression. These effects might be a consequence of barriers set-up by the arrangement of the myocyte connective tissues and the extent of intracellular matrices(Davis HL and Whalen RG, 1995). This diffusional barrier can be surmounted by pre-treatment of the muscle with a hypertonic solution of sucrose, which improves the diffusion of injected substances and produces less variable gene expression in the muscle. Davis and Jasmin (Davis HL and Jasmin BJ, 1993) showed that gene transfer in the diaphram, which is not extensively subdivided by the connective tissue, is more effective than transfer in sucrose-treated murine *tibialis*. Transfection of muscle is also more efficient in regenerating muscle than in normal mature muscle. This regenerative state may be induced by pre-treatment with bupivocaine, a local anaesthetic. Myotoxins like cardiotoxin and notexin have also been used in mice to achieve this effect (Wells DJ, 1993; Davis HL *et. al.*, 1995; Bernstein DI *et. al.*, 1999).

The immune response raised by i.m. vaccinations may be explained upon consideration of the immunobiology of muscle (Hohfeld R and Engel AG, 1994). Fu *et. al.*, (Fu TM *et. al.*, 1997) demonstrated that myocytes are responsible for protein expression after *in vivo*, adminstration. Subsequently the antigenic epitopes are presented to professional

bone-marrow derived APCs by MHC class I restriction, leading to the generation of CTLs. The extent of muscle transfection has been shown to determine the speed of the humoral and CTL response. It is probable that transfection of muscle fibres generates a reservoir of protein which provides a sustained presence of antigen (Whalen RG and Davis HL, 1995). Protein can either be accumulated and then secreted or otherwise released by the muscle fibres. The proteins may also be present in interstitial spaces and circulation for several weeks or months, depending on the nature of the protein. Antibody-antigen complexes could also remain in the lymph nodes for long periods of time. Maintenance of the immune response is more likely to be due to direct transfection of myocytes, than of APCs associated with the muscle, because of the low level and short duration of their expression of a reporter protein (Whalen RG and Davis HL, 1995). The presence of the intracellular and extracellular reservoir of antigen serves to amplify the immune response, a phenomenon similar to a secondary or booster dose.

1.9.2 Mucosal administration of DNA vaccines

Mucosal immunisation frequently results in the stimulation of both mucosal immune responses through the local exposure of immunogens to the MALT. Mucosal surfaces such as the gastrointestinal, respiratory and genital tracts are the principal sites of entry and colonisation for many pathogens. Immunisation *via* the mucosal routes offers enhancement of vaccine efficacy by the simultaneous induction of both mucosal and systemic immune responses. The side effects are minimised by avoiding direct contact between potentially toxic vaccine components and the systemic circulation. Ease of administration (e.g. oral or nasal) reduces the need for trained personnel required for parenteral administration, and this also improves patient compliance, reduces costs, and removes the risk of needle stick injury or cross contamination through repeated use of the same needle (Chen H, 2000), (Partidos CD, 2000). Mucosal administration of DNA vaccines also has the added advantages of DNA vaccination.

The main barriers to the delivery of vaccines to the mucosal inductive sites are enzymatic degradation, mechanical clearance of antigens from the mucosal surfaces, and low uptake efficiency by antigen sampling sites. Strategies for overcoming these barriers include the development of delivery systems or mucosal adjuvants. (Chen H,

2000; McCluskie MJ and Davis HL, 1999; McCluskie MJ and Davis HL, 1999).

Particulate formulations improve delivery to the mucosal surfaces, and endocytosis from the lumen of the gut wall by M cells in the PP results in translocation of these particles to underlying APCs. The rate of particle degradation may be controlled, and thus the release of the antigen to the immune system may be predetermined. Further more, conjugation of ligands to the delivery systems offers the potential of targeting to specific cell types or tissues. Liposomes, microspheres and nanospheres have been used for the mucosal delivery of DNA vaccines.

Administration to mucosal sites is achieved mainly by the oral or i.n. routes (McCluskie MJ and Davis HL, 1999), although the intra-tracheal (i.t.), oral mucosa, tongue (Lundholm P *et. al.*, 1999) or genito-urinary tract (Livingston JB *et. al.*, 1998)have been investigated. Expression of administered antigen gene in mucosal tissues appears to be transient due to rapid epithelial cell turnover. Despite this, mice have been shown to develop long-lived (at least 10 months) immune responses with i.n. immunisation (Okada E *et. al.*, 1997).

Okada *et. al.*, (Okada E *et. al.*, 1997) studied the i.n. administration of DNA vaccine with cationic liposomes and found that both mucosal immunity and a strong CMI were activated. Balb/c mice were inoculated intranasally and intramuscularly with DNA encoding HIV antigens. The i.n. route induced higher titres of HIV-specific IgG antibodies than did the i.m. route. The serum IgG titres were higher than the IgA, while IgA levels in vaginal secretions were higher than IgG. Compared with the i.m. route, i.n. vaccination elicited higher titres of mucosal IgA. The authors also confirmed the activity of faecal IgA by showing that faecal extract inhibited HIV-1 replication. Thus the intranasal administration of the DNA vaccine induced higher levels of secretory IgA antibody in the mucosae of both gut and vagina than i.m. administration. It was also found that both i.n. and i.m. routes stimulated a CTL response to HIV-1.

In a similar series of experiments, Klavinskis *et. al.*, (Klavinskis LS *et. al.*, 1999) reported that nasal adminstration of a luciferase DNA vaccine formulated with cationic

liposomes elicited mucosal immunity in murine genital and rectal tracts. It was determined that extracts of nasal epithelium of recipients of liposome-formulated plasmid had a 30-fold increase in luciferase protein compared with recipients of naked plasmid. The expression of luciferase persisted at the nasal site for at least 28 days in mice immunised with formulated plasmid. The plasmid was located predominantly in the squamous epithelium, with some in the *lamina propria.* Distribution analysis established that intranasal immunisation was associated with DNA delivery to the mucosal surfaces throughout the respiratory and GIT, mostly in the nasal tissue, lung and cervical lymph nodes. Similar distributions were observed with radiolabeled polystyrene microspheres administered to mice intranasally, although a strong dependence on the volume of administration was reported by Eyles *et. al.*, (Eyles JE *et. al.*, 2001). Serum anti-luciferase IgG was higher than IgA in recipients of formulated DNA, while the levels in recipients of naked DNA was negligible. The ratio of IgG to IgA antibody titres was lower in the vaginal fluids (0.1) that in serum (5.3), and the IgA antibodies detected were associated with a secretory component, indicating that s-IgA antibodies were stimulated mucosally by intranasally administered DNA vaccine formulated by complexation to cationic liposomes. CTL responses were also induced in the lymph nodes draining the nasopharynx, and iliac lymph nodes draining the genito-rectal mucosa (Klavinskis LS *et. al.*, 1999).

The precise mechanism by which the mucosal response is generated by mucosal administration is under current investigation, but it is largely accepted that this arises by a combination of efficient antigen presentation by virtue of *in vivo* antigen synthesis. The presence of plasmid DNA in the submucosal lymphoid tissue after mucosal immunisation may result in transfection of various cell types, most likely including APCs and DC in particular, both of which play an important role in the induction of immune responses after parenteral DNA immunisation. The mucosal epithelium contains intraepithelial lymphocytes (IEL) with predominantly CD8+ cells. CD4+ cells are found mostly in the *lamina propria* and serve as regulatory T cells for Th-2 humoral immune responses and IgA production by differentiated plasma cells. Antigen epitopes may be presented by MHC class I or class II to IEL by transfected APCs along with the appropriate co-stimulatory molecules. The resulting production of cytokines, in

particular IL-4 and TGF-β, is thought to promote class switching from IgM+ to IgA+ B cells. CD4+ Th cells and CD8+ CTLs may leave the inductive sites *via* efferent lymphatics, migrate to regional lymph nodes and enter the thoracic duct to reach the bloodstream and finally enter the IgA effector cells where terminal differentiation, synthesis and transport of secretory IgA occur (McGhee JR *et. al.*, 1992; Ada G and Ramsay A, 1997).

Mucosal delivery is most suited for mass immunisation protocols, owing to the relative ease of administration, and noninvasive nature. The possibility of inducing both local and distant mucosal and systemic responses is of particular interest in the prevention of infectious disease.

1.9.3 Cutaneous administration of DNA vaccines

Transdermal delivery is an alternative to more invasive administration routes and has the advantage of lower frequency of dosing, uniform blood levels and increased patient compliance. When utilising the skin as a route of entry for vaccines, the main target sites are within the epidermis, and it is critical for delivery methods to breach the SC and reach the APCs for effective priming of immune responses. There are three possible pathways for the cutaneous barrier to be breached. The precise pathway for any given entity is determined by its physicochemical characteristics and, in the case of vaccination, the method of delivery used.

The paracellular pathway describes the passage of molecules *via* the extracellular lipids, which form continuous pathways surrounding the corneocytes. This route will favour highly lipophilic delivery systems. The transcorneocyte pathway of delivery occurs through the corneocytes, and requires high-energy 'ballistic' methods for effective penetration. The transappendageal pathway is the route compounds travel through the hair follicles and sweat glands. This route allows the stratum corneum (SC) to be avoided by permitting the passage of biomolecules around it to the epithelial cells surrounding the hair follicle. These cells are less of a barrier to penetration by molecules. The transappendegeal route is thought to be the major route by which large molecules enter the skin (Babuick S *et. al.*, 2000).

1.9.3.1 Devices for cutaneous DNA vaccine delivery

The cutaneous delivery of vaccines to the skin has several drawbacks, not least the pain associated with injection, potential spread of disease by repeated needle use, and risk of needle stick injury. The United States Centre for Disease Control (CDC) estimate that 800,000 US healthcare workers annually suffer a needle-stick injury (Hickey PLC, 2001). The use of devices for non-invasive (non-injection) delivery of vaccines to the skin is of great potential benefit.

1.9.3.1.1 Gene gun delivery

Yang *et. al.*, 1990 (Yang N-S *et. al.*, 1990) have described the *in vivo* delivery of genes by particle bombardment. This bio-ballistic or 'gene gun' (g.g.) technique is essentially the electromotive acceleration of gene-coated gold microparticles; the force provided by the acceleration enables efficient penetration of the target tissue. A more elegant method of ballistic vaccine delivery is the gas-powered gene gun. Originally developed for the tranfection of plant cells, this device uses compressed helium gas to accelerate particles through the skin. Since a gene gun delivers plasmids directly into cells, there is an increased efficiency of gene transfer, and immune responses may be elicited with picogram quantities of plasmid DNA (Pertmer TM *et. al.*, 1995). This high velocity particle injection technology is being developed as a drug delivery platform by PowderJect Technologies Ltd., with powder injection devices for single and multiple use currently in the clinical trial stage (Hickey PLC, 2001). The physiology of the skin dictates that the delivered vaccine breaches the SC, and accesses the LC and associated lymphoid tissue within the epidermis. Thus it is important to control the depth of penetration, (P) of particle-formulated vaccine. The Petry equation described in Equation 1.1 shows the key variable controlling P.

The mathematical model of particle penetration shows that particle size, mass and velocity (i.e. momentum) are critical parameters in the delivery process (Equation 1.1). The equation describes a proportionality of particle mass to depth of penetration, suggesting that faster, larger and denser particles are ideal. However, clinical experiments indicate that the skin barrier can stop or deflect organic particulate

materials below 20 μm in mean mass aerodynamic diameter. The presence of fines in a powder formulation may reduce the delivery efficiency, as the these may create an initial barrier to penetration of the skin. Thus, the importance of controlling the particle size distribution is realised. Particle hardness and friability are also critical in the design of formulated systems for the gene gun (Hickey PLC, 2001); the particles must withstand particle-to-particle and particle to wall contact in the high-energy helium jet. The influence of particle morphology and surface smoothness is less well defined. However, it is known that it will affect the interaction of the particle with the helium stream. Very porous or hollow particles, regardless of shape will suffer from reduced delivery efficiency (Burkoth TL *et. al.*, 1999).

$$P = k\left(\frac{m}{A}\right)\log\left(1+\frac{(v_0 - v_t)^2}{\Phi}\right)$$

P = Depth of penetration
k = Petry constant, related to the biochemical properties of the target tissue
m = Mass of particle
A = Plan area of particle
v_0 = Impact velocity
v_t = Threshold particle velocity for penetration
Φ = Penetration coefficient

Equation 1:1 Petry equation relating depth of penetration to key variables in powder injection (Hickey PLC, 2001).

In spite of these limitations, the use of the g.g. for cutaneous DNA vaccine delivery has been shown to be efficient and potent (Feltquate DM *et. al.*, 1997), (Porgador A *et. al.*, 1998), (Roy MJ *et. al.*, 2001). Porgador *et. al.*, 1998 determined that directly transfected DC play a predominant role in presentation of antigen *via* the MHC class I molecules after g.g. DNA delivery, and that only relatively few of these transfected DCs are critical for effective CD8+ T cell priming, and Iwasaki *et. al.*, (Iwasaki A *et. al.*, 1997) demonstrated that APCs of bone-marrow origin played the dominant role in the induction of the CTL response observed.

Roy *et. al.* carried out a phase I clinical trial to test the safety, tolerability, and immunogenicity of particle-mediated DNA vaccine against a Hep B DNA vaccine,

using the PowderJect® delivery device (PowderJect® XR1). The administration of 1 to 4 μg of DNA, expressing Hep B surface antigen, elicited protective antibody responses in 100 % of volunteers. CMI , including CD8+ T cell responses were also detected, and the vaccine was well tolerated (Roy MJ *et. al.*, 2001). The gene gun represents a potent method of DNA vaccine delivery.

1.9.3.1.2 Other cutaneous delivery methods

Other methods of delivering non-particulated DNA formulations *via* the cutaneous route have been developed. Jet injection is a technique that uses air pressure to force liquid through the skin and around cells. The liquid follows the path of least resistance, therefore minimising tissue damage, compared with conventional injection. Jet injections have been shown to be more efficient than conventional injection for the delivery of plasmid, with the efficiency of DNA expression dependent on the force of injection. (Babuick S *et. al.*, 2000),(Anwer K *et. al.*, 1999).

Micro-fabricated microneedles have been shown to increase the permeability of human skin. These arrays of 150 μm-long needles create conduits across the SC, potentially allowing the transport of DNA into the epidermis. The needles only penetrate the SC and the viable epidermis. This removes any potentialo for pain as there is no contact with nerve endings (Henry S *et. al.*, 1998).

Electroporation is the creation of pores in the plasma membrane by the application of a pulse of electric current. This method allows the delivery of DNA into cells. Selby *et. al.* have demonstrated that DNA vaccine potency can be enhanced by iontophoresis (Selby M *et. al.*, 2000), a technique similar to electroporation, but which utilises a constant but weaker electric field.

Sonophoration and photomechanical delivery are techniques which increase the permeability of the skin to macromolecules, utilising ultrasound and laser pulse, respectively. Their use for DNA vaccination has yet to be proved (Babuick S *et. al.*, 2000).

The immunobiology of the skin makes it an attractive and potent route of vaccination and the potential to increase patient compliance and safety, and enhance immune responses to vaccines through the delivery devices gives this method of vaccination a palpable future.

1.10 Effect of DNA vaccine administration route on the immune response

Fyan *et. al.*, 1993 (Fynan EF *et. al.*, 1993) tested the intradermal (i.d., by gene gun), intranasal (i.n.), intramuscular (i.m.) and intravenous(i.v.) routes for the administration DNA vaccines in mice. It was reported that the intradermal route, using the gene gun (g.g.) was the most efficient, achieving protection with 250 - 2,500 times less DNA, compared with DNA in saline delivered by either the i.v., i.p. or i.m. route. A DNA vaccine dose of 0.4 μg delivered by the g.g. was as effective as a 200 μg dose delivered by the i.m. route and 300 μg by the i.v. and i.p. route, achieving a 95 % survival after challenge with a lethal dose. All routes of delivery resulted in low serum antibody titres, which increased rapidly after challenge. Protection occurred in mice that did not have detectable levels of antibodies prior to challenge. The authors also report that none of the routes used stimulated IgA responses. The effectiveness of the g.g. method for the administration of DNA vaccines was also shown in a subsequent study by Raz *et. al.*, (Raz E *et. al.*, 1994) and Feltquate *et. al.*(Feltquate DM *et. al.*, 1997).

The i.m. and g.g. method of delivery was also studied by Pertmer *et. al.*, (Pertmer TM *et. al.*, 1996), who characterised the profile of the immune response with respect to the serum IgG subclasses and cytokine production. Intramuscular inoculation elicited higher serum levels of IgG_{2a} than IgG_1, with the g.g. route showing the reverse. The administration of 100 μg i.m. and 1 μg by g.g. resulted in similar serum IgG levels. However, the IFN-γ production profiles by antigen-stimulated splenocytes were similar in both groups. The g.g. delivery did result in higher IL-4 production after antigen-stimulation of splenocytes, but only after the third immunisation, while the IL-4 levels of cells derived from i.m. inoculated mice were insignificant. The authors also reported that cross boosting with the alternative route had no influence on the IgG subclass ratios.

The tendency of g.g.-mediated DNA immunisation to elicit predominantly IgG_1 subclasses and for i.m. immunisation to result in either a mixed, or predominantly IgG_{2a} response could reflect significant differences in the amount of soluble antigen synthesised, following DNA delivery by these two methods. The level of antigen load could influence the role that the B-cells may play in antigen presentation and their tendency to shift the immune response to a Th-2 profile. Delivery of DNA vaccines by g.g. may lead to greater levels of antigen expression and influence the quality of the response. A 700-fold reduction in the dose of g.g. delivered DNA resulted in a 500-fold reduction in tranfection levels, but only a 3-fold reduction in the IgG titres, and the IgG_1:IgG_{2a} ratios remained steady at more than 10:1 (Pertmer TM *et. al.*, 1996). It is plausible that the direct introduction of the DNA into cells is an important factor. Boehm *et. al.*, (Boehm W *et. al.*, 1998) found that s.c. and i.m. routes were suitable for inducing a CTL response, whereas the i.p. or i.v. routes were not.

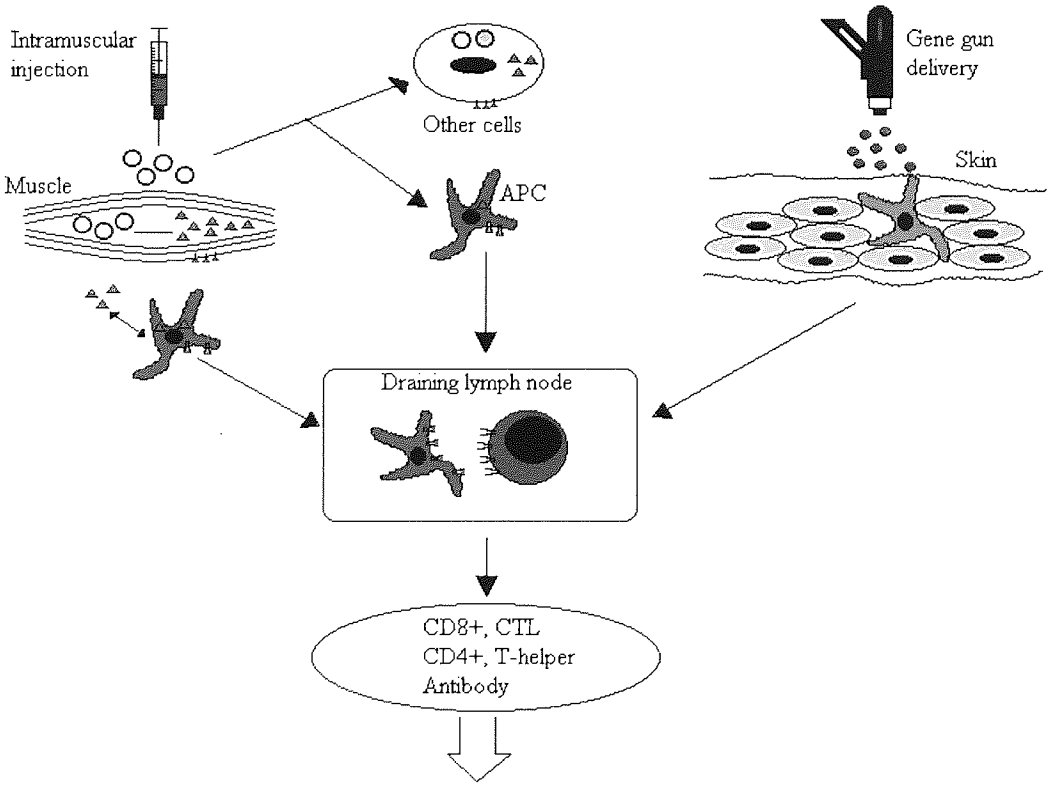


Figure 1.9: The cellular aspects of immune induction after intramuscular or subcutaneous immunisation with DNA vaccines.

Serum antibody titres were high in mice immunised by i.m. and s.c. routes, and undetectable in mice immunised by the i.v. and i.p. route.

Shroff *et. al.* administered to groups of 5 Balb/c mice, plasmid DNA vaccine encoding the glycoprotein D of Herpes Simplex Virus type 2 (pHSV-gD2), in combination with bupivacaine *via* the i.m., i.n., i.vag, i.p. and i.d. routes (Shroff KE *et. al.*, 2000). The authors reported that animals immunised by the i.d. route showed the highest level of systemic cellular response, although all routes showed strong cellular responses. All delivery routes yielded gD2 specific serum antibody, but the i.d. and i.m. routes resulted in the highest serum antibody responses. There was no quantitation of antibody subclasses. However, it was reported by the authors that both the i.m. and i.d. generated mucosal IgA. To determine if the plasmid was present in the Peyer's patches (PP) after immunisation *via* different routes, spleen, mesenteric lymph nodes (MLN), and PP were isolated and analysed for plasmid using PCR. Plasmid was detected in MLN and PP at 1 and 4 hours after immunisation by the i.m., i.d. and i.n. routes, corroborating the mucosal IgA responses reported.

Thus, the general trend from studies on the route of administration is that there is a Th-1 bias of the immune response by i.m. administration, while the mucosal, dermal and cutaeneous routes effect a mixed Th-1/Th-2 response. This has also been shown by Oliveira *et. al.*, (Oliveira SC *et. al.*, 1999), who postulated that the larger amount of DNA administered during i.m. vaccination may effect the Th-1 bias by the inherent adjuvancy of CpG motifs present in larger quantities. But Pertmer *et. al.*(Pertmer TM *et. al.*, 1996) reported that a reduction in the mass of DNA administered by i.d. route did not affect the Th-1/Th-2 bias. Further, Hasan *et. al.* (Hasan UA *et. al.*, 2000) concluded that the type of T helper cell response is not dependent on the route of administration, as immunisation of mice by a varicella zoster virus glycoprotein E DNA vaccine by the s.c. and i.m. route both resulted in strong Th-2 responses. It is possible that the type of antigen may play a crucial role in the type of immune response after DNA vaccination (Hasan UA *et. al.*, 2000). McCluskie *et. al.* (McCluskie MJ *et. al.*, 1999) showed that the observed effects of the route of DNA vaccine administration was time dependant. The authors noted that 4 weeks after the priming of mice with Hepatitis B surface antigen DNA vaccine, only IgG_1 antibodies following g.g., predominanalty IgG_1 following i.d., and mixed IgG_1/IgG_{2a} following i.m. delivery were observed. These all shifted toward a Th-1 response over time such that i.d. became mixed Th-1/Th-2 and

i.m. became Th-1-like, although they remained Th-2 like for the g.g. delivery route.

1.11 Regulatory issues regarding DNA vaccines

Whilst great potential can be envisaged for the future of DNA vaccines, it is prudent to give due consideration to the safety and potential hazards of this new technology. Several authors have alluded to and addressed this issue (Griffiths E, 1995), (Minor PD, 1995), (Mor G *et. al.*, 1997), and guidelines have been produced by regulatory bodies to aid manufacturers and clinical researchers (Zoon KC, 1996).

1.11.1 Physical properties of DNA vaccines

DNA vaccines are considered as biological products and are therefore subject to regulatory procedures for production and use. The following aspects of plasmid DNA vaccines have been highlighted in an FDA document, which gives preliminary guidance on the manufacture and preclinical evaluation of these entities (Zoon KC, 1996). Listed as a source of concern are: the origin of the DNA incorporated into the vector, including eukaryotic promoters and enhancers; termination and polyadenylation sites; antibiotic resistance markers and homology of the plasmid DNA to known sequences in the human genome. The knowledge of the human genome sequence, recently completed, will facilitate sequence homology determination. The use of β-lactamase inhibitors (e.g. ampicillin) is not encouraged, as these can stimulate severe allergic reactions in susceptible individuals (Zoon KC, 1996).

For the bulk plasmid product, the DNA content in the DNA vaccine and the presence of other cell-derived contaminants such as ribonucleic acid (RNA) and proteins should be evaluated. The ratio of absorbance of ultraviolet light at 260 and 280 nm wavelengths (A260/A280) is a useful measure of DNA purity (Manchester KL, 1996). Agarose gel electrophoresis (AGE) may be used to determine the homogeneity of size and tertiary plasmid structure. Hybridisation techniques may be employed to increase the sensitivity of AGE to contaminating RNA and protein. The use of silver stained gels will provide a sensitive detection of protein contaminants (Zoon KC, 1996).

The complete DNA sequence of each plasmid construct intended for licensure should be

determined, as any changes in the plasmid DNA vector, exclusive of the antigen gene intended for expression, generally results in the plasmid vaccine being considered a new product. However, a change in the inserted gene, with no change in the plasmid DNA vaccine vector, may be considered as a modification requiring minimal testing. So that if the same plasmid vector is used to express a series of related proteins, then the safety issues for each DNA plasmid vaccine are only those presented by the insert and its expressed product (Zoon KC, 1996).

1.11.2 Preclinical immunogenicity and safety evaluation

Generally, the design of preclinical studies should reflect the intended use of the final vaccine product; so that relevant animal models are chosen for *in vivo* studies. Preclinical studies should assess the immune response, including seroconversion rates, geometric mean antibody titres and if possible, CMI in vaccinated animals. Information on the duration of antigen expression,and whether long-term expression will result in either tolerance or autoimmunity should also be assessed. Animal challenge/protection studies with the corresponding infectious agent are encouraged early in development to demonstrate the rationale for the use of the investigational vaccine (Zoon KC, 1996).

Toxicity studies should assess local and systemic reactions, including dose-ranging and dose-escalation with consideration of the anticipated therapeutic or clinical dose. Toxic effects on potential haematopoietic and immune organs should be determined. Local site reactogenicity studies should include evaluation of injection site tissue. The distribution of the vaccine in, and its affinity for specific organs or tissues should be investigated. The duration of vaccine immunogen expression and persistence of the vector in somatic cells containing the plasmid DNA vaccine should be defined. The determination of the vaccine's immunotoxic effect should be incorporated into the study design, either by inducing tolerance to the antigen encoded or by inducing autoimmunity (Zoon KC, 1996).

Of great concern is the possible integration of plasmid DNA into the genome of the vaccinated subject. The integrated vaccine DNA sequence may result in insertional mutagenesis through the activation of oncogenes or inactivation of tumour suppressor

genes. Additionally, an integrated plasmid might result in chromosomal instability through the induction of chromosomal breaks or rearrangements. The performance of a preclinical study to assess integration should focus on the potential of the plasmid DNA to combine with endogenous host DNA sequences. The effects of either over-expression or aberrant expression of antigen should be considered. Plasmid DNA vaccines should also be evaluated for migration to gonadal tissue and possible germline alterations in both male and female animals (Zoon KC, 1996).

These guidelines are designed to ensure product safety and productivity without stifling innovation in this new field of vaccinology. Some of issues raised in the guidelines have been addressed, most notably the risk of integration of the plasmid into the host genome.

The majority of muscle tissue is composed of highly differentiated, fused and multinucleated myotubules that are considered terminally differentiated and retain no capacity to re-enter mitosis. Hence, integration of plasmid DNA into muscle genomic DNA was presumed to be an extremely rare occurance. Wolff *et. al.*, (Wolff JA *et. al.*, 1992) showed that plasmid DNA injected i.m. persisted extrachromosomally in the muscle tissue 1 year after administration. A more comprehensive approach was taken by Martin *et. al.*, who conducted, a preclinical safety study in mice to determine the structural nature of plasmid DNA sequences persisting in total muscle DNA following a single i.m. injection of a plasmid (Martin T *et. al.*, 1999). The authors found that the integration of plasmid DNA after direct injection of plasmid into muscle is a low probability event. Continued presence of plasmid in the genomic fraction after purification was consistent with covalent linkage of plasmid DNA to genomic DNA, but there was no direct evidence of this. It was concluded that the level of integration, if it did occur, would produce a theoretical mutation rate 3000 times lower than the spontaneous mutation rate (Martin T *et. al.*, 1999). Thus, plasmid integration remains a theoretical safety concern until further evidence, using more sensitive techniques, provide direct quantitation of integration rate to corroborate or refute this concern.

Other safety aspects of DNA vaccine have been addressed by Bagarazzi *et. al.* who

immunised adult, pregnant and infant chimpanzees with plasmid vaccines and found them to be safe and well tolerated in all groups (Bagarazzi ML *et. al.*, 1998). Regarding the potential to induce autoimmune disease, Mor *et. al.* examined the effect of DNA vaccines in lupus-prone B/W mice; this strain of mice was chosen because they are at high risk for the development of DNA-induced autoimmunity. Using ELISPOT assays, the authors reported an increase in the B cells secreting IgG antibodies against mammalian double-stranded DNA, but no anti-muscle cell auto-antibodies were detected. Long term studies of normal and lupus-prone mice showed that repeated administration of DNA vaccines neither induced nor accelerated myositis or systemic autoimmune disease. While the possibility remains that a subset of DNA vaccines may stimulate an autoimmune response, the authors concluded that the level of autoantibody production elicited by conventional DNA vaccines was not sufficient to induce active autoimmune disease (Mor G *et. al.*, 1997).

It is clear that as the information and understanding of the DNA vaccinology increases, the perception and requirements for safety assurance will adapt to suit the current status of knowledge.

1.12 Aims and objectives of the present study

Current vaccines consist of either the whole attenuated pathogen, or its antigenic subunit, and despite the significant contribution of these to the reduction of disease, there are limitations to their effectiveness in terms of the types of immune responses induced, patient compliance issues, (route and frequency of administration) and physical stability.

The mechanism of immune induction by DNA vaccines generates cell mediated and humoral immune responses, and this new technology holds great promise. However, the issue of dosage form is important, not only for patient compliance, but also for the control and design of the type of immune response elicited. Since its advent nearly a decade ago, there have been vast improvements and increase in the knowledge and application of DNA vaccine technology to improve immune responses to a wide range of antigens and pathogens. The majority of these studies have utilised the i.m. route for

administration of the DNA vaccines.

In order to consider other routes of administration, either a different formulation or dosage form of the DNA vaccines is required, not only to ensure stability of the DNA molecule, but to allow the control and design of the type of immune response.

Against this background, the aim of this study was the formulation DNA vaccines into biodegradable microspheric particles. In order to achieve this aim, the following main objectives were set;

- To design and fabricate microspheres containing DNA vaccines. For the vaccine formulations to be used in a clinical setting, the ability to transfer the fabrication technology to a large scale is important. This objective was set to investigate the use of more than one technology in the design of the formulation and manufacturing process.

- To characterise the fabricated microspheres with respect to their size and surface characteristics, and assess the *in vitro* biological integrity of the vaccine formulations.

2. Production of plasmid DNA and validation of activity

2.1 Introduction

For plasmid DNA to be used as the active principle in formulated microspheres, it is necessary for it to be available in bulk quantities. Many methods have been developed to purify plasmid DNA from bacteria; there are generally three major steps involved in the purification procedure. These are the growth of the bacterial culture, harvesting and lysis of the bacteria, and purification of the plasmid DNA (Sambrook J *et. al.*, 1989).

2.1.1 Bacterial growth and culture conditions for plasmid DNA purification

The growth of bacterial cells may be influenced by a number of factors; aeration and temperature, the choice of culture medium, and incubation times. Bacterial growth in liquid culture occurs in three phases; a short lag phase, a log phase characterised by exponential rate of growth, and a stationary phase in which there is no net increase in biomass. To ensure adequate aeration, it is recommended that the culture volume constitute no less that 25 % (optimally 10 %) of the total volume of the culture vessel, and the culture vessel should be shaken vigorously, at approximately 250 rpm. A temperature of 37°C is optimal for the growth of *E. coli*. (Doyle K, 1996).

Culture incubation times affect both the yield and quality of the plasmid DNA isolated. A low culture density results in low plasmid yield, while an excessively high density will reduce the plasmid purity. As a general guide, a growth time of 12-16 hours is advocated (Sambrook J *et. al.*, 1989).

2.1.2 The selection of bacterial strain

The genotype of the host bacteria in which the plasmid is to be cloned is also important. The *E. coli* strain used in this procedure was DH5α®. This strain contains mutated forms of an *end*A, and *rec*A gene. The mutated form of *rec*A prevents recombination of introduced DNA with the host DNA, ensuring the episomal stability of the inserts. The wild-type *end*A gene produces a 12 kDa periplasmic endonuclease I. Strains with the mutated form of the gene show improved stability and yield of plasmid (Doyle K,

1996).

The plasmid obtained was transformed into a DH5α® strain of *E. coli* for subsequent large scale cloning and amplification. Plasmid was harvested by alkaline lysis of the cultured cells and the structural and biological activity of the plasmid was confirmed by agarose gel electrophoresis and protein expression assays, respectively.

2.1.3 Agarose gel electrophoresis

Agarose is a linear polymer extracted from seaweed (Sambrook J *et. al.*, 1989). Agarose gels are cast by dissolving agarose in the desired buffer until a clear, transparent solution is obtained. The solution is then poured into a mould and allowed to harden. Upon hardening the agarose forms a matrix, the density of which is determined by the concentration of the agarose. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates toward the anode. The rate at which it migrates is mainly governed by:

The molecular size of the DNA: Large molecules migrate slowly because of the greater frictional drag and because they 'worm' their way through the pores of the gel less efficiently than smaller molecules.

Agarose concentration: There is a linear relationship between the logarithm of the electrophoretic mobility of the DNA (μ) and the gel concentration (τ), which is described by the equation:

$$\log \mu = \log \mu_0 - (K_r * \tau)$$

where,

μ_0 = free electrophoretic mobility of DNA

K_r = is the retardation coefficient. This is a constant related to the properties of the gel and the size and shape of the migrating molecules.

Equation 2:1: Mathematical description of the electrophoretic mobility of DNA through agarose gel (Sambrook J *et. al.*, 1989).

Table 2.1 illustrates the resolving power of different concentrations of agarose.

2.1.4 Factors affecting the migration of DNA through an agarose gel

1) Conformation of the DNA: Supercoiled (SC), nicked circular (OC), and linear (L) DNA of the same molecular mass migrate through agarose gels at different rates.

2) Applied voltage: To obtain the maximum resolution of DNA fragments greater than 2kb in size, agarose gels should be run at no more than 5V *per* cm of electric field.

3) Presence of intercalating dyes: EtBr included in the gel reduces the electrophoretic mobility of linear DNA by about 15 %. The dye intercalates between stacked base pairs, extending the length of the linear and nicked circular DNA molecules and making them more rigid.

Table 2.1: Resolving powers of different concentrations of agarose gel (Sambrook J *et. al.*, 1989).

Concentration of agarose in gel (% $^{m}/_{m}$)	**Efficient range of separation of linear DNA molecules (kb)**
0.6	1 - 20
0.9	0.5 - 7
1.5	0.2 - 3
0.3	5 - 60
0.7	0.8 - 10
1.2	0.4 - 6
2.0	0.1 - 2

3) Composition of the electrophoresis buffer: The electrophoretic mobility of the DNA is affected by the composition and ionic strength of the electrophoresis buffer. Several buffers are available for these purposes, and the most common are shown in table 2.2 .

TAE is the most commonly used buffer, but its buffering capacity is comparatively low. Both TPE and TBE have significantly higher buffering capacity. However, the resolving power of TAE for supercoiled DNA is better than TBE and TPE, which makes it more suitable for electrophoresis of plasmids.

Table 2.2: Commonly used buffers for agarose gel electrophoresis (Sambrook J *et. al.*, 1989)

Buffer	Working solution		Concentrated stock solution (*per* litre)	
Tris- acetate (TAE)	1 x	0.04M Tris-acetate 0.001M EDTA	50 x	242g Tris base 57.1 mL glacial acetic acid 100 mL EDTA 0.5M (pH8.0)
Tris-phosphate (TPE)	1 x	0.09M Tris-phosphate 0.002 M EDTA	10 x 85%	108 g Tris base 15.5 mL phosphoric acid (1.679g/mL) 40 mL EDTA 0.5M (pH 8.0)
Tris-borate (TBE)	0.5 x	0.045M Tris-borate 0.001M EDTA	5 x	54 g Tris base 27.5 g boric acid 20 mL EDTA 0.5M (pH 8.0)
Alkaline	1 x	50 mN NaOH 1mM EDTA	1x	5mL 10N NaOH 2mL EDTA 0.5M (pH 8.0)

2.2 Materials and Methods

2.2.1 Transforming of DH5α®

A single colony of *E. coli* strain DH5α® was isolated from a freshly grown non-selective nutrient agar plate for 12-16 hours. The single colony was used to inoculate a 100 mL of LB broth containing no antibiotic. The bacteria were cultured at 37°C for 3 hours with shaking at 230 cycles *per* minute.

The cells were transferred into ice-cold sterile 50-mL polypropylene tubes, stored on ice for 5 minutes, then recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. The tubes were inverted for 1 minute to drain the pellet of media, the cells were resuspended in 10 mL of ice-cold 0.1M $CaCl_2$ and recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. The last traces of fluid were drained by standing the tubes inverted for 1 minute. The cells were then resuspended in 2 mL of ice-cold 0.1M $CaCl_2$.

Using a chilled, sterile pipette tip, a 200 μL aliquot of each cell suspension of competent cells was transferred to a 1.5 mL microfuge tube, and a 10 μL aliquot of DNA (no more than 50 ng) added to each tube. The contents of the tube were swirled gently and stored on ice for 30 minutes. Competent cells with 10 μL of sterile water added in place of the DNA solution were used as a negative control. The tubes were placed in a water bath,

pre-heated to 42°C, for exactly 90 seconds, chilled on ice for 2 minutes, then immediately incubated at 37°C for 45 minutes in a water bath to allow the bacteria to recover and express the antibiotic resistance protein encoded by the plasmid.

Each sample of bacterial cell culture was then gently spread unto the surface of LB agar plates containing the 100 μg/mL ampicillin using a sterile plastic rod. After leaving at room temperature for the culture to be absorbed, the plates were incubated at 37°C for 16 hours. Colonies present on the agar were viable because of their ability to express the antibiotic resistance, a phenotype attributed to the presence of the antibiotic resistance gene on the plasmid of interest.

2.2.2 Cloning and amplification of plasmid in transformed bacteria

The respective quantities of tryptone, yeast, sodium chloride, and agar (see table 2.3) were weighed into a 50 mL culture flask, and the pH adjusted to 7 with 5N NaOH. The mixture was autoclaved at 121°C, 15 pounds *per* square inch, for 30 minutes left to cool to 60°C and ampicillin (or appropriate antibiotic marker) added to a final concentration of 100 $\mu g.mL^{-1}$. The agar was poured into sterile agar plates and left to solidify, then inoculated with the transformed *E. coli* bacteria containing the plasmid of interest. The plates were incubated at 37°C for 12 hours.

2.2.2.1 Preparation and inoculation of growth medium

Table 2.3: Composition of LB broth for the propagation of *E. coli* for plasmid cloning and harvesting.

	LB Broth			
Tryptone (g)	5	6.25	25	20
Yeast (g)	2.5	3.125	12.5	10
Sodium Chloride (g)	5	6.25	25	20
d/d Water	500	625	2500	2000

The respective quantities of tryptone, yeast and sodium chloride were dissolved, and made-up to volume with distilled water and divided equally into four, 4L-capacity culture flasks. The mixtures were autoclaved at 121°C for 30 minutes, cooled to 60°C and ampicillin added to a concentration of 100 μg/mL.

A single colony was picked from the selective agar plate and used to inoculate a 10 mL starter culture of LB broth containing the selective antibiotic which was cultured at 37°C for 8 hours. The starter culture was used to inoculate the larger LB broth by a 1 in 900 dilution, and this was grown at 37°C for 12 - 16 hours.

2.2.2.2 Extraction and purification of plasmid

The bacterial cells were harvested by centrifugation at 6000*g* for 15 minutes at 4°C, and completely resuspended in 125 mL of resuspension buffer(50mM Tris chloride, pH 8; 10mM EDTA; 100 μg/mL RNase A). A further 125 mL of lysis buffer(200mM sodium hydroxide, 1 % SDS) containing RNAse was added, and the mixture inverted several times to ensure thorough mixing. After a 5 minute incubation at room temperature 125 mL of chilled neutralisation buffer (3M potassium acetate, pH 5.5) was added and immediately mixed thoroughly, resulting in the formation of a cell lysate containing precipitated genomic DNA, proteins and cell debris which was filtered to obtain a cleared lysate containing plasmid DNA. A proprietary buffer was added to the lysate at this point to remove endotoxins, and the plasmid loaded onto an ion-exchange column, washed with 600 mL of wash buffer (1M sodium chloride; 50nM MOPS, pH 7; 15 % isopropanol), and eluted into 75 μL of elution buffer (1.25M sodium chloride; 50nM Tris chloride, pH 8.5; 15 % isopropanol). The plasmid solution thus obtained was precipitated with isopropanol and centrifuged to obtain a pellet, which was washed in 70% ethanol, air dried, and resuspended in a suitable volume of TE buffer.

2.2.3 Restriction digestion of plasmid DNA

A 1 μL sample of the DNA solution was made up to 18 μL of the restriction enzyme buffer in distilled water, and 5 μL of the Hind III enzyme added to the mixture in microfuge tube. A control experiment was run concurrently, containing no HindIII restriction enzyme. After mixing by pipetting and brief centrifugation, the microfuge tube was placed in a 37°C water bath for 2 hours. The reaction was stopped by a 2 minute incubation of the reaction tubes in a 60°C water bath and the fragments analysed by agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis using a ethidium bromide or SYBR Green ™

A 0.5 g mass of agarose was weighed into a 125 mL glass beaker, and 50 mL of electrophoresis buffer added and mixed well. The mixture was weighed, and microwaved until the agarose completely dissolved. The water lost by boiling was replaced with distilled water. After leaving to cool to about 60°C, 5 μL of EtBr solution 10 mg.mL^{-1} (or 5 μL of SYBR Green ™ stock solution) was added to the agar solution to give a final concentration of 1 μg.mL^{-1} (or 1 in 10,000 dilution of stock SYBR Green ™) The agar solution was gently poured into an electrophoresis rig and left to set with the appropriate comb. The samples were mixed with loading buffer (0.25% bromophenol blue; 0.25% xylene cyanol FF; and 15 % Ficoll type 400, Pharmacia; at 6x concentration) in water. The solidified gel was immersed in TAE buffer in an electrophoresis tank. The comb was then carefully removed to reveal the wells into which the samples were loaded. The gel was set at 80V and run at constant voltage for 60 minutes.

The DNA bands were visualised by placing the gel onto a UV transilluminator which emits electromagnetic radiation at a wavelength of 330 nm, the excitation wavelength of ethidium bromide (EtBr). The light emitted by the fluorescing DNA-EtBr complexes was captured with a UVP camera.

To analyse the crucial stages of the plasmid purification procedure, the following samples were analysed by agarose gel electrophoresis.

- cell lysate after removing cell debris and genomic DNA
- buffer after loading plasmid onto the ion-exchange column
- ion-exchange column wash buffer
- eluted DNA

Any nucleic acid present in the samples was precipitated with isopropanol, washed with 70% ethanol solution, dried and resuspended in TE (pH = 8), then analysed by gel electrophoresis as described above.

2.2.5 Validation of biological activity of plasmid by *in vitro* transfection of human embryo kidney epithelial (293) and murine macrophage (RAW) cells

An epithelial cell line derived from human embryo kidney, and a mouse -derived macrophage cell line were used to asses the biological activity of plasmid samples *in vitro*.

2.2.5.1 Human embryo kidney epithelial cell line (293) sub-culture routine

Cells were seeded at a density of 3 - 5 x 10^5 cells cm^{-2} in a 5 mL tissue culture flasks, using Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FCS (complete media). When confluent, the cells were aspirated, washed once with PBS (pH = 7.4) pre-heated to 37°C and covered with 0.5 mL of trypsin/EDTA for no more than 2 minutes. When cells were detached from the bottom of the flask 5 mL of complete medium at 37°C was added, and the cells suspended by gentle pipetting action. The cell suspension was spun at 1,000 rpm for 5 minutes and the supernatant containing trypsin/EDTA aspirated. The cell pellet was re-suspended in 3 mL of complete medium, and 1 mL of the cell suspension mixed with 5 mL of complete medium in a 5 mL tissue culture flask. The sub-cultured cells were incubated at 37°C with 5% CO_2 until confluent.

2.2.5.2 Murine macrophage (RAW) cell line sub-culture routine

Cells were seeded at a density of 3 - 5 x 10^5 cells cm^{-2} in 60-mm cell culture dishes, using DMEM supplemented with 10mM HEPES buffer and 5% heat-inactivated FCS (placed at 56°C for 30 minutes). When confluent the cells were aspirated, washed once with PBS (pH = 7.4) and covered with 3 mL of medium. Using a cell scraper the cells were physically detached from the bottom of the cell culture dish, and pipetted gently to dislodge any cell aggregates. A millilitre of the cell suspension thus obtained was added to 4 mL of fresh media in a 5 mL cell culture dish. The sub-cultured cells were incubated at 37°C with 5% CO_2 until confluent.

2.2.5.3 Transfection procedure in 293 and RAW cell line using PEI and SuperFect™

On the day before the transfection experiment the cells were seeded at a density of $1x10^5$ in the wells of a 24-well cell culture plate and incubated at 37°C in 5% CO_2. The transfection reagents used were SuperFect™ and polyethylenimine(PEI) at increasing nitrogen to phosphate (N:P) ratios of 0 (DNA only), 4, 8 and 12. To prepare the PEI-DNA complexes, a 250 $\mu g.mL^{-1}$ solution of DNA in distilled water was prepared, and a volume of PEI solution containing a mass of PEI equivalent to the required N:P ratio was added and immediately vortexed for 5 seconds. The solution was incubated at room temperature for 30 minutes to form stable complexes. SuperFect™ transfection reagent was used according to the manufacturer's instructions: one microgram (1 μg) of plasmid DNA was diluted to a total volume of 60 mL in serum-free medium (SFM), and 5 μL of the SuperFect™ added and immediately vortexed for 10 seconds. After a 10 minute incubation at room temperature, 350 μL of full media was added to the DNA:SuperFect™ complex.

The cells were aspirated and washed once with PBS pre-heated to 37°C, and the complexed DNA solutions gently placed onto the cells. The cells were incubated at 37°C and 5% CO_2 for 3 hours, and the medium containing the complexes removed and replaced with fresh medium containing 10% FCS and supplemented with antibiotic. After 24 hours at 37°C and 5% CO_2, the cells were harvested and assayed for luciferase expression.

2.2.5.4 Luciferase assay

The cells were aspirated and washed three times with PBS. A minimal volume of cell lysis solution (0.1% Triton X-100, 100mM potassium phosphate, 2mM dithiotreitol) was added to each well (50 μL *per* well of a 24-well plate), and the lysed cells scraped and transferred into a 1.5 mL microfuge tube. The tubes were briefly centrifuged to pellet the cell debris,and the cell lysate collected and immediately frozen at -70°C until analysis. To assay for the luciferase expression, 10 μL of each cell lysate was placed into the well of a 96-well black plate. The addition of 100 μL of luciferase assay reagent

(luciferin) resulted in a chemiluminescence, the intensity of which was measured for 10 seconds in a luminescence plate reader. A standard curve of luciferase concentration against the relative luminescence was produced by a serial dilution of luciferase enzyme.

2.2.6 Quantitative assay of plasmid DNA

DNA was assayed using fluorometric and photometric techniques.

2.2.6.1 Fluorometric

To quantify low concentrations of DNA solution, PicoGreen® dsDNA Quantification Reagent was used. A standard curve was produced using standard concentrations of 1000, 750, 500, 250, 125, 12.5, and 1.25 ng/mL in PBS. The reagent was prepared from a 200x concentrate by a 1:200 dilution in PBS. Using 96-well clear plates, 50 μL of the standard DNA solution was added to each well, and 50 μL of the working concentration of PicoGreen® reagent added to the DNA solutions. The samples were mixed well by shaking briefly in a microplate reader, left to incubate for 5 minutes at room temperature then measured at excitation and emission wavelengths of 480 nm and 535 nm, respectively.

2.2.6.2 Spectrophotometric

Double-stranded DNA displays an adsorption maximum at 260 nm. To assay DNA concentrations, 500 μL of the DNA solution in either TE buffer or PBS was placed into a 700 μL capacity quartz cuvette, and this was placed into a spectrophotometer, and the absorption at 240, 260 and 280 nm measured. Samples were diluted where necessary to ensure that the absorption was between 0.3 and 0.7, as recommended (Manchester KL, 1995). The concentration of the DNA solutions were then calculated by multiplying the absorption at 260 nm by 50, then by any dilution factors. The ratio of the absorptions at 260 nm and 280 nm is indicative of the purity of the DNA sample, a value between 1.8 to 2.0 being favourable (Glasel JA, 1995), (Huberman JA, 1995), (Manchester KL, 1995).

2.2.7 Radiolabelling of DNA

Ready-To -Go™ DNA labelling beads were purchased from Amersham Pharmacia Biotech and used to label DNA with alpha-^{35}S-labeled deoxycytosine triphosphate ([α-^{35}S]dCTP). The procedure followed was according to the manufacturer's protocol. Into a 1.5 mL microfuge tube was placed fifty nanograms (50 ng) of linearised plasmid DNA in 45 μL of distilled water. The aqueous DNA was denatured by placing the microfuge tube in a 100°C water bath for 3 minutes, and immediately placed on ice for 2 minutes to prevent re-annealing. A 5-μL aliquot of [α-^{35}S]dCTP was added to the denatured DNA solution and mixed thoroughly by pipetting. The mixture was added to a microfuge tube containing the Ready-To-Go™ DNA labelling beads (Amersham Pharmacia Biotech, Bucks, UK) which consist of the triphosphates of deoxyadenosine (dATP), deoxyguanosine (dGTP), deoxythymidine (dTTP), Klenow fragment, and random oligodeoxyribonucleotides and placed at 37°C for 18 to 24 hours. The random oligomers anneal to random sites on the DNA and serve as random primers for DNA synthesis by the Klenow fragment. The nucleotides present, including the [α-^{35}S]dCTP, are incorporated into the DNA. Unincorporated [α-^{35}S]dCTP was removed by centrifugation through a MicroSpin™ column (Amersham Pharmacia Biotech, Bucks, UK), and the separation of free label from labelled DNA confirmed by a denatured polyacrilamide gel electrophoresis (PAGE). All procedures involving the use of radioactivity were carried-out as dictated by the UK Ionising Radiation Regulations, 1985.

2.3 Results and discussion

2.3.1 Transforming of DH5α® with a luciferase plasmid

The presence of viable colonies on the selective agar plate is indicative of a successful transforming procedure, as the absence of the β-lactamases encoded by the plasmid vector prevents bacterial growth in the ampicillin-rich agar. A single colony was isolated and cultured for 8 hours in LB selective broth to clone the transformed bacteria and this culture was diluted into 2500 mL (1:900) of fresh selective LB broth and left shaking vigourously for 15 hours. The plasmid was then harvested as described.

2.3.2 Authentication of plasmid by agarose gel electrophoresis of native and restricted plasmid

The figure below shows the DNA bands of the purified plasmid and the corresponding linearised plasmid, by which the true size may be estimated using a comparison with the linear fragments of the Hind III ladder. The luciferase plasmid (pGL3-Control) consists of 5256 base pairs (bp), and has a Hind III restriction enzyme site at position 245 bp (see figure).

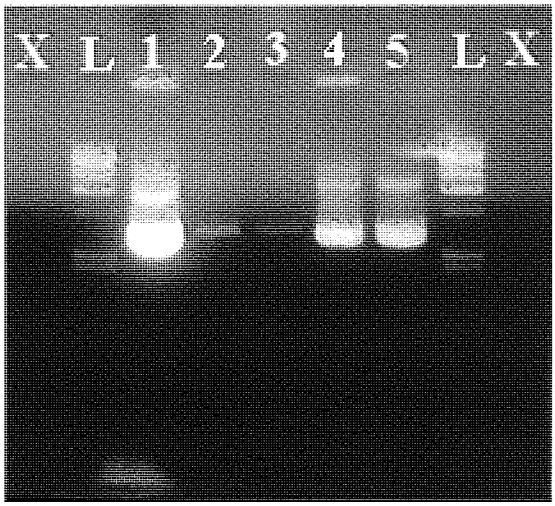


Figure 2.1: Agarose gel electrophoresis of purified plasmid DNA, pCMVluc. The gel depicts the different stages of the plasmid purification process; presence of DNA was evaluated in the cleared lysate (lane 1), the 'flow through' (lane 2), the washings of the loaded anion exchange column (lane 3), the eluted plasmid DNA (lane 4). Lane 5 is the control DNA, before cloning and amplification. L = λ/HindIII size marker. X = empty well.

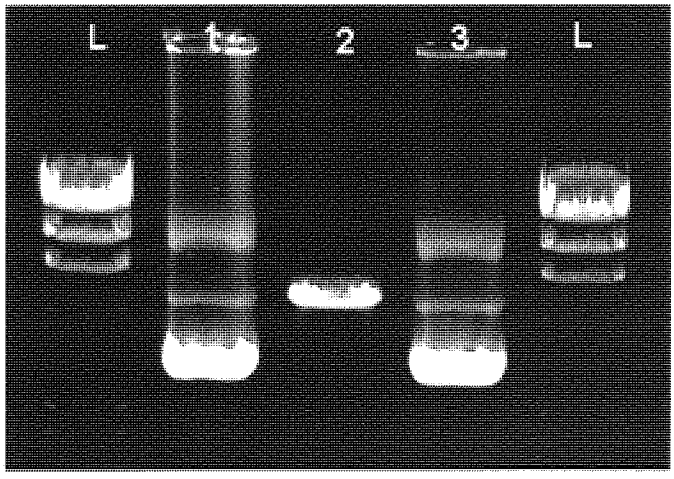


Figure 2.2: Agarose gel analysis of plasmid DNA purified from transformed *E. coli* (lane 1); and from larger scale amplification and harvesting (lane 3). The size of the plasmid relative to the ladder size markers was determined and confirmed after linearisation with a restriction enzyme, Hind III, which cuts the plasmid backbone once (lane 2). L = DNA size markers.

The bands indicate that the purified plasmid has the same linear electrophoretic mobility as that used for the transforming procedure. The unrestricted (native) supercoiled plasmid samples also show identical positions before and after transformation. This, together with the fact that a single viable colony from a selective agar plate was cloned to obtain the bacterial culture from which the plasmid was purified confirms that the plasmid obtained is that required; i.e. the luciferase plasmid.

2.3.3 Luciferase standard curve of luminescence

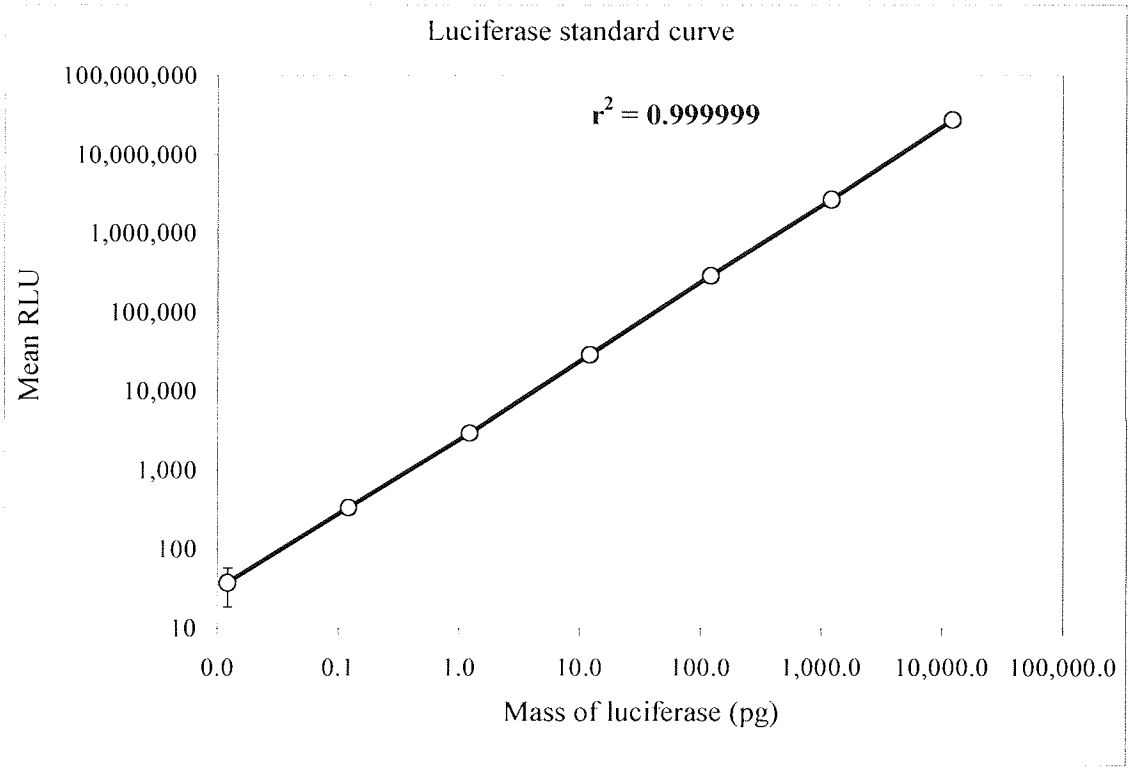


Figure 2.3: Luciferase standard curve. The range of linearity of the luminescence was determined using increasing masses of the luciferase enzyme, using a microtitre plate reader.

There was a good correlation between the mass of luciferase *per* well and the luminescence over 6 orders of magnitude ($1x10^{-14}$ to $1x10^{-8}$). From this graph the masses of luciferase expressed *per* mg of cellular protein was calculated.

2.3.4 Transfection of epithelial kidney embryo (293) cells with luciferase plasmid to validate biological activity.

To further characterise the purified plasmid, the biological activity was assessed by *in vitro* transfection. Figure 2.4 below shows the levels of luciferase expression in 293 cells 24 hours after transfection, using SuperFect as a transfection reagent. A standard curve was produced using the pure luciferase enzyme at increasing concentrations. The luciferase concentration was observed to be directly proportional to the luminescence between 10 fg and 10 ng of luciferase enzyme *per* 20 μL sample (see figure 2.3).

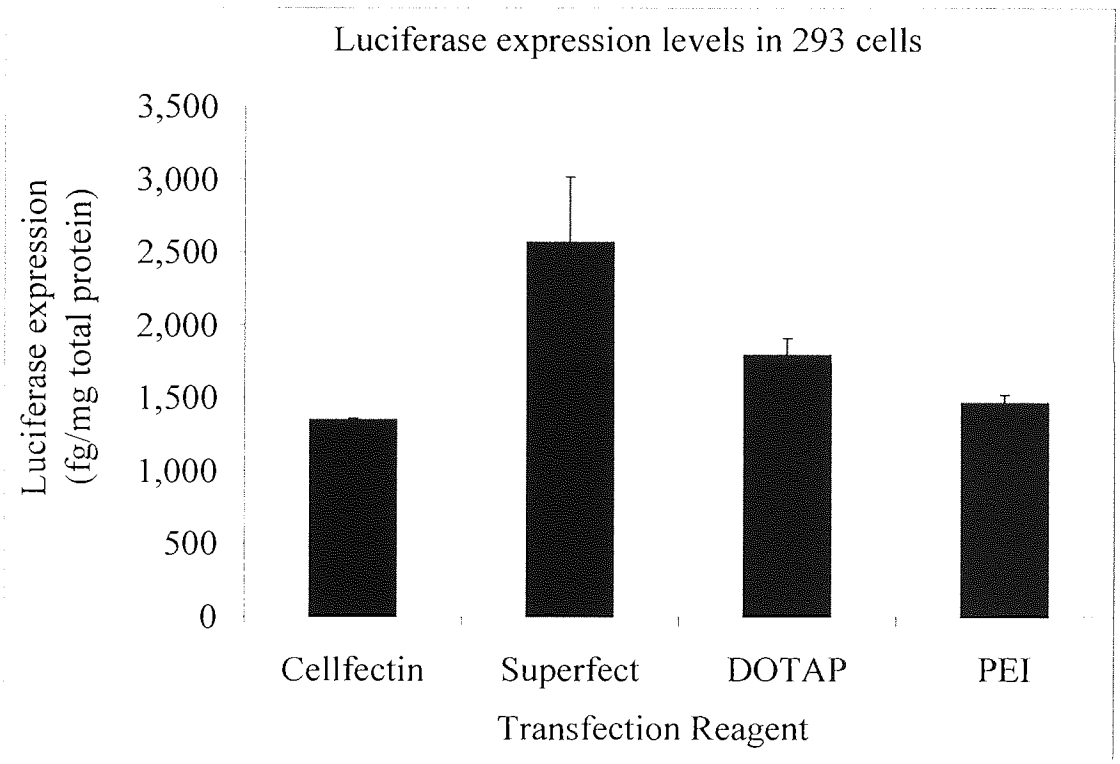


Figure 2.4: Luciferase expression by 293 cells, 24 hours post-transfection. Of all the agents used, SuperFect™ was found to be the most effective transfection reagent in 293 cells.

The results further confirm that the purified plasmid is biologically active, and encodes the luciferase enzyme. SuperFect™ was the most efficient of all the transfection reagents tested. It is intersting to note that the efficiency of trasfection using PEI was similar to that of CellFectin™.

2.3.5 Radiolabelling of plasmid DNA

The quality of radiolabelled DNA was assessed by PAGE. Figure 2.5 below shows that the free label was effectively removed from the labelled DNA, and that DNA extracted from microspheres was free of unincorporated [α-^{35}S]dCTP. This confirms that the homogenisation process did not remove the incorporated label. Hence all radioactivity detected was a direct measure of the labelled DNA and not free label.

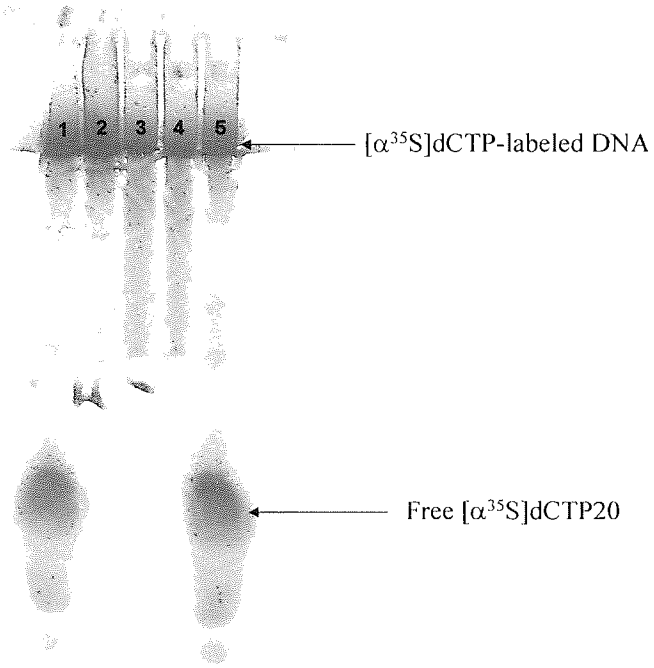


Figure 2.5: Radiograph of [α-^{35}S]dCTP -labelled DNA. The purified DNA showed no presence of free [α-^{35}S]dCTP, so that all scintillation assays were a direct measure of labelled DNA and no free label. Lane 1 and 5 are DNA samples before separation from the free label, and show the presence of the [α-^{35}S]dCTP. Lanes 2, 3 and 4 are DNA samples after separation, after homogenisation and after extraction from microspheres, respectively.

2.3.6 Quantitation of DNA samples by fluorescence and spectrophotometry

Figure 2.6 is a typical calibration curve obtained with PicoGreen® dsDNA quantitation reagent. The linearity was well within acceptable limits over the concentrations assayed. All subsequent samples of unknown DNA concentrations where diluted where necessary to obtain relative fluorescence values within the range of linearity.

2.4 Conclusions

The transforming of *E. coli* was successful and resulted in the cloning of the plasmid within the bacteria during culture. Large-scale amplification of the transformed bacteria and the harvesting procedure yielded structurally and biologically intact plasmid DNA for the further formulation, as determined by agarose gel electrophoresis and protein expression in an epithelial cell line. The method of transfection of the 293 cell line confirmed that SuperFect™ was an effective transfection reagent for the assessment of biological activity of the plasmid in that cell line.

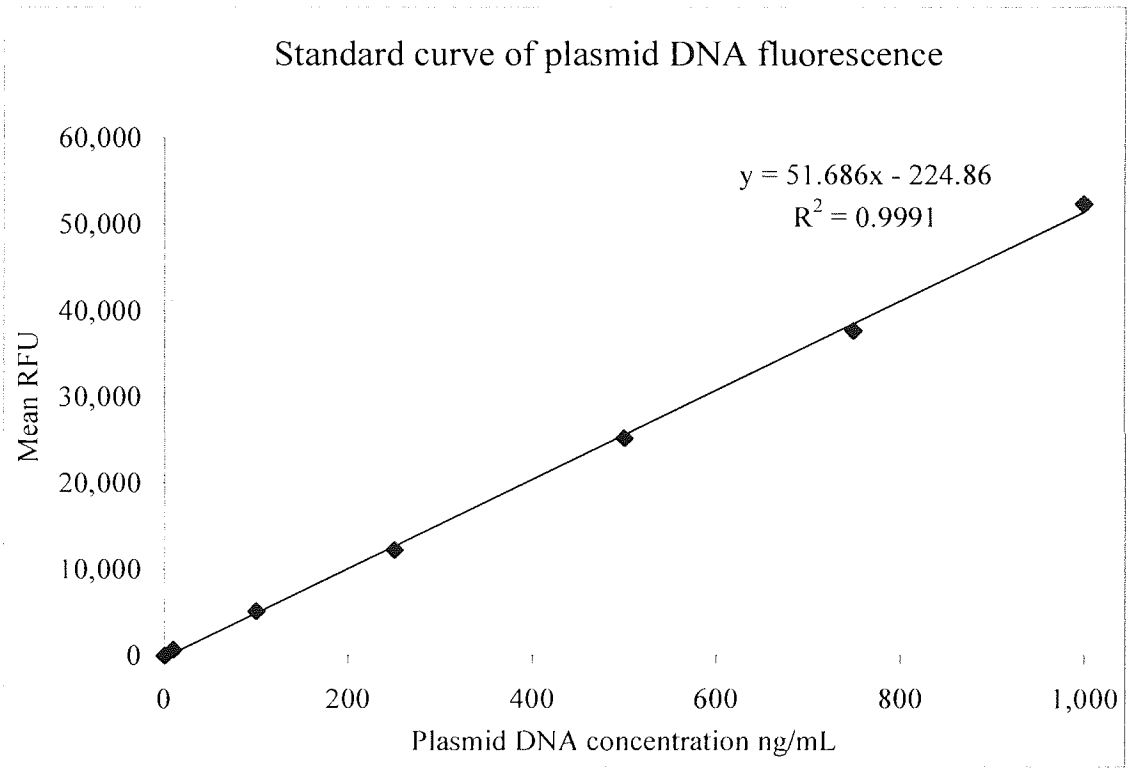


Figure 2.6: Standard curve of DNA solutions against their respective relative fluorescence units (RFU). There was a linear correlation between the DNA solutions at the specified concentrations, and the RFU.

3. Stability of plasmid DNA to homogenisation, sonication, and lyophilisation processes

3.1 Introduction

The levels of structure of DNA may be described as primary, secondary and tertiary structures. The primary structure is essentially the linear sequence of nucleotides that comprise the molecule. The secondary structure describes the polynucleotide right-handed double helix. Bacterial plasmids are primarily isolated as covalently closed circular DNA molecules in negatively supercoiled (SC) forms. Nicking of one of the strands of a supercoiled molecule results in an open circular (OC) conformation. Should the nicking occur in both strands a linear (L) form of the molecule is created. Figure 3.1 shows the different forms plasmid DNA.

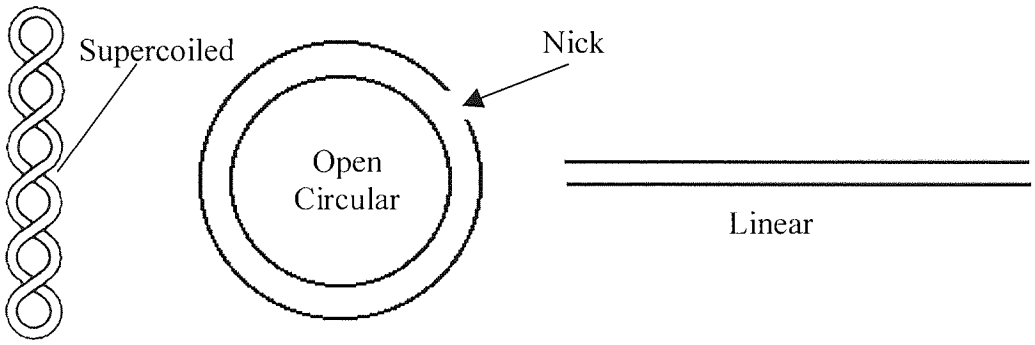


Figure 3.1: Conformations of plasmid DNA

Like any pharmaceutical product it is necessary to define the purity of the DNA in terms of any potential degradation products. The chemical degradative process of DNA has been studied (Lindahl T, 1993; Evans RK *et. al.*, 2000) as well as its physical stability (Middaugh CR *et. al.*, 1998; Levy MS *et. al.*, 1999). The question of whether the OC form of DNA should be taken as a degradation product was considered by Middaugh *et. al.* (Middaugh CR *et. al.*, 1998). It has been shown that the OC form may contain more than one nick, as the newly created OC DNA continues to accumulate nicks, apurininc sites and modified bases over time (Evans RK *et. al.*, 2000), so that the reduction in biological activity of this form may range from insignificant to complete loss. It is thus apparent that the SC form is the most stable, and that the purity of the DNA in a

formulation may be inferred both biologically and physically from the content of this isoform.

The visualisation of the different conformations of the plasmid is achieved by electrophoresis of the DNA sample through a gel which separates the isoforms by their size and shape, owing to their different electrophoretic mobilities.

3.2 Materials and methods

3.2.1 Homogenisation of DNA solution and water-in-oil emulsion

A 5.6 μg mass of plasmid DNA in 10 μL was made-up to 1 mL in sterile PBS, chloroform or a 5%$^{m}/_{v}$ polymer solution. The mixture was placed in a 7 mL glass container and homogenised at 10,000rpm for 2, 5 or 10 minutes using a Silverson SL2 homogeniser (Silverson Machines Ltd, Bucks, UK) fitted with a 10.3 mm tubular mixing unit. The resulting emulsion was placed in a 1.5 mL microfuge tube and centrifuged at 15,000 rpm for 5 minutes to separated the two phases. The upper aqueous phase was carefully pipetted and analysed for its structural integrity by agarose gel electrophoresis.

3.2.2 Sonication of DNA solution and water-oil-emulsion

A 6 μg mass of plasmid DNA in 10 μL was made-up to 1 mL in PBS, chloroform or a 5%$^{m}/_{v}$ polymer solution. The mixture was placed in a 7 mL glass container and probe sonicated for 5, 20 or 60 seconds, using a titanium alloy probe with a 9.5 mm tip diameter (MSE soniprep, Sanyo) The resulting emulsion was placed in a 1.5 mL microfuge tube and centrifuged at 15,000 rpm for 5 minutes. The structural integrity of plasmid DNA in the aqueous layer was determined by agarose gel electrophoresis.

3.2.3 Lyophilisation of DNA solution

A 6 μg mass of plasmid DNA in 10 μL was made-up to 200 μL with distilled water or sucrose solution at DNA : sucrose mass ratios of 0, 0.5, 1, 10 or 100. The solutions were placed into lyophilisation vials and frozen rapidly by placing in liquid nitrogen (-

95.8°C). The frozen samples were placed onto the stainless shelves of a freeze-drier (VirTis®, UK), preset at a temperature of -20°C. The freeze-drying chamber was evacuated of air to achieve of pressure of less than 200 mTorr and the samples lyophilised for 72 hours at a condenser temperature of -40°C or less. The dried samples were re-hydrated by addition of distilled water and the structural integrity of the plasmid DNA assessed by agarose gel electrophoresis.

3.2.4 Ethanol precipitation of plasmid DNA samples

A volume of 3M sodium acetate, pH 5 (Sigma UK) was diluted in the aqueous plasmid DNA sample to a concentration of 0.3M, in a microfuge tube. A volume of pre-chilled absolute ethanol (molecular biology grade; BDH Biochemicals, Poole, UK) equalling twice that of the DNA solution was added, and the mixture was vortexed and placed at -20°C for 20 min for the DNA to precipitate. The mixture was then centrifuged for 15 minutes at 15,000 rpm to pellet the DNA precipitate, and the ethanolic supernatant carefully removed by aspiration. The pellet was washed by resuspension in 500 μL of ethanol 70 $^{v}/_{v}$%. The DNA was pelleted by centrifugation and aspirated as before. The pellet was air dried for 20 minutes, resuspended in a small volume of TE buffer (pH 8),and analysed by agarose gel electrophoresis.

3.2.5 Agarose gel electrophoresis

A 0.5 g mass of agarose powder (Gibco BRL, Paisely, UK) was weighed into a beaker, and 50 mL of 1x TAE was added to form a 1%$^{m}/_{v}$ suspension, which was weighed, then heated in a microwave until completely dissolved. The loss of water by evaporation from the solution was replaced with TAE by weight and the solution was left to cool. When the temperature of the agarose solution was less than 60°C (gel is still a liquid solution), 5 μL of EtBr 10 mg/mL was added and mixed thoroughly. The agarose solution was poured into a gel mount to set in the presence of a comb to form the wells. On setting, the solidified gel was covered with TAE buffer, the comb removed and the wells loaded with the DNA sample in loading buffer. The voltmeter was set at 80 volts, providing an electric field of 5 V/cm, and run at constant voltage for 60 minutes.

3.2.6 Quantification of optical density of DNA bands on agarose gel.

The digital image of the agarose gel was analysed by using the Scion Image™ software (Scion Corporation Maryland, USA), and the optical density of each band determined. An example of the analysis is displayed in appendix (figure 11.1).

3.3 Results and discussion

The conformational stability of the plasmids after homogenisation, sonication and lyophilisation was determined by viewing on agarose gel. The portion of the total plasmid mass in the open circular (OC) and supercoiled (SC) conformation was determined, where applicable, by quantifying the density of the DNA bands visualised after EtBr fluorescence, using the Scion™ Image software package (Scion Image, Maryland, USA).

3.3.1 Quantitation of DNA band densities

The processed DNA samples were analysed by agarose electrophoresis and the gel image digitally captured using the UVP™ Gel documentation system. This image was analysed by Scion™ Image Software, which converted the band intensities into peak areas, and these were compared to determine the amount of SC DNA as a percentage of total DNA. Figure 3.2 shows the linearity exhibited by this method. Known masses of the plasmid DNA were analysed as described and the peak areas of the band intensities plotted against the masses of DNA.

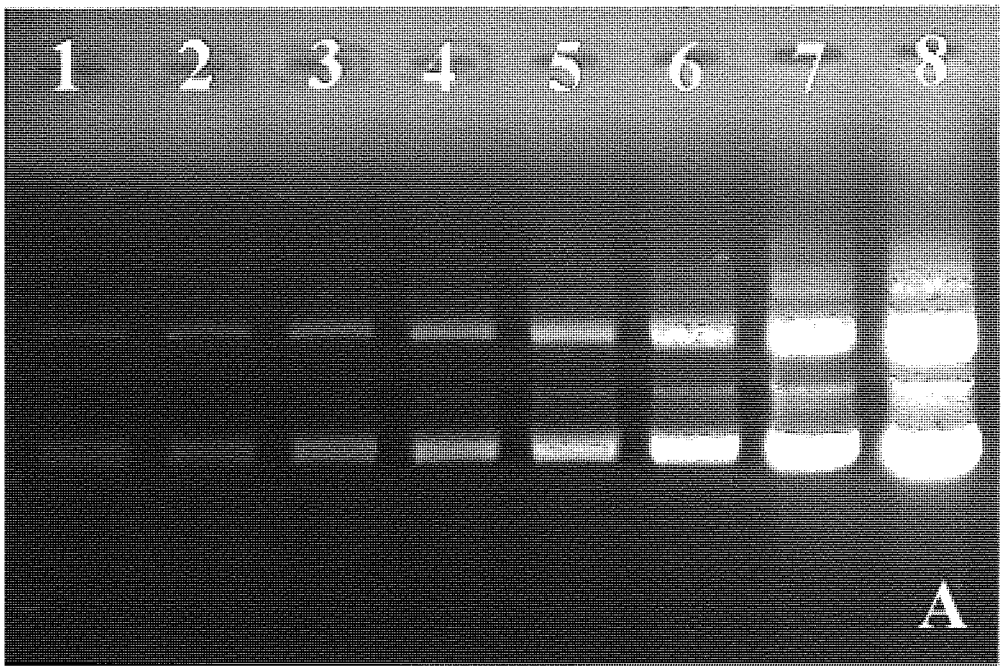
3.3.2 Effect of homogenisation of plasmid DNA in aqueous and organic solution on the plasmid conformational integrity

Figure 3.3 illustrates the effect of homogenisation of plasmid DNA on its conformational stability. The plasmid solutions were homogenised at high speed for 2, 5 and 10 minutes. It can be seen that homogenisation generally caused a reduction in the SC content of the DNA, however there was no further decrease in the proportion of SC plasmid with homogenisation between 2 to 10 minutes. Homogenising the plasmid in PBS for 2 minutes at high speed caused a large reduction in the percentage of SC in the plasmid mass (from ~85 % to less than 10 %). Emulsifying the plasmid PBS solution in

chloroform reduced the reversion of the SC conformation, effecting a 50 % reduction in the SC form. Addition of stearylamine to the disperse organic phase had the same effect on the plasmid stability as the $CHCl_3$ alone.

The homogenisation of the DNA in either solution or emulsion resulted in a significant reduction of the SC content, with the aqueous solution displaying a 4 fold reduction compared with the emulsion. Levy *et. al.,* (Levy MS *et. al.*, 1999) investigated the shear damage to the SC form of plasmid DNA of the size range 13 to 29 kb. The plasmids were subjected to controlled shear, and it was found that a 29 kb plasmid lost 100% of its SC after 20 seconds of shear, while a 13 kb retained 100%.

Figure 3.3 indicates that by 2 minutes most of the damage has occurred, and further homogenisation results in no further damage, possibly because of the small concentration and size of the remaining SC DNA. This is consistent with the findings of Levy *et. al.,* (Levy MS *et. al.*, 1999). Inclusion of an organic disperse phase protected the SC DNA, resulting in less damage within the first 2 minutes. The enclosure of the aqueous phase within the core of the organic disperse phase could explain this finding, with the organic phase absorbing the shear forces. The initial damage probably occurred prior to the formation of the protective emulsion, when the DNA solution was in direct contact with the shear forces due to homogenisation. the presence of a gas-liquid interface during the homogenisation might also be responsible for the observed damage. The samples were not homogenised in an air-tight environment, and it has been reported that the presence of an air-liquid interface has a marked effect on the damage caused by shear (Levy MS *et. al.*, 1999). This explains the marked damage seen in the aqueous DNA samples, and the protection by the water-in-oil emulsion, where the aqueous phase was effectively in an air-tight enclosure.



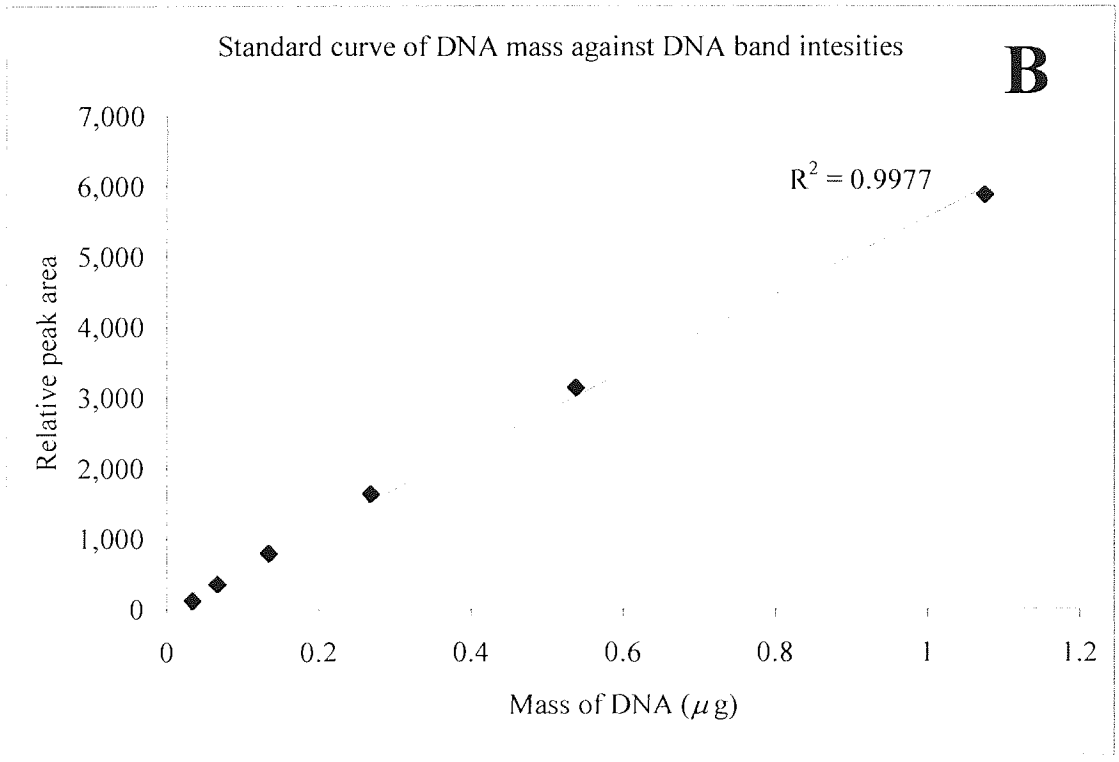


Figure 3.2: Limit of linearity of the DNA band intensities after analyses by Scion™ Image software. Figure A is a sample of the agarose gel from which the optical densities were determined. Lane 1 = 0.034 μg; lane 2 = 0.067 μg; lane 3 = 0.134 μg; lane 4 = 0.268μg; lane 5 = 0.538 μg; lane 6 = 1.075 μg; lane 7 = 2.15 μg; lane 8 = 4.3 μg. The measured optical densities of the bands had a linear correlation with the mass of DNA up to about 1 μg (lane 6), as shown by the standard curve in figure b.

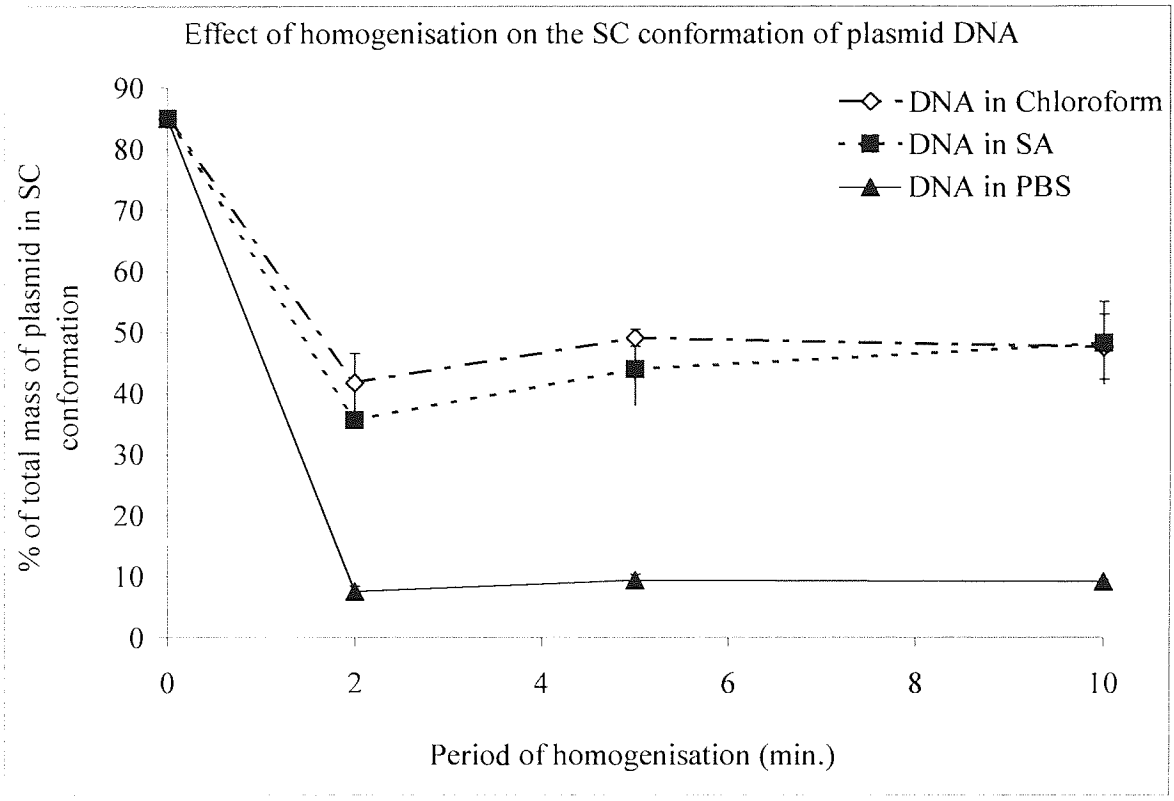


Figure 3.3: Conformational stability of plasmid DNA after high-speed homogenisation for 2, 5 and 10 minutes in PBS, or as the aqueous phase in a water-in-oil emulsion. The disperse phase of the emulsion was a chloroform solution of stearylamine or chloroform on its own. Generally, increasing the homogenisation time had no detrimental effect on the DNA conformation. Emulsifying the aqueous plasmid solution protected the DNA form the effects of homogenisation. All experiments were performed in triplicate, and the error bars represent the standard error of mean.

3.3.3 Effect of sonication of plasmid DNA in aqueous and organic solution on the plasmid conformational integrity

PBS solutions containing plasmid were sonicated for 5, 20 and 60 seconds. This was repeated for emulsified PBS plasmid solutions, the disperse phase consisted of chloroform or chloroform with stearylamine. The aqueous phase was then obtained and electrophorised on a $1\%^{m}/_{v}$ agarose gel with EtBr incorporated. The DNA bands were visualised by UV transillumination at 320 nm and the image digitally captured with GrabIt® software. Figure 3.4 shows the DNA bands after sonication.

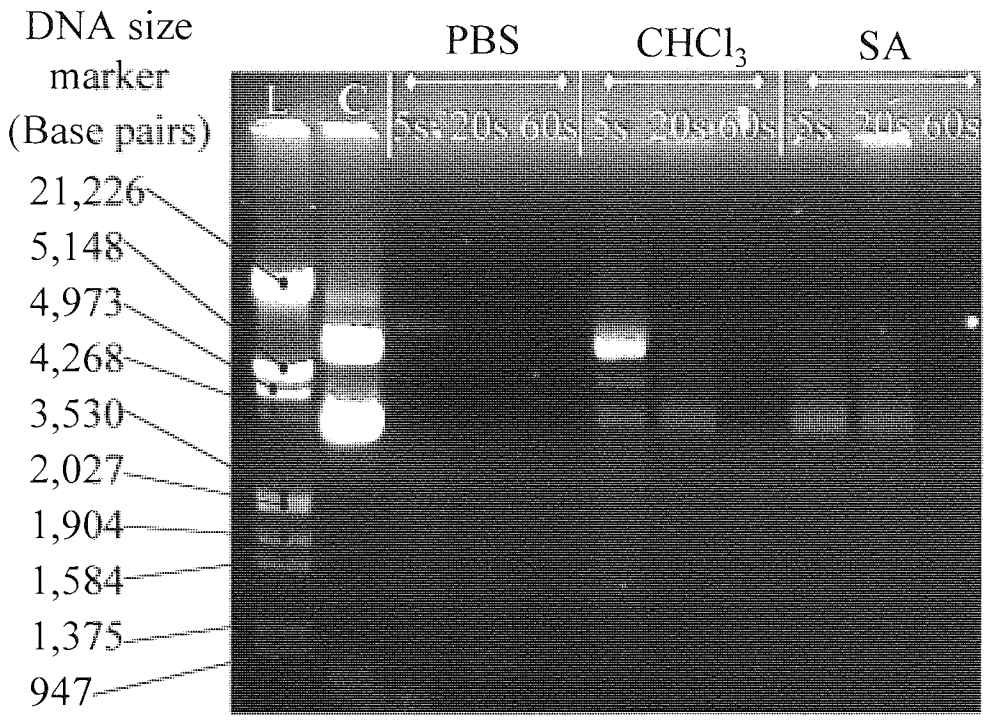


Figure 3.4: Plasmid DNA after sonication in different media, electrophorised on a 1% agarose gel and stained with EtBr. The gel is labelled with the medium in which sonication occurred (PBS, $CHCl_3$, and an organic stearylamine solution), and the period for which they were sonicated (5, 20 and 60 seconds). Samples were in duplicate. C = control DNA , and L = l/HindIII + EcoRI size marker.

Plasmid DNA was sonicated to study the effect of this process on its stability. The gel picture shows that 5 seconds of sonication was detrimental to DNA stability, regardless of the medium. The sample in PBS alone showed the most degradation after 5 seconds, and after 20s there was no DNA visible. There appeared to be a retention of the supercoiled form of the plasmid when sonication occurred in the water-in-oil emulsions with chloroform or an organic solution of stearylamine as the disperse phase. The degradation also appears to be time dependent, as the loss of the DNA bands were progressive with time in all media. The OC and any linear form originally present is first to disappear, and the SC conformation displays a level of resistance to the sonicative degradation.

3.3.4 Effect of lyophilisation of plasmid DNA in aqueous and organic solution on the plasmid conformational integrity

Figure 3.5 is a graphical representation of the retention of SC conformation after lyophilisation for 72 hours. The SC conformation was determined by the quantification of the density of bands after electrophoresis and digital image capture.

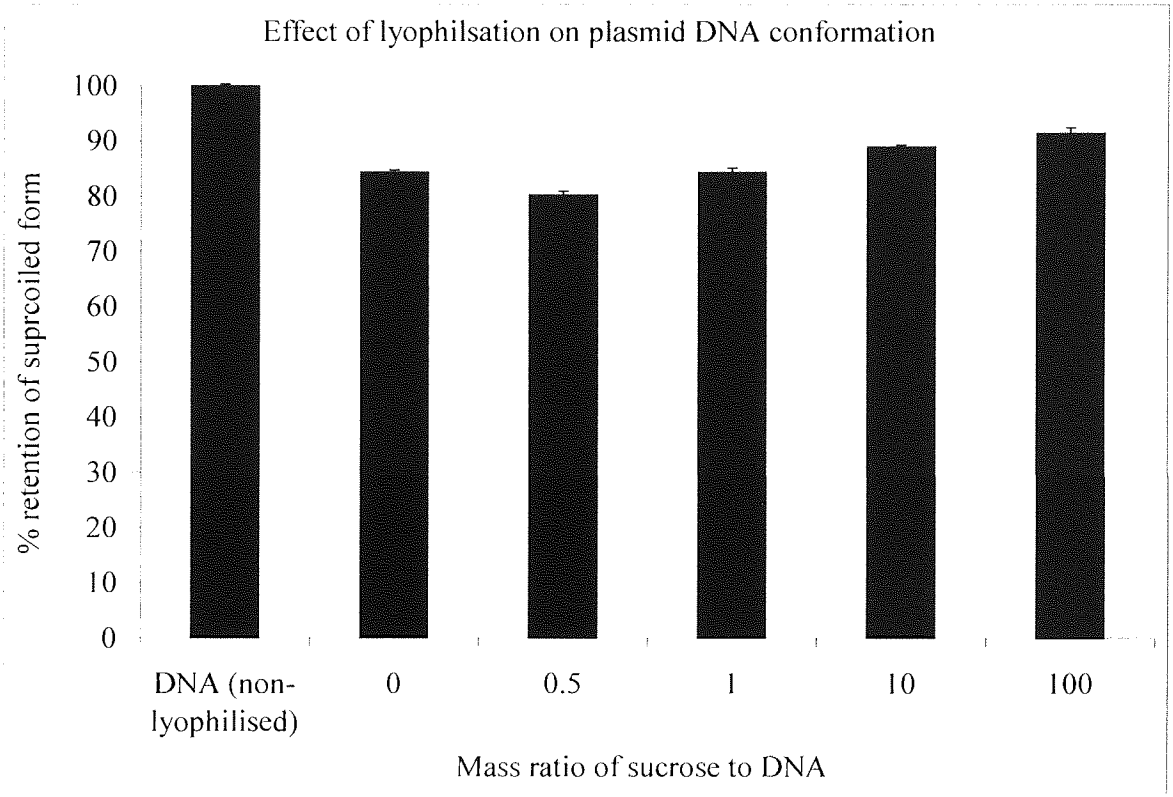


Figure 3.5: The conformational stability of plasmid DNA after 72 hours lyophilisation as measured by quantification of the band intensity after gel electrophoresis. There is a 10 to 20% reduction in the SC content of the plasmid after lyophilising for 72 hours, compared with the unprocessed DNA. Experiments were run in duplicate.

The quantified content of SC plasmid in each of the samples after lyophilisation is shown graphically in figure 3.5. There is a trend of increased stabilisation with increasing sucrose, however this is only slight, as a 100 fold increase in the ratio of sucrose results in less than 10% increase in SC content after lyophilisation. Generally, there was a 20% drop in the SC content of the plasmid compared with the non-lyophilised sample, and this increased to an 8% drop on the inclusion of sucrose as a

cyoprotectant at mass ratio of 100.

The microencapsulation process involves the creation of a primary water-in-oil emulsion by homogenisation or sonication. The stability of the DNA to these processes was investigated to ascertain which was more suitable for the microencapsulation process. Figure 3.4 (page 113) is a picture of the sonicated samples after agarose gel electrophoresis. This process was severely damaging to the DNA, with the PBS displaying no protection from damage after 5 seconds, compared to the samples in emulsion. Curiously, the loss of DNA observed appears to begin with the linear and OC forms, with the SC DNA still present alone in progressively reducing quantities as the sonication times increase. This suggests an inherent stability of the SC DNA to this type of degradative force. The emulsion conveyed some protection to the aqueous phase, presumably because of the airtight conditions with which the protection from shear damage during homogenisation is credited.

Lyophilisation is the process of removing water molecules form a solid, frozen sample, under vaccum. It was prudent to investigate whether this process was detrimental to the SC content of plasmid DNA. Desiccation of biological material is potentially harmful to its structure and activity, and the incorporation of cryoprotectants has been shown to be beneficial (Anchordoquy TJ *et. al.*, 2001). The tertiary structure of DNA is maintained in part by hydrogen bonds provided by water molecules and the effect of removing this entity was investigated. Figure 3.5 describes the results obtained. There was slight damage to the SC DNA by the process, which appears to be reduced by the inclusion of sucrose as a lyoprotectant. It is thought that disaccharides act as lyoprotectants by replacing the water molecules lost, so that the desiccated end-product has a level of protective hydration.

3.4 Conclusions

The major processes of the microencapsulation process investigated appear to have at least some degradative effect on the DNA structure, as measured by the reduction in the SC DNA content of plasmid samples. Sonication was the most damaging process, and can be replaced by homogenisation to produce the required primary water-in-oil

emulsion. Homogenisation was harmful to the DNA, but less so compared with sonication and the fact that the emulsion protected the DNA in the aqueous core of the primary emulsion makes it the process of choice for generation of the emulsion. Lyophilisation effected a slight reduction in the SC DNA, and this may be reduced by the inclusion of lyoprotectants.

4. Microspheric formulation of plasmid DNA polyplexes

4.1 Introduction

The double emulsion method of microsphere formation was first described by Ogawa *et. al.,* for the encapsulation of leuprolide acetate (Ogawa Y *et. al.*, 1988). It has since been used for the encapsulation of water soluble molecules, including peptides and proteins, and more recently, plasmid DNA (Ando S *et. al.*, 1999; Capan Y *et. al.*, 1999; Hsu Y-Y *et. al.*, 1999; Lunsford L *et. al.*, 2000; Wang D *et. al.*, 1999). Formulation of DNA vaccines into microspheres is desirable because the particulate nature enhances uptake by APCs, leading to strong cell-mediated immunity and Th-1 profiles required for responses against intracellular pathogens. This particulate nature also enhances responses by the mucosal and intradermal route, again, primarily due to increased uptake by APCs. In this study the microencapsulation of plasmid DNA was investigated; plasmid DNA was also complexed with polyethylenimine (PEI) prior to encapsulation.

4.2 Polyethylenimine

The polyethylenimines are synthesised by ring-opening polymerisation of ethylenimine *via* a nucleophillic addition mechanism, resulting in a molecules with a high positive charge density. Every third atom is an amino nitrogen that can be protonated, so that PEI retains a substantial buffering capacity at virtually any pH. The degree of protonation depends on the pH value and salt concentration (Chang DR, 1986), (Li D *et. al.*, 1998). These molecules are normally used as speciality cationic polyelectrolytes in papermaking and water purification industries as flocculating agents (Li D *et. al.*, 1998). However, Boussif *et. al.*(Boussif O *et. al.*, 1995) demonstrated its versatility as a vector for gene transfer. It was postulated that the high charge ratio of the molecule will allow a high degree of DNA binding, and the reservoir of protonatable amines at physiological pH may offer an endosome buffering capacity. It has since been used in several transfection strategies as an effective gene delivery agent (Zanta MA *et. al.*, 1997; Baker A *et. al.*, 1997; Bandyopadhyay P *et. al.*, 1998; Coll J-L *et. al.*, 1999; Klemm AR *et. al.*, 1998). The structure of PEI is illustrated in figure 4.1 below:

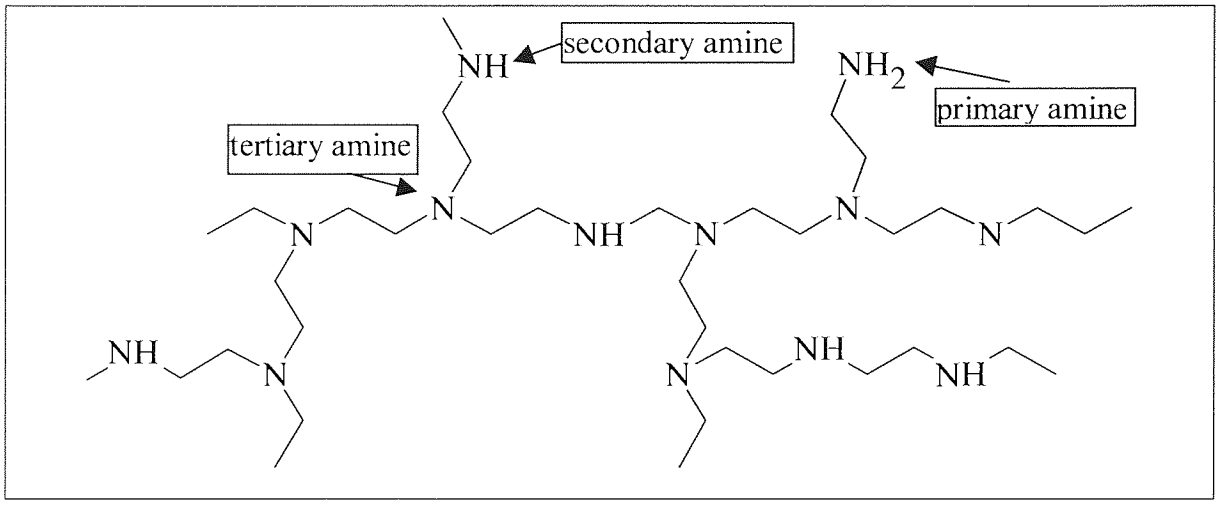


Figure 4.1: Structure of PEI showing the range of primary, secondary and tertiary amines (Kabanov AV *et. al.*, 1998).

In this study plasmid DNA was complexed with PEI and encapsulated. The DNA-PEI microparticulated complexes were characterised with respect to their size and surface charge. The inclusion of PEI in the microspheres was postulated to increase the encapsulation efficiency and ultimately provide increased endosomal buffering capacity so as to increase the transfection capability of the encapsulated plasmid DNA.

4.3 Materials and methods

4.3.1 Microencapsulation of plasmid DNA

A 5 $\%^{m}/_{v}$ solution of either PLA_{2kDa} (L104, Boehringer Ingelheim, Germany) or PLGA 50:50, Mw 63,000 (Alkermes Polymers, USA) was made-up by dissolving 250mg of either PLA_{2kDa} or PLGA into 5 mL of $CHCl_3$ or DCM. For rhodamine labelling, 100 μL of a 1 $\%^{m}/_{v}$ aqueous solution was added at this stage. The solution was placed into a 10 mL glass beaker and homogenised with a solution of plasmid and $PVA_{13\text{-}23kDa}$ for 2 minutes to form a single water-in-oil-emulsion. The emulsion was homogenised in 50 mL of a 5 $\%^{m}/_{v}$ $PVA_{13\text{-}23kDa}$ solution for 5 minutes. The resulting double emulsion was stirred gently for 3 to 4 hours, until the organic solvent evaporated and the microspheres were dried. The microspheres were harvested and washed by centrifuging the suspension at 20,000 rpm for 30 minutes and re-suspending in distilled water. This procedure was repeated 3 times, and the pellet resuspended in 1 mL of distilled water, placed into a glass vial and frozen rapidly in liquid nitrogen, then lyophilised for 48 to

72 hours to obtain a dry white powder.

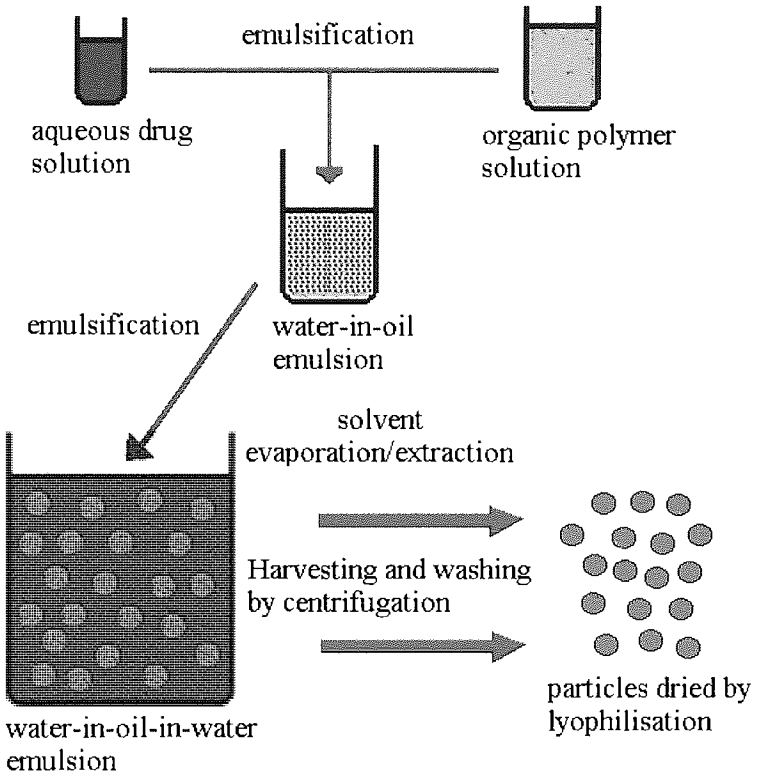


Figure 4.2: Diagrammatic representation of the microencapsulation process by the double emulsion solvent evaporation method, as originally described by Ogawa *et. al.*, (Ogawa Y *et. al.*, 1988). The resulting microspheres were harvested by centrifugation, and washed three times with double-distilled water and lyophilised to obtain a dry powder.

4.3.2 Complexation of DNA with polyethylenimine

A stock solution of 1 $\%^{m}/_{v}$ PEI adjusted to pH 7.4 with HCl was prepared in double-distilled water and filtered through a 0.2 μm filter. This stock was stored at 4°C. The ratio of plasmid DNA to PEI was expressed as the amine nitrogen to DNA phosphate ratio (N:P ratio). The optimal N:P ratio was determined by picogreen fluorescence quenching assays and confirmed by agarose gel electrophoresis.

To form complexes for encapsulation, a volume of the stock PEI solution containing a mass equivalent to an N:P ratio of 8 was slowly added to the aqueous DNA with vortexing, and the complexes incubated at room temperature for 30 minutes. The DNA: PEI complexes were then microencapsulated as described.

4.3.3 Determination of the optimal N:P ratio of DNA:PEI complexes

4.3.3.1 Picogreen fluorescence quenching assays

DNA-PEI complexes of N:P ratios 0, 2, 4, 8 and 10 were made-up in double-distilled water. Equal volumes of a 20 mg/mL poly-DL-aspartic acid (PAA) solution was added to separate aliquots of the complexes and incubated at 4°C overnight. The samples were then placed into the wells of a clear 96 well microtitre plate in 50 μL aliquots, and 50 μL of picogreen dye added to each sample, as described in section 2.2.6.1 to determine the concentrations of uncomplexed DNA, compared with an uncomplexed control sample.

4.3.3.2 Agarose gel electrophoresis of complexes

The DNA-PEI complexes of N:P ratios 0, 2, 4, 8, and 10 with and without PAA were loaded into the wells of a 1 $\%^{m}/_{m}$ agarose gel cast with EtBr and run at 80V for 1 hour.

4.3.3.3 *In vitro* cell transfection of RAW and 293 cell lines

RAW and 293 cells were plated in separate wells of a 24 well plate and cultured to ~80 % confluency as described in section 2.2.5.1 and 2.2.5.2. The cells were transfected with PEI at N:P ratios of 0, 2, 6, 8, and 10 and the transfection efficiencies determined by assaying for luciferase expression as described in section 2.2.5.4.

4.3.4 Characterisation of microspheres

The microsphere formulations obtained as a dry white powder were characterised with respect to their surface morphology, size distribution and surface charge (zeta potential). The structural integrity of the encapsulated DNA was also assessed by agarose gel electrophoresis.

4.3.4.1 Size distribution of microspheres by laser diffraction

The size distributions of all particles were determined by laser diffraction, the principle of which will be briefly discussed.

4.3.4.1.1 Theory of light diffraction by particles

Small particles illuminated by a beam of light scatter the beam in all directions. The intensity of the scatter in any particular direction is calculable using a number of approximate theories, and depends on the size of the particles and their optical properties. This scatter is referred to as the diffraction of light by the particles. Since the light beam illuminates many particles simultaneously, the end result is a distribution of the particles sizes, and cannot provide an accurate number or amount of particles. It should also be noted that the complexity of the calculations requires the use of assumptions and simplifications to arrive at a size distribution from the diffracted laser pattern, so that the use of different algorithms and assumptions leads to different distribution data for the same sample (Washington C, 1992).

The most complete theory describing the angular distribution of light scattered from a particle of arbitrary size is referred to as the Mie theory, and can only be used in absolute terms for particles of simple shape such as spheres, spheroids and infinitely long cylinders. The data output obtained consequently assumes that particles are spheres, and provides the corresponding equivalent diameters (Washington C, 1992).

To simplify data analysis from scattering experiments a number of approximate light scattering models are available, including Rayleigh, Rayleigh-Gans-Debye (RGD), Fraunhofer and anomalous scattering.

Rayleigh treats the particles as points, equivalent to extremely small particles into which light does not penetrate. This leads to a very simple scattering pattern identical to that obtained from Mie theory for particles of very small diameter. The Rayleigh theory suggests that the scattering pattern and intesity from very small particles will be independent of particle shape, since the particles are treated as point scatterers.

RGD scattering extends the Rayleigh theory, making the assumption that the particle refractive index is close to that of the medium in which it is suspended. This allows the scattering pattern to be calculated for particles slightly larger than those for which the

Rayleigh theory is applicable. RGD also predicts that the diffraction pattern is independent of the particle shape.

Fraunhofer scattering is a simple diffraction theory which allows the scattering patterns to be derived for large particles without reference to the optical properties of the material. The theory assumes that light that has penetrated the particle does not contribute to the scattering pattern, or alternatively that the particles are completely opaque, thus, the scattering can be described as though the particles were a collection of opaque disks. Consequently it is increasingly in error for smaller particles, but is useful for rapid calculations of the scattering from particles of several micrometers and larger.

The term anomalous scattering is normally used to describe effects due to light which has penetrated the particle. It therefore requires a knowledge of the optical properties of the particle material. This model is considered as a modified Fraunhofer theory which requires the relative refractive index to be supplied, and hence can analyse data from particles smaller than those measurement by Fraunhofer methods alone. It does not, however, cover the whole range of sizes that can be examined by the Mie theory, and generates increasing errors for particles of diameter near to, or smaller than the wavelength of light.

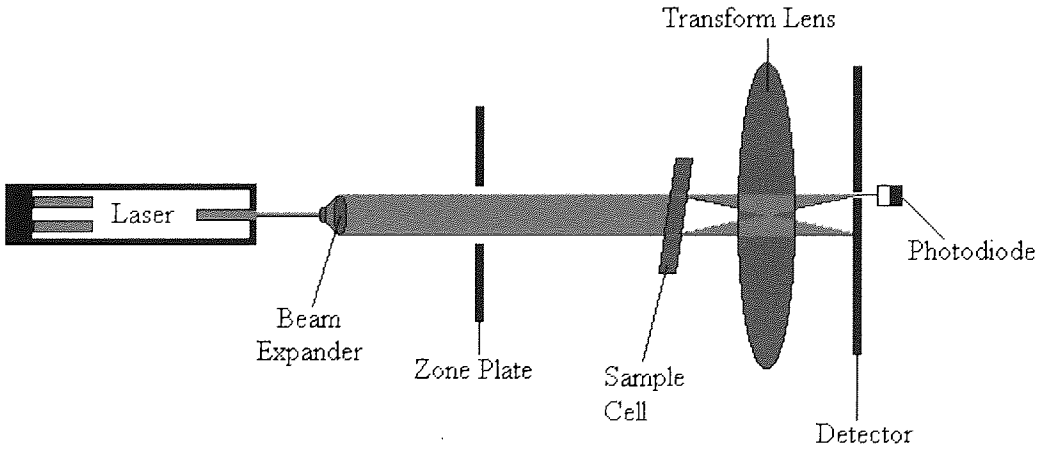


Figure 4.3: Schematic of operation of laser diffraction particle sizer (Washington C, 1992).

The operation of the Malvern Mastersizer *X* is illustrated in figure 4.3. A helium-neon lamp provides the laser. The beam diameter of is normally about 1 mm, so a beam

expander is used to produce a uniform parallel beam of 5 - 10 mm, to allow a useful sample volume to be illuminated. The zone plate allows through only the central intensity maximum of the laser beam, removing unwanted scatter diffraction due to the laser optics. The expanded beam then passes through the sample in the cell. The cell is mounted at a slight angle to the beam, so that reflections from the cell windows are not returned to the laser to be multiply reflected. The transform lens takes in scattered light and produces an output image in which each scattering angle corresponds to an annular ring in the detector plate. Consequently the signal at a particular position in the detector corresponds to the intensity of light that has been scattered through a particular angle, regardless of the position of the scattering particle in the suspension. The resulting scatter pattern is then presented as an approximation to the true size distribution, using algorithms based on the chosen scattering model.

In this study the Mie theory alogorithm was used. The refractive indices of the scatter medium (water) was set at 1.3300, and that of PLGA 50:50, 1.4620 (Chambers M, 2000)

4.3.4.1.2 Interpretation of laser diffraction results

4.3.4.1.2.1 Equivalent spheres and diameters

The use of a single parameter to describe the size of a particle is not practical, as particles may have irregular shapes. The problem is solved by quoting the particle size of a non-spherical particle as the diameter of a sphere which is equivalent to the particle. This is termed the equivalent diameter of the particle. The equivalence of the sphere to the particle may be in terms of volume, in which case the volume equivalent sphere is the sphere which has the same volume as the irregular particle, and is characterised by the volume equivalent diameter. There are a number of ways of defining equivalent, including surface, projected area and Stokes' equivalent diameter (Allen T, 1997).

4.3.4.1.2.2 Measure of central tendency

The mean, median and mode are measures of central tendency of the range of particle sizes. The mode is the most commonly occurring value in a distribution, and is the peak

of the distribution curve. The median divides the distribution into two equal parts, so that 50 % of the particles are larger, and 50 % are smaller than the median. The mean is the centre of gravity of the distribution. The fundamental size distribution derived by the laser diffraction method is volume based. This means that when the result lists, for example 11% of the distribution in the size category 6.97-7.75 μm this means that the total volume of all particles with diameters in this range represents 11% of the total volume of all particles in the distribution. From this, the mean diameters in terms of the number, surface area and length, or ratios of these may be derived.

The distribution data obtained from the laser diffraction gives a range of particle sizes spread about the mean. If the particles in a sample are all of the same size, it is said to be monodisperse, while if a range of particle sizes exists, it is said to be polydisperse. A way of specifying the spread of particle sizes in a sample is to quote the quartiles (25 % and 75 % points) or the interquartile range (difference between 25 % and 75 % points). Thus, for two given samples, the one with the narrower size distribution will have a smaller interquartile range. The span and uniformity of the dispersion are measures of the deviation of the particle sizes from the mean (Allen T, 1997).

4.3.4.1.3 Procedure for size determination

The size distributions of the microsphere formulations were determined by laser diffraction, using the Malvern Mastersizer™ *X* (Malvern Instruments, Malvern, UK). To prepare samples for size distribution determination, ~5 mg of each formulation was dispersed into distilled water (filtered with a 0.22 μm-pore filter). Where dispersion of the particles was problematic, the dry powder was placed into a small mortar and triturated into a smooth paste with a 0.01 $\%^{m}/_{v}$ solution of sodium dodecyl sulphate (SDS). The paste was then diluted with double-distilled water, to form a suspension, a sample of which was placed into the sample presentation unit of the Mastersizer to obtain an obscuration between 10 and 20% (a large enough sample of particles to give a statistically significant measure of the size distribution), and the size distribution of sample determined from the detected diffraction pattern resulting from the passage of a laser beam through the sample. The results are expressed in volume diameters, and the deviation from the mean expressed as the uniformity.

4.3.4.2 Determination of the zeta potential of microspheres

4.3.4.2.1 Theory of surface charge, zeta potential

The surface of a particle will be composed of chemical constituents that are likely to be ionisable, so electrons and positive ions can be lost into the solution until some equilibrium level of ionisation is reached. In general a charged surface tends to gather counter ions close to it. The ions closest to the charged surface are strongly bound and are carried along with the movement of the particle, while the more loosely bound ions are replaced with particle movement. This gives rise to an electrical double layer, consisting of the inner which may include adsorbed ions, and diffuse regions where ions are distributed as influenced by electrical forces and random thermal motion. The two parts of the double layer are separated by the Stern plane, at about a hydrated ion radius from the surface, thus counter ions may be held at the surface by electrostatic attraction and the centre of these hydrated ions form the Stern plane. A schematic diagram of the double layer is illustrated in figure 4.4 a and b.

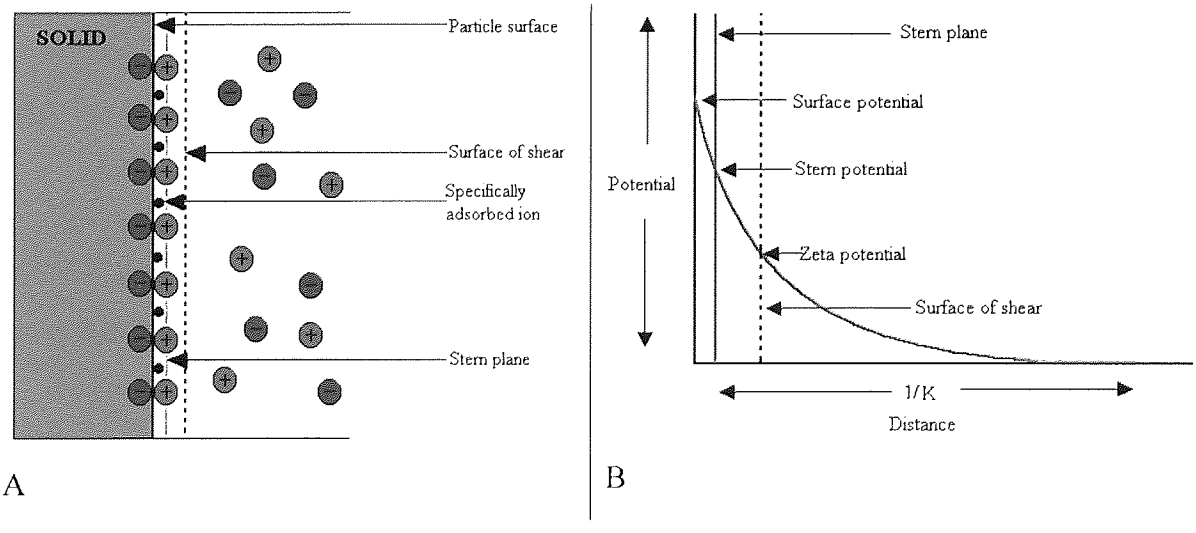


Figure 4.4: Schematic representation of the electric double layer (a), and the changes in potential with distance from the particle surface (b) (Aulton ME, 1988).

The potential changes linearly from the surface potential to the Stern potential, then decays exponentially to zero in the diffuse region. In addition to ions in the Stern layer a certain amount of solvent will be bound to the ions and the charged surface. This

solvating layer is held to the surface and the edge of the layer, termed the surface or plane of shear, and represents the boundary of relative movement between the solid and the liquid. The potential at the plane of shear is termed the zeta potential. Because the surface of the solvating layer is ill defined, the zeta potential actually represents a potential at an unknown distance from the particle surface (Aulton ME, 1988).

The surface charge and therefore the zeta potential of particles is influenced by the pH of the medium, as well as the ionic strength. The pH of the medium will determine the state of ionisable functional groups on the particle surfaces, so that a positive charge due to amine groups will be reduced or neutralised at basic pH.

4.3.4.2.2 Measurement of zeta potential

Electrophoresis is the measurement of colloidal particle movement when placed in an electric field. The measurement can be used to determine the sign of the charges on the particles and also their electrophoretic mobility, which is related to the surface charge and zeta potential. An effect of the electric field on the electrophoresis cell is the electro-osmosis, and occurs because the glass cell walls have a negative charge. However, there is a point in the cell at which the electro-osmotic solvent flow is zero, and the measured particle velocity is then the true electrophoretic mobility.

As the mobility must be measured in the stationary layer, a small volume in the cell is defined by the crossing point of two laser beams, where interference fringes are created. Particles within this interact with the fringes to produce scattered light which oscillates at frequencies determined by the speed of the particles. The light scattered by the particles experiences a shift in frequency, or a Doppler effect, and this is detected by a photomulitiplier. A frequency spectrum is obtained which is converted to the electrophoretic mobility, from which the zeta potentials are calculated using the Smoluchowski equation (Aulton ME, 1988), (Washington C, 1992).

$$\mu = \varepsilon \bullet \xi / \eta$$

where:

μ = electrophoretic mobility
ε = permitivity
ξ = zeta potential
η = viscosity of measurement medium

Equation 4:1: The Smoluchowski equation, describing conversion of electrophoretic mobility to zeta potential.

4.3.4.2.3 Procedure for zeta potential measurement

The zeta potential of the microsphere formulations was determined by a conversion of the electrophoretic mobility of the particles to a zeta potential using a Smoluchowski factor (F) of 1.5. This was done using the Malvern Zetamaster™. To prepare samples for zeta potential determination 5 mg of the formulation was dispersed in distilled water and diluted to obtain a sample of appropriate concentration for the measurement of the electrophoretic mobility. Sonication and addition of surface active agents was avoided to prevent possible changes to the particle surface charge prior to determination.

4.3.4.3 Assessment of microspheres morphology

The morphology of the microsphere formulations was qualitatively assessed by scanning electron microscopy (SEM). To prepare samples for SEM, the dry microspheres were spread onto adhesive carbon pads pressed onto aluminium stubs and gold-coated by 10mA current for 1.5 minutes under an argon atmosphere using a Gold Sputter Coater (Emscope SC 500, Ashford, Kent, UK). The coated samples were mounted onto a scanning electron microscope (Cambridge Stereoscan S90B, Leo Microscopy Ltd., Cambridge Instruments ,UK) and viewed at an acceleration voltage of 25 kV using a tungsten filament.

4.3.4.4 Extraction of plasmid DNA from microspheres, and determination of loading efficiency

A mass of microspheres between 10 and 20 mg was accurately weighed and dissolved in 5 mL of either $CHCl_3$ or DCM in a 15 mL polypropylene tube. The plasmid DNA was extracted by the addition of 500 μL of TE buffer. The mixture was vortexed

briefly, then left shaking gently for 20 minutes to allow partition of the DNA into the aqueous phase. The two phases were separated by a 20-minute centrifugation at 3,000 rpm, and the aqueous phase obtained by careful pipetting. An intermediate layer representing a stable emulsion at the interface was also collected in the process, and the extraction procedure repeated twice. Residual solvent in the collected aqueous phase was driven-off by leaving the aqueous samples shaking at 37°C for 10 minutes.

The concentration of plasmid DNA in the aqueous phase was determined by the PicoGreen™ (Molecular Probes) fluorescence assay, as described in section 2.2.6.1. The structural integrity of the DNA was assesses by agarose gel electrophoresis, as described in section 2.2.4.

4.3.5 Microscopic assessment of *in vitro* particle uptake by RAW cells

The uptake of empty rhodamine-labelled microspheres was studied qualitatively in an *in vitro* macrophage cell line. The cells were cultured in DMEM (# 31966-021, Life Technologies) and seeded at a density of $5x10^5$ cells *per* well of a 24-well cell culture plate, each well containing a glass coverslip (thickness no.1, diameter 13mm). At a confluency of about 50 – 60 percent after 24 hours, 500μL of suspension of the microsphere formulations in serum-free medium containing approximately 20μg of microspheres was places into each of the wells and incubated for 24 hours under standard conditions (37°C, 100% humidity, and 5% carbon dioxide). An identical experiment was performed with the cells incubated at 4°C. Cells were washed three times with sterile PBS, fixed unto a microscope slide and viewed under a fluorescence microscopy (excitation 450 – 490nm; emission 520nm). The image of the particles and cells under the fluorescence microscope was captured digitally, and are shown in figure 4.12. Confocal laser microscopy images were also obtained, and these are shown in figures 4.13 and 4.14.

4.4 Results

4.4.1 Fluorescence quenching assays

Th intercalation of the picogreen dye with DNA molecules results in strong fluorescence when excited at 490 nm, with emission at 535 nm. Picogreen molecules

are prevented from DNA intercalation when the DNA molecules are bound to PEI, hence the fluorescence is progressivley 'quenched' as the ratio of PEI molecules increases, until all the DNA molecules are bound to PEI and there are no DNA molecules available for picogreen intercalation. Figure 4.5 shows this trend with increasing N:P ratios.

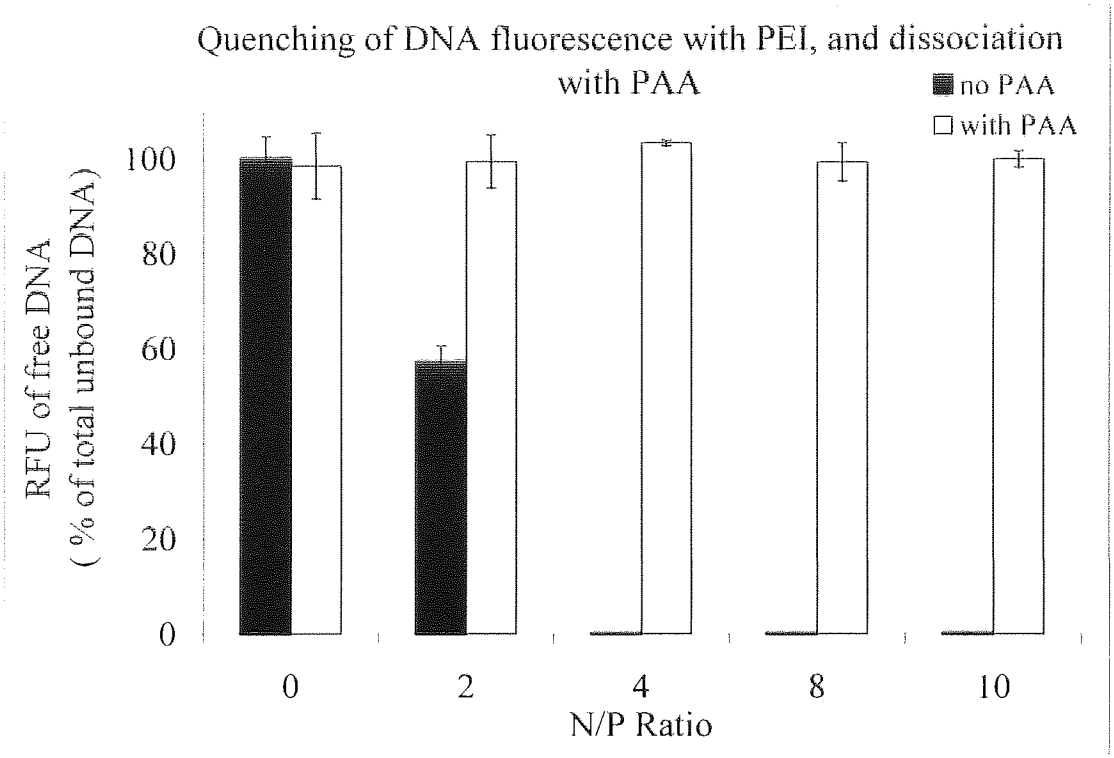


Figure 4.5: Quenching of fluorescence on addition of PEI at increasing N:P ratios. The black bars (no PAA) indicate that at an N:P ratio of 4 there is a complete complexation of DNA, hence there is no fluorescence. The addition of PAA to the complexes liberates the DNA from the PEI and restores the fluorescence to the original value, as shown by the white bars.

At an N:P ratio of 4, there is complete complexation of the DNA in solution and no fluorescence is observed. The addition of PAA to the complexes at all N:P ratios resulted in 100 % liberation of the DNA molecules. PAA is a negatively charged molecule which effectively competes for the positively charged residues of the PEI and consequently removes and liberates the DNA molecules from the DNA-PEI complex.

4.4.2 Agarose gel electrophoresis of complexes

Visualisation of DNA-PEI complexes, and those incubated with PAA was achieved by

agarose gel electrophoresis. The effect of increasing the N:P ratio on the DNA migration was observed, and shown in figure 4.6.

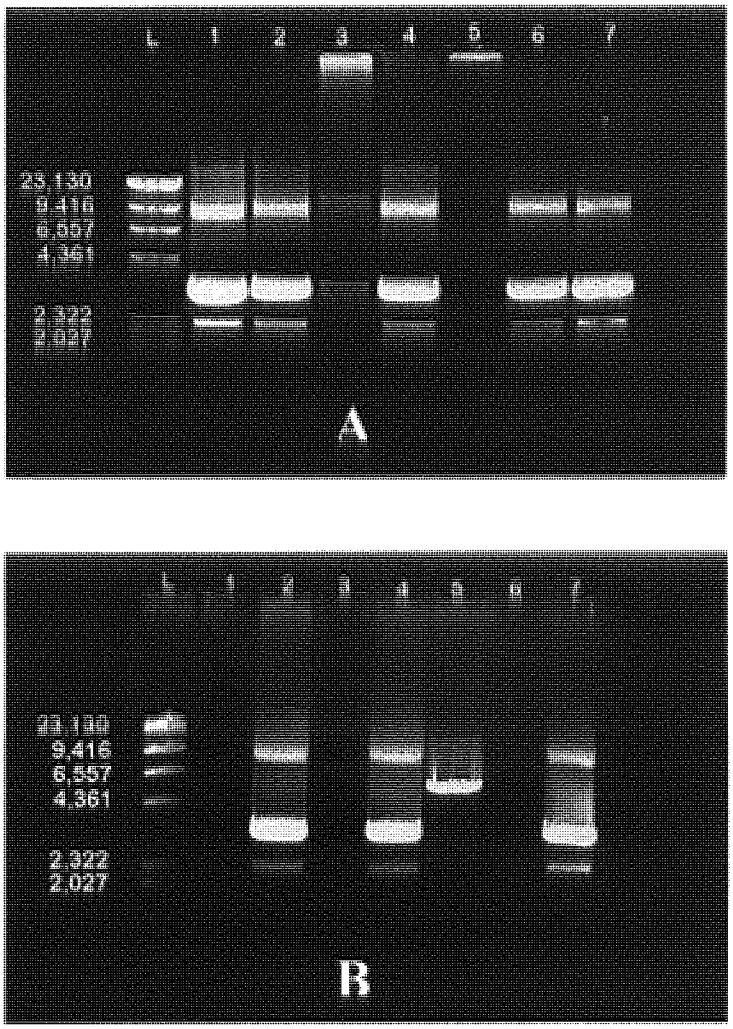


Figure 4.6a and b: Agarose gel electrophoresis of DNA complexed to PEI and liberated by addition of PAA. (Figure 4.6 a: Lane 1= N:P 0; Lane 2 = N:P 0 + PAA; Lane 3 = N:P 2; lane 4 = N:P 2 + PAA; lane 5 = N:P 4; lane 6 = N:P 6 + PAA. Figure 4.6 b: lane 1 = N:P 8; lane 2 = N:P 8 + PAA; lane 3 = N:P 10; lane 4 = N:P 10 + PAA; lane 5 = linearised DNA control; Lane 6 is an empty lane; 7 = non-complexed DNA control).

There was complete complexation of the DNA by PEI at an N:P ratio of 4 or more. Whether there was a loose association between the DNA and PEI molecules at ratios below 4, or there was some complexation but not with all the molecules of DNA may be elucidated with the fluorescence quench assay. However, it can be seen that the process of complexation with PEI and liberation with PAA did not affect the structural conformation of the plasmid molecules.

4.4.3 Luciferase expression in cells transfected with PEI at various N:P ratios

The effect of increasing the N:P ratio of DNA-PEI complexes on the transfection levels of 293 and RAW cells *in vitro* is shown in figure 4.7 below:

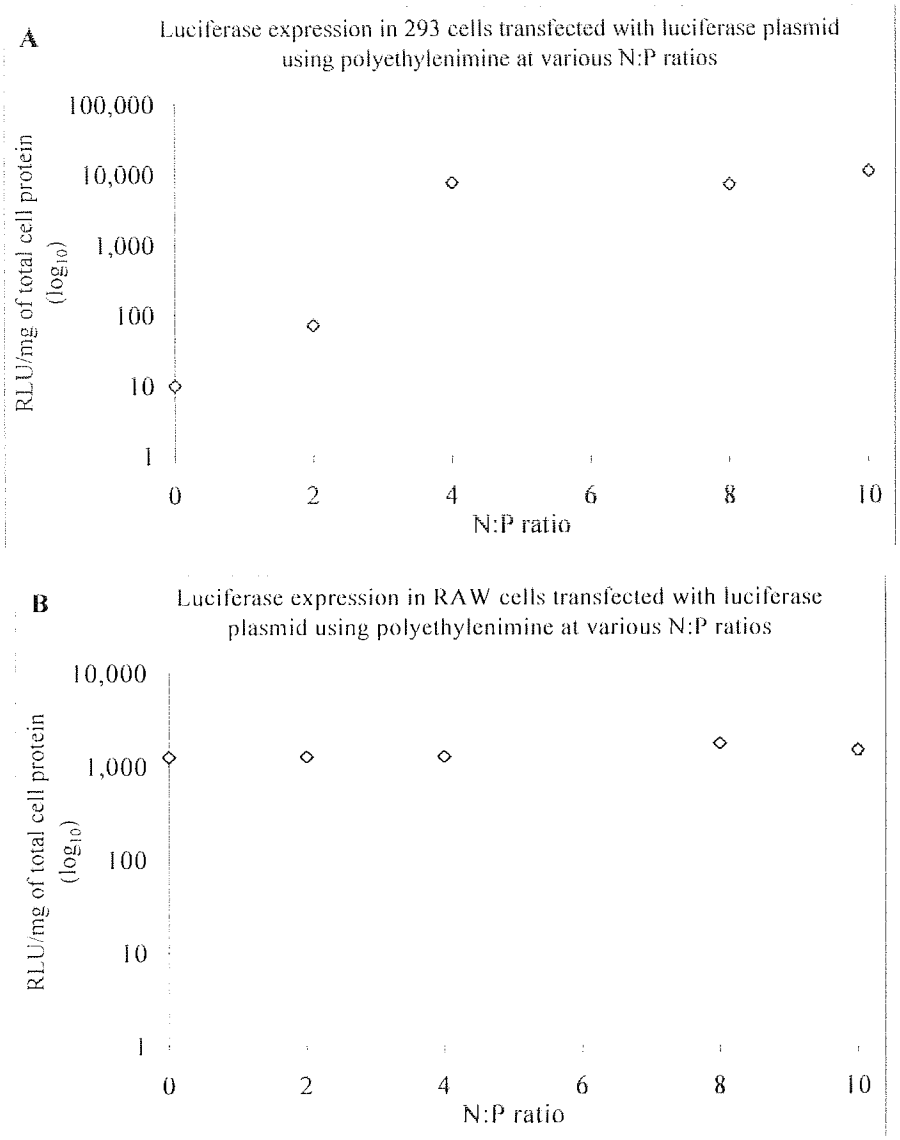


Figure 4.7a-b: Luciferase expression in 293 cells (a) and RAW cells (b) *in vitro*.

The 293 cells were amenable to transfection at lower N:P ratios compared with RAW cells, and showed transfection levels about 10 times higher than in RAW cells. The lowest N:P ratio effecting maximal transfection levels was that which produced maximal complexation of DNA (see figures 4.6 and 4.7). The fluorescence quench

assays showed100 % complexation of DNA to PEI. This was visually confirmed with the agarose gel analysis of the complexes, which also showed the complete lack of free, uncomplexed DNA at an N:P ratio of 4. It was observed that maximal transfection levels in 293 cells were at the same ratio.

The effect of increasing N:P ratios in RAW cells on the transfection levels showed the same general trend observed with 293 cells; an increase in luciferase expression levels with increasing N:P ratios, but with an important difference. In the RAW cells, maximal expression levels was seen at an N:P ratio of 8, and any further increase in this ratio reduced the expression. By broad comparison, RAW cells were not as conducive to transfection by the DNA-PEI complexes compared with 293 cells, and the formation of complexes alone was not sufficient to provide maximal transfection levels. There is a requirement of additional PEI to increase the transfection of these cells, and at an N:P ratio of 8 this appears to have been achieved. The reduction in the transfection levels at an N:P of 10 is most likely due to the toxicity of PEI to the cells mediated through its high positive charge density.

RAW cells are an immortalised macrophage cell line, and have retained the phagocytic phenotype of their origins. During phagocytosis the complexes are internalised into phagosomes or endosomes located close to the plasma membrane. These peripheral or early endosomes have a pH of 6 - 6.5. These early endosomes are transported further into the cells, where there is a slight drop in pH to about 5.5 and are now called late endosomes. It is at this stage that fusion of the late endosomes and primary lysosomes occurs to form phagolysosomes, characterised by a further drop in the pH to 4.5, the optimal pH for hydrolytic enzymes present in these vesicles which degrade DNA and other susceptible molecules to smaller fragments or their basic constituents. Herein lies the advantage of PEI as a transfection agent; the buffering capacity of the amine groups effectively protects the bound DNA from the degradative optimal low pH of the phagolysosomes, and aids the escape of DNA from these vesicles to the nucleus. This major barrier explains the generally lower transfection levels seen in RAW cells, and also accounts for the steady maximal luciferase expression levels at higher N:P ratios. The increased number of PEI molecules increases the buffering capacity, and

presumably maintains the vesicular pH at a value which is more favourable to the DNA molecules.

The 293 cells retain their epithelial cell phenotype so do not share the macrophage degradative properties, and internalisation of complexes results in the delivery of intact DNA molecules to the nucleus for transcription and translation. The lack of any need for buffering in the lysosomal vesicles precludes any increase in the expression levels on increasing the N:P ratio past that which is optimal for the electrostatic association of the DNA with PEI, hence, maximum expression is observed at a lower N:P ratio. All subsequent formulations were prepared with an N:P ratio of 8.

4.4.4 Morphological analysis of microspheres loaded with plasmid DNA prepared by solvent evaporation

The microspheres were viewed by scanning electron microscopy after lyophilisation. A further formulation with DNA-PEI complexes at an N:P ratio of 80 was included to determine the effect of a large quantity of PEI on the microsphere morphology. Pictures of the microspheres as viewed by SEM are shown in figure 4.8.

The microspheres show similar morphology and size, regardless of the amount of PEI or complexes incorporated. Polyethylenimine has no effect on the formation or the morphology of microspheres at the levels used.

4.4.5 Size distribution and zeta potential of microspheres

The size distribution of the microspheres was determined by laser diffraction, and graphs of the distributions are shown in figure 4.9. All particles formulated were below 10 μm, with evidence of some aggregated particles extending the range to *circa* 20-30 μm. For comparison, a microsphere formulation containing an excess of PEI (N:P ratio of 80) was prepared and its characteristics compared with that of the two formulations.

Encapsulation of DNA-PEI complexes at N:P ratio of 8 had little effect on the median size of the microspheres when compared with those encapsulating free DNA. There is however an increase in the deviation from this median size, represented by the

broadness of the distribution graph. By comparison, addition of large amounts of PEI shifts the median size of the microspheres to about 3.5.

The surface charges of microspheres were calculated from a measurement of their zeta potentials, shown in table 4.1. There was no effect of the PEI on the surface charge of the microspheres at a N:P ratio of 8, suggesting that there was little surface location of the complexes, and the zeta potential remained equal to that of the microspheres containing free DNA. Incorporation of complexes with 10 times the amount of PEI resulted in a slight increase in the positive zeta potential from ~-6 to -+3 mV. This suggests some surface location of either the PEI or the DNA-PEI complex on the microspheres, but the magnitude of the increase of the zeta potential compared with the increase in PEI indicates that most of the PEI was located inside the microspheres, otherwise a larger positive surface potential would be observed.

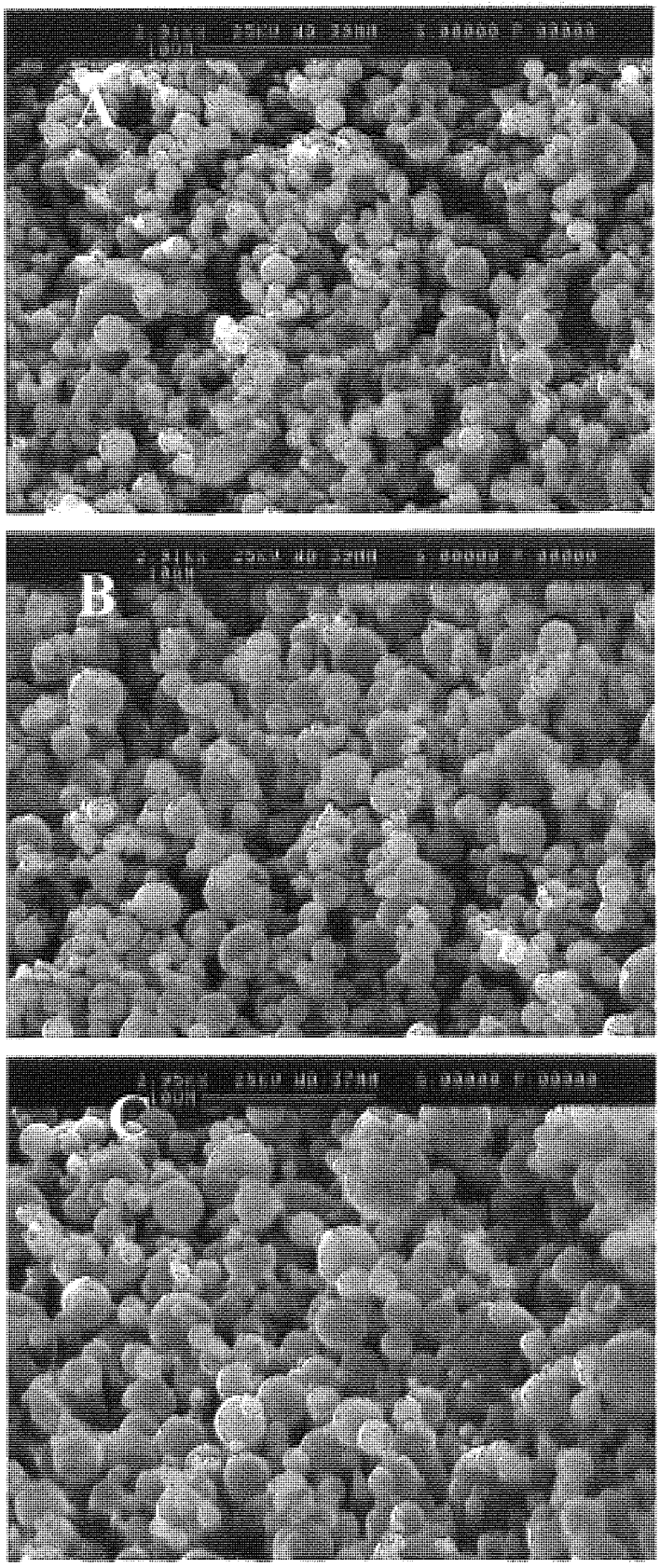


Figure 4.8a-c: SEM photographs of PLA $_{2kD}$ microspheres prepared by solvent evaporation, containing naked DNA (a); DNA-PEI complex at a N:P ratio of 8 (b); DNA-PEI complex at an N:P ratio of 80 (c).

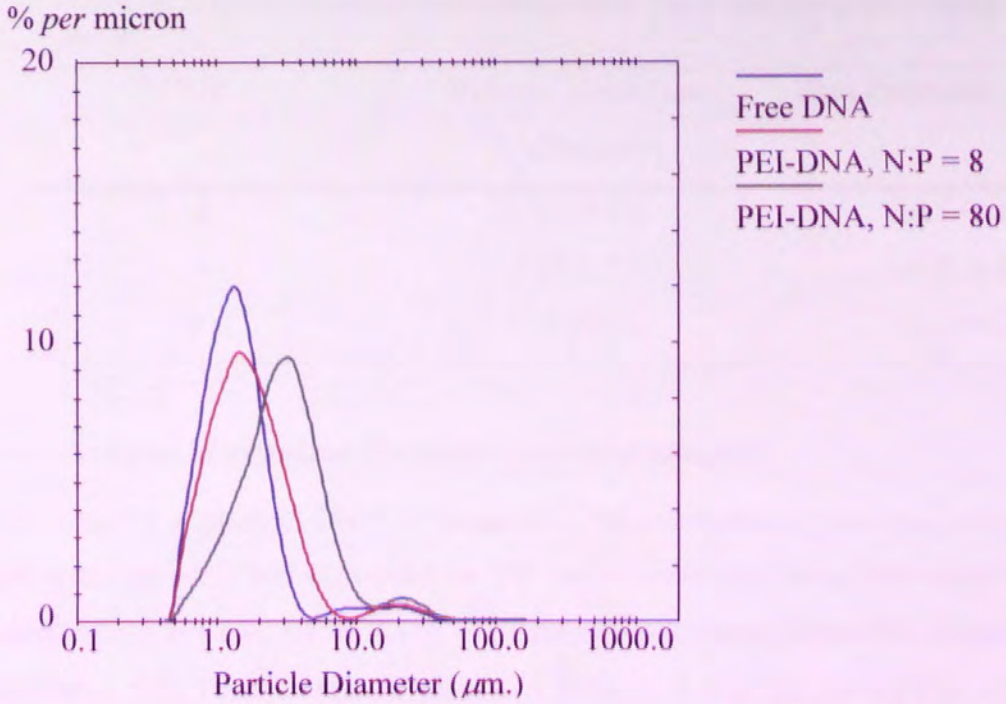


Figure 4.9: Size distributions of PLA $_{2kD}$ microspheres prepared by solvent evaporation encapsulating naked DNA (N:P = 0,blue line); DNA-PEI complex, N:P = 8 (red line); and DNA-PEI complex, N:P = 80 (green line).

The lack of heavy influence of PEI on the surface charge of the particle makes it a useful formulation excipient. PEI has a major low-pH buffering capacity, and so can protect DNA in either lysosomes or phagolysosomes and increase the transfection efficiency. Delivery of the PEI to cells *in vitro* has also been shown to inhibit the fusion of lysosomes to endosomes (Klemm AR *et. al.*, 1998). The encapsulation of PEI in microspheres offers the possibility of delivering higher concentrations intracellularly, without the risk of toxicity to the plasma membrane, as the high positive charge is masked by the microspheres. Specific cell types like APCs may be targeted by the attachment of ligands to the microsphere surface to increase uptake and MHC class I presentation of the expressed antigen.

Table 4.1: Mean volume diameters of PLGA 50:50 63 kDa microspheres containing DNA-PEI complexes at various N:P ratios. The uniformity is the deviation from the mean diameter, and is an indication of the polydispersity of the microspheres. The distribution graph is shown in figure 4.9 above.

N:P	Volume mean /μm (Uniformity)	Zeta Potential / mV (sd)
0	1.30 (0.97)	-6.7 (0.1)
8	1.56 (1.1)	-6.5 (0.1)
80	3.56 (0.78)	+3.3 (0.2)

4.4.6 Analysis of extracted DNA for structural integrity

The integrity of plasmid DNA extracted from the microspheres was analysed by agarose gel electrophoresis and visualised by UV transillumination using EtBr as a fluorescent intercalating dye (see figure 4.10). The extracts containing DNA-PEI complexes were incubated with PAA to liberate the DNA from PEI. The gel shows that encapsulated DNA was associated with the PEI, and required liberation from the complexes, as the extracts not incubated with PAA failed to migrate through the gel (see lanes 4 and 5 of figure 4.10). There was, however, a general reduction in the SC form of the plasmid DNA after extraction and no difference was seen between the DNA encapsulated as either free or as PEI complexes (see lanes 2 and 3 respectively, figure 4.10). It would appear that the encapsulation process itself, and not the addition of PEI, is detrimental to the plasmid structural integrity.

4.4.7 Determination of plasmid loading efficiencies in microspheres

Plasmid DNA was radiolabelled as described in section 2.2.7 and microencapsulated as free DNA or DNA-PEI complexes. About 100 mg of each formulation of microspheres was accurately weighed into a plastic scintillation vial and digested with 2M NaOH. The level of radioactivity of the samples was determined as described in section 2.2.7. The amount of DNA in each sample was determined *per* mass of microspheres, and the encapsulation efficiency calculated. Figure 4.11 below shows the influence of complexation on the encapsulation efficiency.

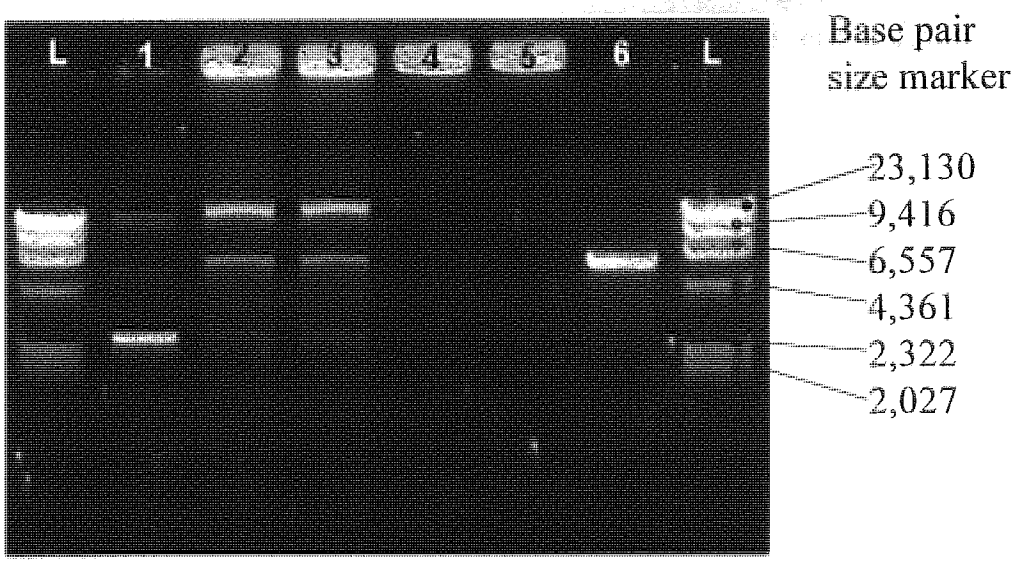


Figure 4.10: Agarose gel electrophoresis of plasmid DNA extracted from PLA_{2kDa} microspheres. Extracted samples were incubated with PAA overnight to liberate DNA from PEI. The integrity of the extracted DNA samples was compared with that of non-encapsulated DNA control (lane 1) and a linearised DNA control (lane 6). Lane 2 and 3 show samples extracted from microspheres and incubated with PAA, and lanes 4 and 5 show the extracted samples without incubation with PAA. L = λ / HindIII ladder.

The complexation of DNA with PEI results in a 4-fold increase in the encapsulation efficiency in PLA $_{2kD}$ microspheres prepared by solvent evaporation. It is likely that the particulate nature of the complexes prevented any leaching of the DNA from the inner aqueous phase during the drying phase of the water-in-oil-in-water double emulsion. Microsphere formulation with PEI increased the encapsulation efficiency to ~20 %. Although this is 4 times higher than the formulation without PEI, it is still quite low. Methods available to increase this involve the use of higher theoretical DNA loading, but the possible aggregation of the PEI-DNA complex at higher concentrations precludes this method.

A similar approach to plasmid DNA formulation reported by Capan *et. al.*, (Capan Y *et. al.*, 1999), involved the encapsulation of PLL-DNA complexes in PLGA 50:50 microspheres. Complexation had no effect on either the efficiency of plasmid DNA entrapment, size distribution or the morphology of the particles. The negative surface charge was however reduced at increased levels of PLL, as measured by the surface (zeta potential).

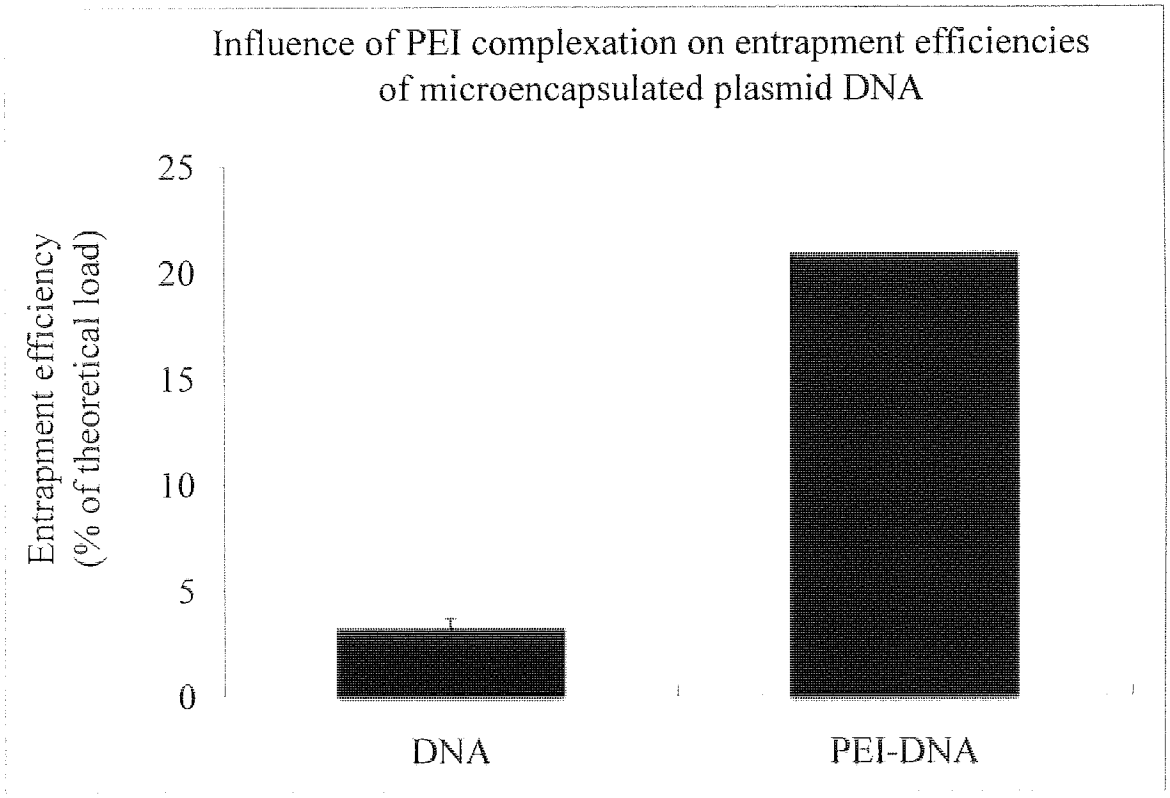


Figure 4.11: Encapsulation efficiencies of plasmid DNA and PEI-DNA complexes in PLA_{2kDa} microspheres.

4.4.8 Uptake of microspheres by macrophage (RAW) cells

Rhodamine-labelled microspheres with DNA or DNA-PEI complexes were incubated with RAW cells to asses any influence of the complexes on the *in vitro* uptake by APCs. The results are shown qualitatively in figure 4.12.

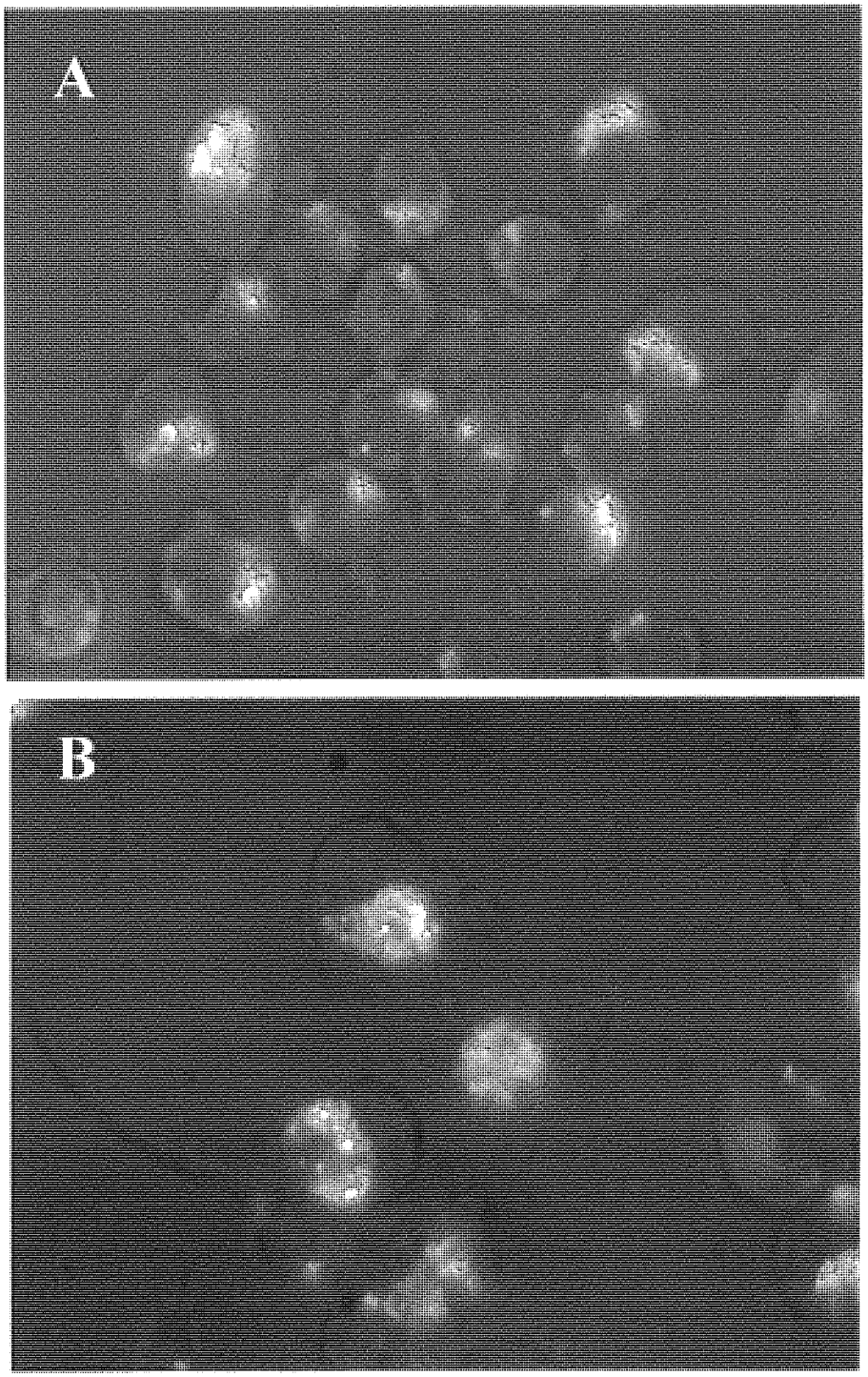


Figure 4.12: Internalisation of rhodamine-labelled PLA_{2kDa} microspheres loaded with either plasmid DNA (A) or DNA-PEI complexes (B) by RAW cells *in vitro*. (Magnification = 630x)

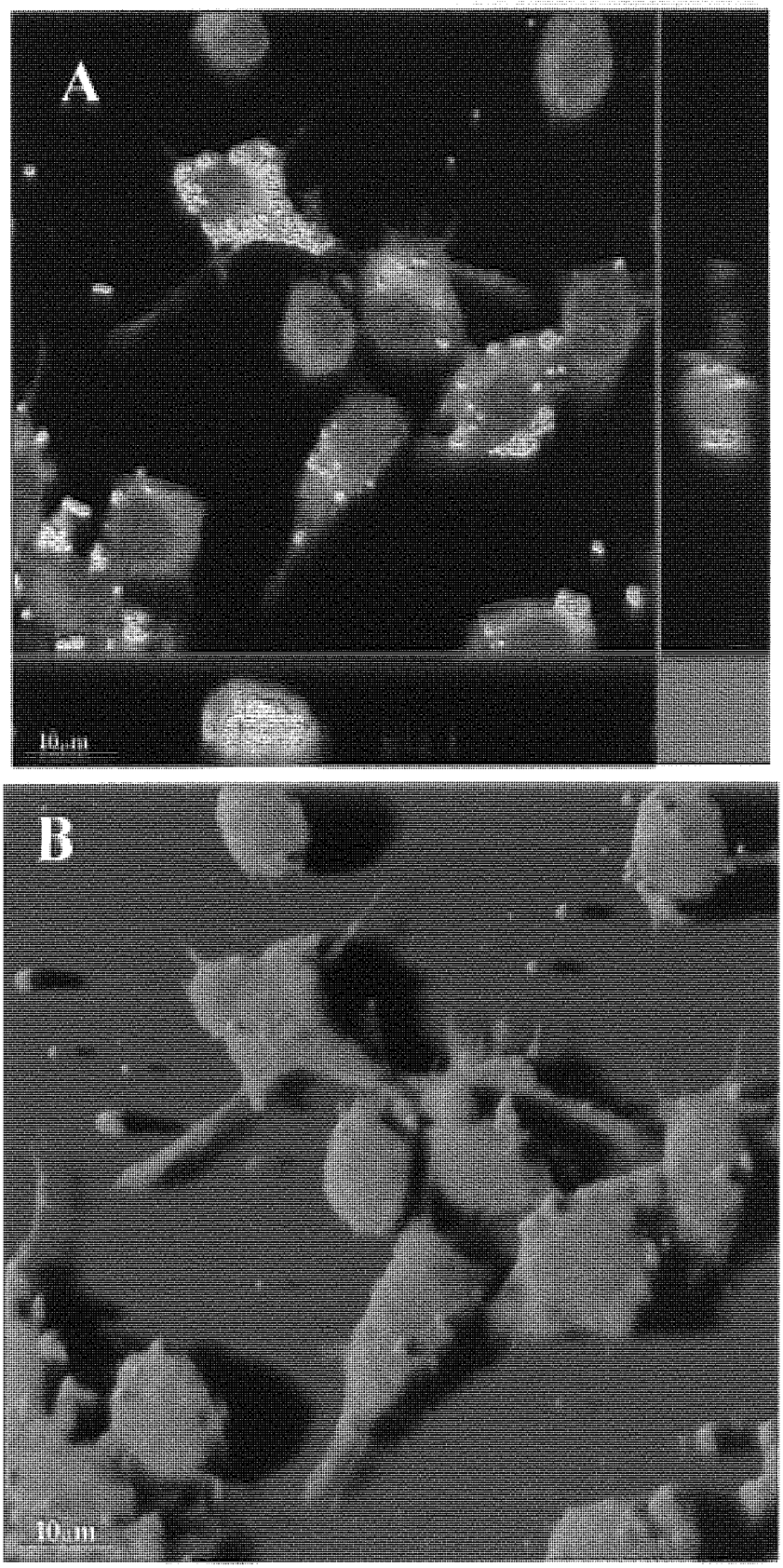


Figure 4.13: Confocal microscopy images of macrophage (RAW) cells after incubation with microspheres for 24 hours at 37°C. The pictures depict the cross sectional (A) and 3-dimensional (B) view of the same sample of cells.

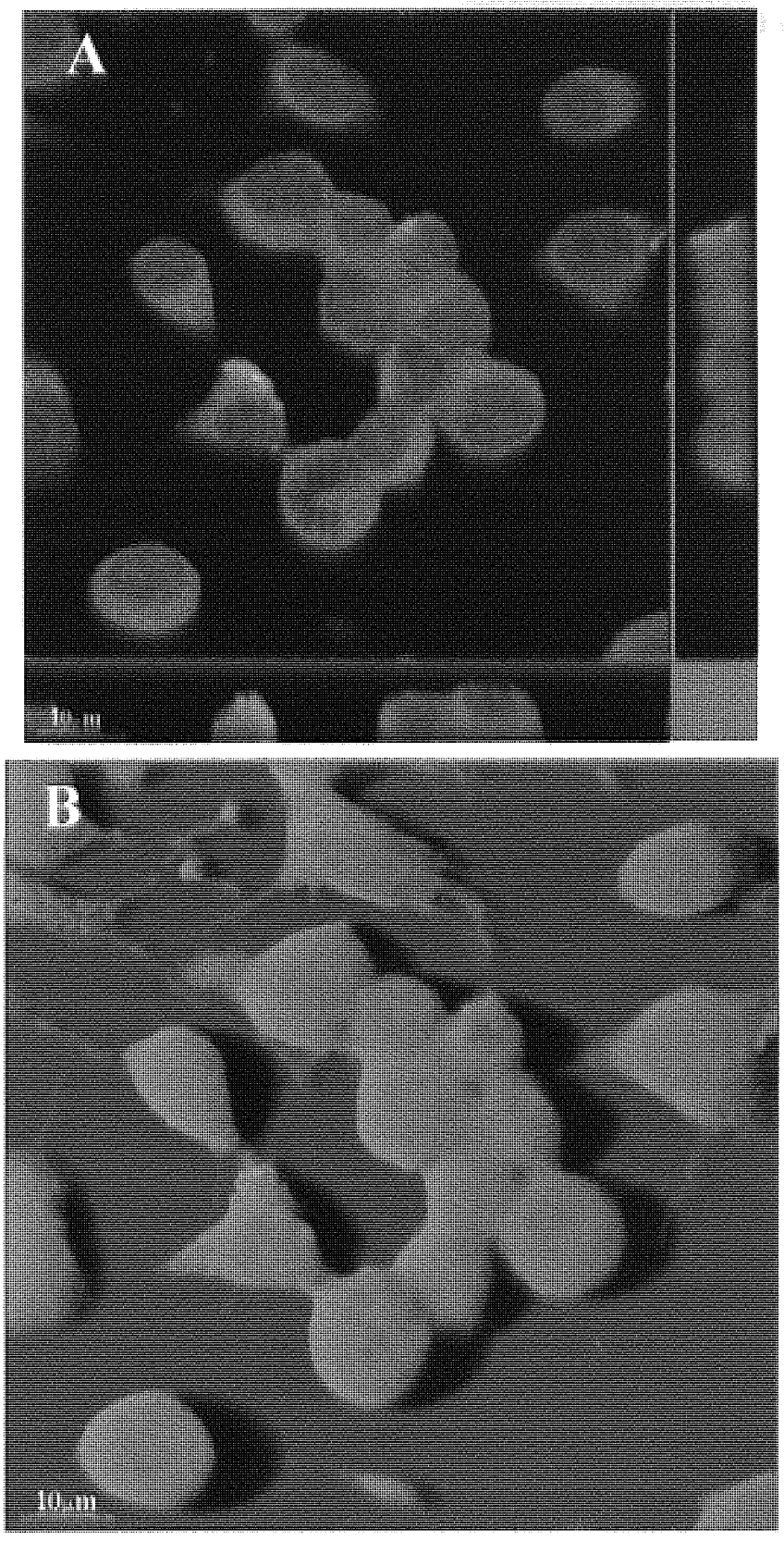


Figure 4.14: Confocal microscopy images of macrophage (RAW) cells after incubation with microspheres for 24 hours at 4°C. The pictures depict the cross sectional (A) and 3-dimensional (B) view of the same sample of cells.

The microspheres were taken-up by the RAW cells, as seen by the presence of the discrete packets of particulate fluorescence coinciding with the inner sections of the cell outlines. The pictures represent an overlay of the image of the RAW cells viewed by light microscopy and the fluorescence microscopy showing the presence of microspheres by the rhodamine label. There was no qualitative difference between the uptake by RAW cells of microspheres with free DNA (figure 4.12 a), and microspheres with DNA-PEI complexes (figure 4.12 b).

Confocal images of RAW cells after incubation with the particles are displayed in figure 4.13. These confirm the intracellular location of the particles. Figure 4.14b shows that there was no internalisation of particles when the cells were incubated at 4°C.

4.5 Conclusions

Plasmid DNA may be entrapped into PLA_{2kDa} microspheres at encapsulation efficiencies which may be increased by the complexation with PEI. The PEI molecule appears to have had the inherent property of increasing the transfection levels in phagocytic cells by its acid-buffering capacity. This DNA-PEI microsphere formulation is readily taken-up into a RAW cell line *in vitro*, and it is expected that this trend will be seen *in vivo*. The mucosal administration of particulate formulations of DNA vaccines is highly desirable as this form encourages delivery to the mucosal inductive sites, and protection from degradative enzymes in low-pH environments may be prevented by complexation with PEI. The size distributions observed are within the range which make the microspheres amenable to uptake by APCs, and the zeta potential of surface charge may be altered by formulation with appropriate polymers to alter the hydrophobicity, further improving the uptake capabilities of the microspheres.

One drawback of the encapsulation of PEI-DNA complexes is the possibility of aggregation at high concentrations, so that DNA loading of the microspheres is inherently low. Methods available to stabilise the complexes as discrete packets at high concentrations will serve to improve this aspect of the formulation.

5. The use of stearylamine as an agent in DNA vaccine microsphere formulations.

5.1 Introduction

5.1.1 Stearylamine

Stearylamine (SA) is an naturally occurring amphipathic molecule consisting of an hydrophobic C_{18} domain and an amine molecule providing a positive charge. Figure 5.1 illustrates the chemical structure.

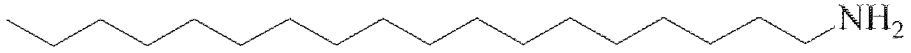


Figure 5.1: Chemical structure of Stearylamine

This hydrophobic nature coupled with the positive charge make is an attractive reagent for plasmid DNA formulation, and to that end, Wang *et. al.*, have investigated it's effect as a liposome transfection reagent (Wang D *et. al.*, 1996). It was found that for certain fibroblast cell lines (e.g. CV-1 and NIH3T3), liposomes formulated with SA had significantly higher transfection efficiencies compared with the commercial standard, Lipofectin ™. It was also noted that the action of SA liposomes was not affected by the presence of serum, a useful trait for an *in vivo* transfection reagent.

Particulated vaccines have been shown to have increased immunogenicity, and this has been ascribed to uptake by APCs. The uptake of particles by cells is influenced by the surface charge and hydrophobicity of the particle. Cell membranes have a natural negative charge due to the presence of glycoplipid molecules. Positive surface charge enables electrostatic adsorption of the particles to the cell membrane, and this leads to increased uptake of the particle. This is the basis for the use of cationic liposomes for cell tranfection (Hui S-W and Zhoa Y-L, 1995), (Schwendener RA *et. al.*, 1984), (Behr JP *et. al.*, 1989). Particles with hydrophobic surfaces have also shown increased uptake *in vitro* (Tabata Y and Ikada Y, 1990), (Tabata Y and Ikada Y, 1988). Herein lies the rationale for the use of SA in microsphere formulations of plasmid DNA. The hydrophobic and positive charge nature of the molecule are desirable properties. The use of SA as a reagent in microspheres has not yet been investigated.

5.2 Materials and methods

5.2.1 Microsphere preparation and characterisation

The method of microsphere preparation was as previously described. SA was added as a solid mass to PLGA or PLA polymer at the required mass percentage (ranging from 2.5 $\%^{m}/_{m}$, to 15 $\%^{m}/_{m}$), and dissolved in chloroform. All other preparation and characterisation procedures were as described in section 4.3.

5.2.2 Quantitative evaluation of particle uptake by macrophage cells *in vitro*

The range of activity of internalised microspheres was initially established to test the feasibility of the method. Microspheres were prepared as previously described, incorporating radiolabeled DNA (section 2.2.7). RAW cells were seeded in the wells of a 6 well plate, as described (section 2.2.5.2), and achieved 70 % confluency after 24 hours. The cells were washed once with PBS, overlaid with a suspension of microspheres at a concentration of 100 μg or 10μg *per* 2 mL of serum-free media, and incubated at 37°C, in 5 % CO_2 and 100 % relative humidity for 24 hours. The cells were washed three time with PBS and lysed with 0.2M NaOH. All washings and lysates were collected and the radioactivity determined by scintillation as described previously (section 2.2.7).

To study the effect of SA on the uptake of microspheres, RAW cells were seeded at a density of $17x10^6$ cells *per* 100 mm cell culture dish. The cells were 70 % confluent after 24 hours. Microspheres were suspended in SFM at a concentration of 3 mg *per* 10 mL. The cells were washed once with PBS, and 10 mL of the microsphere suspension (3mg of microspheres) carefully placed onto the cells. Microspheres formulated with 0, 2.5, 5 and 10 % SA were investigated, and all experiments were run in triplicate. After 24 hours at 37°C, in 5 % CO_2 and 100 % relative humidity, the cells were washed three times with PBS and lysed with 0.2M NaOH. All washings and lysates were determined for their radioactivity by scintillation as described in section 2.2.7.

5.3 Results and discussion

5.3.1 Effect of stearylamine on microsphere morphology

The morphology of the microspheres was assessed by scanning electron microscopy, as described in section 4.3.4.3. The influence of SA on the general morphology is shown by the photographs in figure 5.2. Qualitatively, increasing the SA concentration in the polymer tended in produce more uniform particles, but up to a point. At a 12.5 $\%^{m}/_{m}$ concentration of SA, there is a disruption of the microsphere morphology, as shown in figure 5.2c.

The disruption of the microsphere shapes at a high SA concentration suggests the possibility of a saturatable interaction between components of the microspheres. Thus, the following hypothesis might be postulated; SA interacts with plasmid DNA during microsphere formation, and this interaction prevents the disruption of microspheres by SA. Since microspheres are formed in the absence of SA, the SA-DNA interaction is not a prerequisite for either the favourable morphology observed by SEM, or to the ultimate formation of microspheres. The reason that the interaction is postulated to be saturatable lies in the fact that the disruption appears to be gradual, and is dependent on the increasing SA concentration. It is not likely to be due to the reduction in PLGA, as this is effectively in excess at the SA concentration when disruption occurs. The only other culpable constituent of the microsphere formulation is the plasmid DNA, the quantity of which constant in all formulations, and bears negatively charged phosphate groups which have the potential to interact by electrostatic association, with the amine groups of SA. It is therefore possible that generally, SA in excess, or 'free' SA present in the formulation has the tendency to promote malformation of microspheres. Inclusion of DNA in the formulation removes or reduces the detrimental effect of the SA by electrostatic interaction, and a further increase in the SA beyond a point where SA is in excess of the DNA-interaction capacity leads to the disruption of microsphere morphology. In support of this concept, it was noted that formulations of microspheres with higher loadings of plasmid DNA were formed at SA concentrations which disrupted microsphere morphology at lower DNA loadings (figure 5.3); i.e., the increased level of DNA reduced the presence of free or non-associated SA, promoting the formation of microspheres at higher SA concentrations.

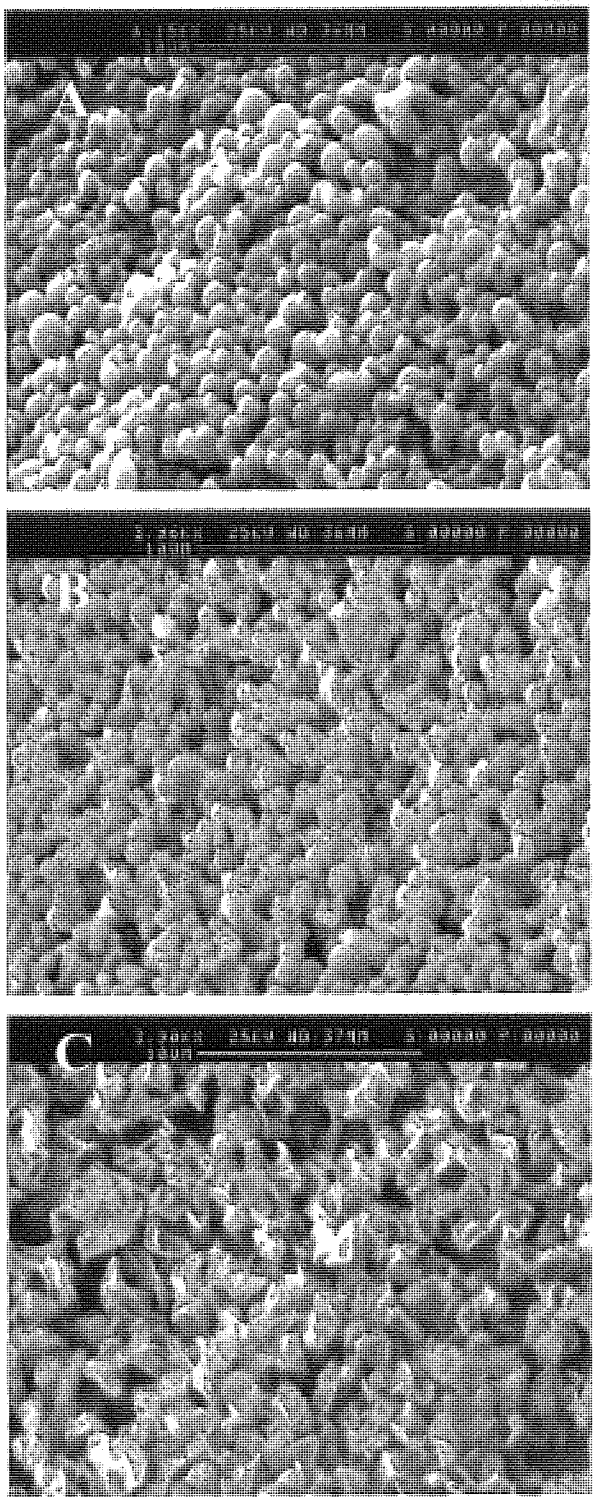


Figure 5.2a-c: Plasmid-loaded PLGA 50:50 microspheres formulated with stearylamine at concentrations of 2.5 $\%^{m}/_{m}$ (A), 5 $\%^{m}/_{m}$(B), and 12.5 $\%^{m}/_{m}$(C), viewed by SEM.

5.3.2 Effect of DNA concentration on microsphere morphology

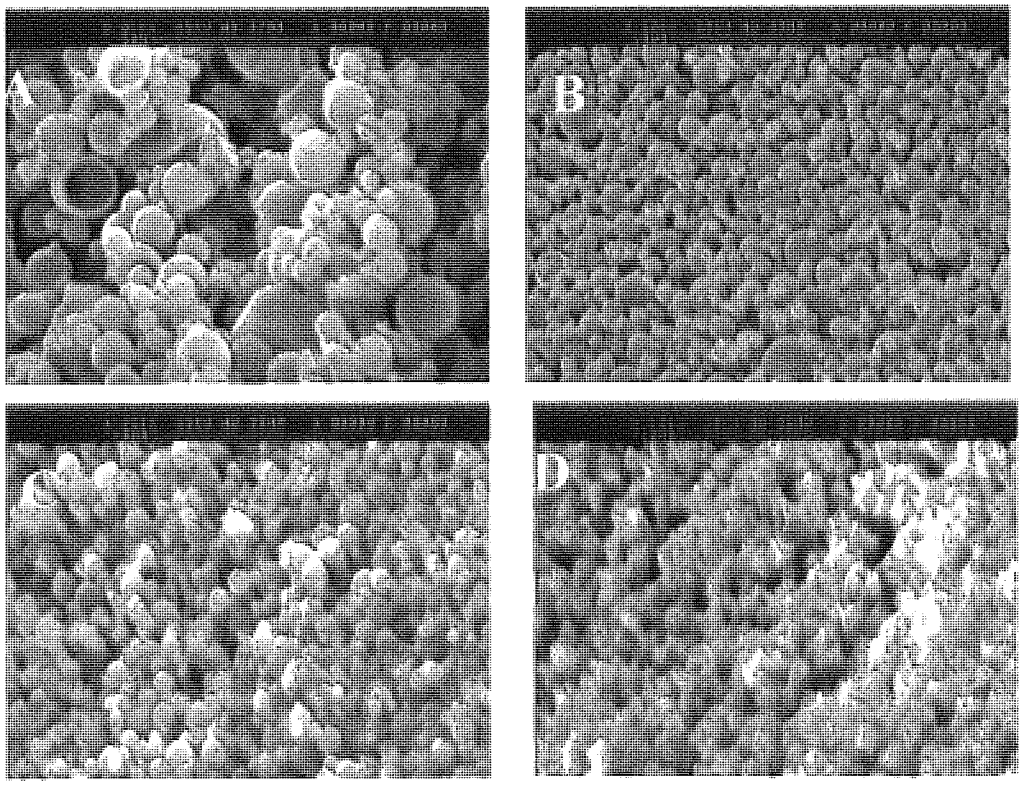


Figure 5.3: SEM photographs of PLGA 50:50 microspheres loaded with plasmid DNA at high and low concentrations, and formulated with and without stearylamine. Microspheres were prepared containing a theoretical plasmid DNA loading of 1mg/100mg of polymer, using no stearylamine (A) or 10 $\%^{m}/_{m}$ stearylamine (B); and 0.1mg/100mg of polymer, using no stearylamine (C) and 10 $\%^{m}/_{m}$ stearylamine (D).

Thus, there appears to be an optimal concentration of SA in the PLGA polymer beyond which the microsphere morphology is disrupted. Further investigations into the precise mechanisms of DNA-SA, or PLGA-SA interactions might explain the effect of SA on the morphology of the microspheres observed.

5.3.3 Effect of stearylamine on the surface charge of microspheres

The surface charges of the microspheres were determined by measurement of the zeta potentials. To investigate its effect on the microsphere surface charge, microspheres were prepared with increasing concentrations of SA, and the results are shown in figure 5.4.

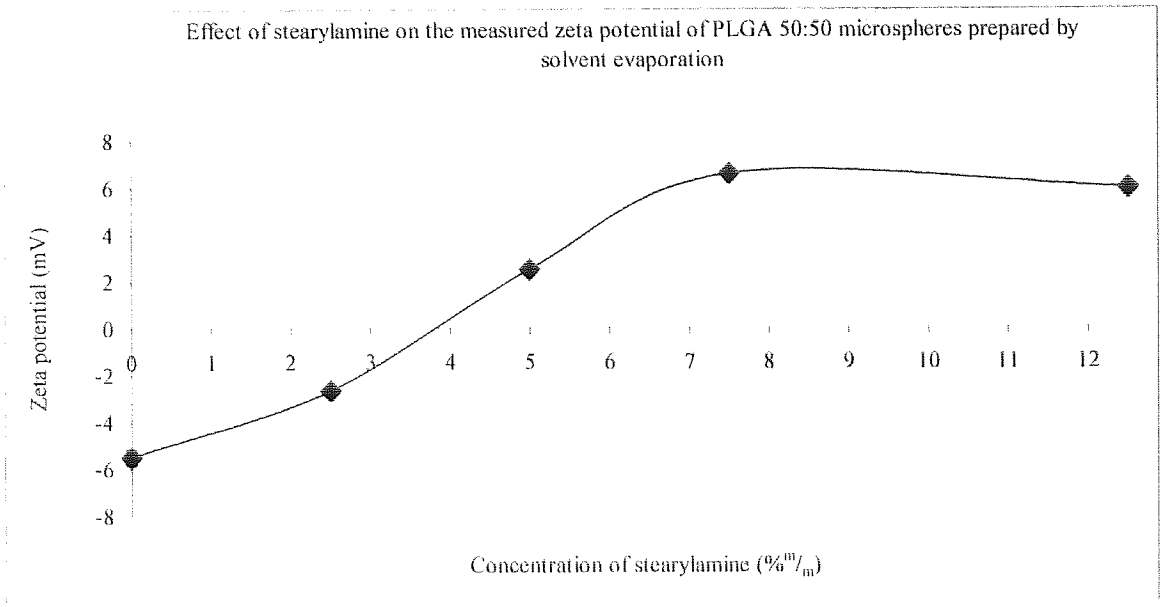


Figure 5.4: Surface charge of microspheres loaded with plasmid DNA formulated with stearylamine at different concentrations. The zeta potentials are shown, as measured by their electrophoretic mobilities. Each point represents the mean of 5 readings, with the standard deviations represented by the error bars.

There was a steady increase in the zeta potential of the microspheres with increasing SA. Since the zeta potential is a property of the surface of the particles and not the bulk, this measurement is an indication of the surface location of the SA. The graph suggests that beyond a SA concentration of 7.5 $\%^{m}/_{m}$, there is no further increase in the zeta potential. This might reflect a saturation of the surface of the microspheres with SA molecules.

A net positive charge on the surface of the microspheres is desirable, this property increases the association of the particles with cell surfaces, and hence promotes the uptake of the formulated DNA by APCs. That the positive charge is observed only at higher concentrations of the SA, where the morphology of the microspheres is compromised might be a limitation of the formulation as it is at present. Further modifications might allow increased surface location of the SA to effect an increased positive surface charge at lower concentrations.

5.3.4 Effect of stearylamine on the size distribution of PLGA 50:50 microspheres

The size of the microspheres bears important consequences on the *in vivo* fate of the

formulations. Size distributions were determined by laser diffraction, and are represented in figure 5.5. The volume distributions are presented, and the fundamental size distribution obtained is volume based. This means that when the result reads, for example 10% of the distribution of a certain size, this means that the total volume of all particles with diameters in this range represents 11% of the total volume of all particles in the distribution. The significance of this is realised when the following consideration is made; a spherical particle with a diameter of 2 μm has a volume of 12.6 μm^3, and a 4 μm spherical particle has a volume of 100.5 μm^3. In other words there is an 8-fold increase in the volume of the sphere when the diameter is doubled. Thus, the volume distribution is inherently biased towards the larger-sized particles, as the total volume of a relatively small number of large particles exceeds that of a large number of small particles.

The volume distributions of the particles show that the inclusion of SA in the microsphere formulations causes a shift towards the larger end of the distribution (figure 5.5). This apparent increase in the size of the microspheres was not reflected in the SEM photographs, which all show that the microspheres were around 1 μm. To ensure that there was no or little aggregation of the particles, all samples were dispersed in a dilute solution of SDS or Tween 20, and sonicated prior to sizing. It is therefore not likely that the apparent increase in the size is due to aggregation, however this cannot be totally ruled out, as the addition of the SA might increase the hydrophobicity of the microspheres, increasing the propensity for aggregation.

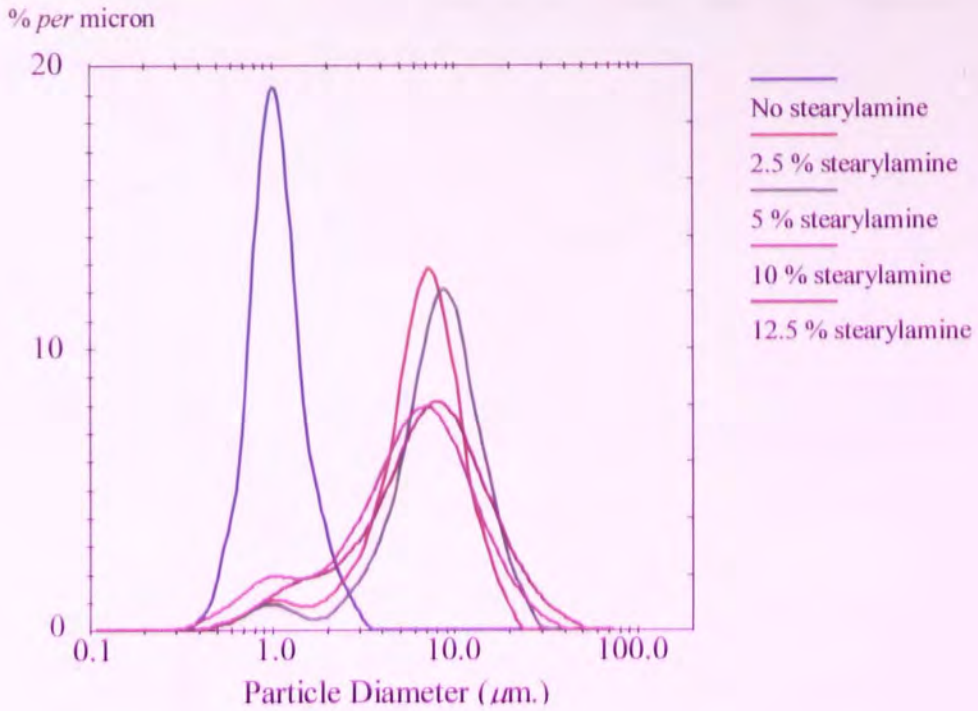


Figure 5.5: Effect of SA on the volume distributions of DNA-loaded microspheres. The diagram shows the frequency plots of microspheres with no stearylamine (**blue line**), 2.5% stearylamine (**red line**), 5% stearylamine (**green line**), 10 % stearylamine (**pink line**) and 12.5 % stearylamine (**brown line**). There is a distinct shift of the distribution to the larger size range on addition of stearylamine. This was however not reflected in the SEM pictures (see figure 5.3).

Another important consideration is the optical properties of the polymers comprising the microspheres. As detailed in section 4.3.4.1.1, the pattern of the scattered laser light is converted to the size distribution based on the presentation unit, which describes the relative refractive index of the material from which the microspheres are made. Addition of SA to the PLGA may alter the refractive index, so that the presentation unit used for the conversion of the scattered light to the size distribution does not account for the altered refractive index of the PLGA-SA blend.

Figure 5.6 shows the number distribution of the microspheres, which gives a better representation of the number of particles within a given size range. The number distribution is less susceptible to the effects of the volume of larger particles. It can be seen that the addition of SA does not affect the number distribution. This suggests that the increase in the volume distribution seen upon SA addition may be ascribed to a few

large particles. The number distribution reflects the size observed in the SEM photographs of the microspheres.

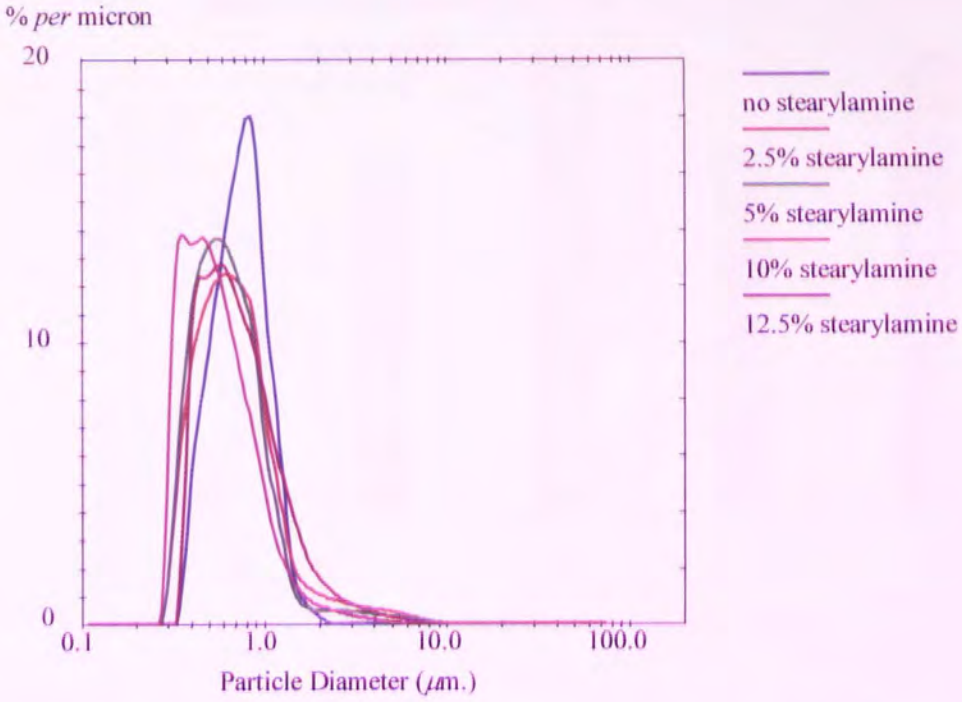


Figure 5.6: Effect of SA on the number distributions of DNA-loaded microspheres. The diagram shows the frequency plots of microspheres with no stearylamine (**blue line**), 2.5% stearylamine (**red line**), 5% stearylamine (**green line**), 10 % stearylamine (**pink line**) and 12.5 % stearylamine (**brown line**).

Figure 5.7 details the effect of the addition of SA on the mean volume and number diameters. These values are derived from the volume distribution data shown in figure5.5, and the uniformity has been included in the chart to describe the spread of the diameters about the mean. The number mean diameters reflect the number distribution; addition of SA has little effect.

Given the stated limitations of the method specific to this formulation, the sizes obtained cannot be taken as absolute values, but are useful for relative comparisons of the effect of SA on the particle size distributions. The SEM photographs confirm the particles to be spherical, with diameters closer to those determined by the number distribution, and not the volume distribution.

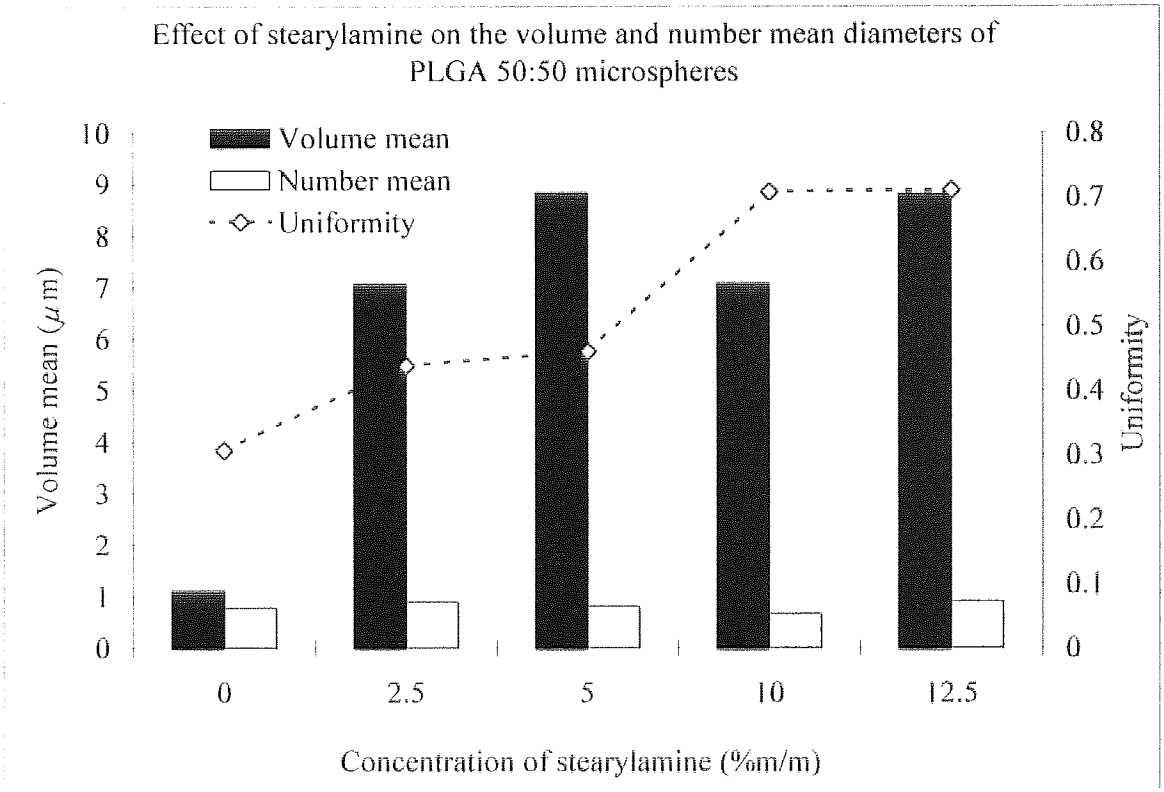


Figure 5.7: Effect of stearylamine on the volume mean diameters of microspheres loaded with plasmid DNA. Addition of stearylamine increased the volume mean diameters of the microspheres, as measured by laser diffraction, and this is reflected in the size distribution graphs shown in figure 5.5. There was also an apparent increase in the uniformity, which is a measure of the absolute deviation of the particle sizes from the mean.

5.3.5 Effect of stearylamine on the encapsulation efficiency of plasmid DNA in microspheres

Microspheres were formulated using increasing concentrations of SA. The effect of the SA on the DNA entrapment efficiency is are shown in figure 5.8. The inclusion of SA in the microsphere formulation generally increased the efficiency of encapsulation from 50 to more than 70 %. The largest increase was seen in the formulation with 2.5 $\%^{m}/_{m}$ SA, beyond this concentration, the encapsulation efficiencies were not significantly higher, but were above that of the SA-free formulation (see figure 5.4). This gives some credence to the possibility of DNA-SA interaction during microsphere preparation, most possibly during the homogenisation of the primary water-in-oil emulsion, when the charged amine end-group of the SA might partition into the aqueous phase, and interact with the phosphate group of the DNA backbone. This association may be stable enough

to prevent the loss of DNA into the larger external

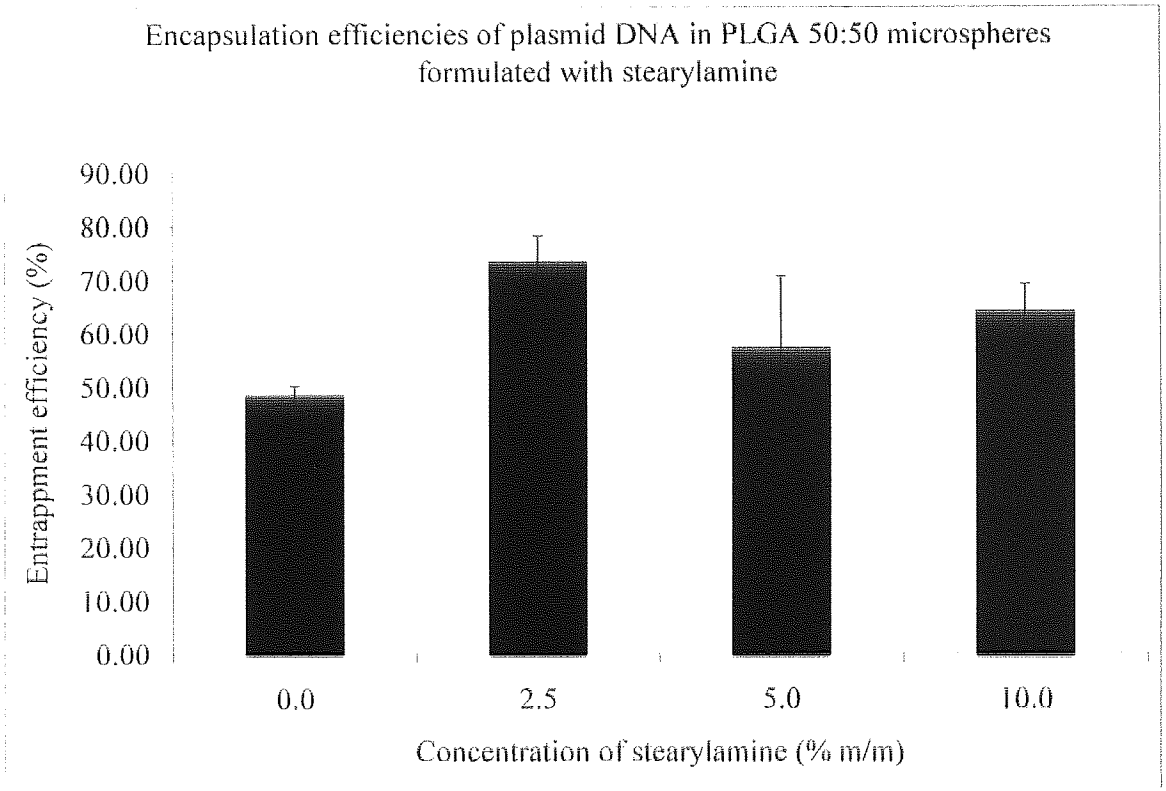


Figure 5.8: Efficiency of plasmid DNA entrapment in microspheres formulated with stearylamine at different concentrations. Each formulation was prepared in triplicate, with each bar representing the mean entrapment efficiency, and standard error represented by the error bars.

aqueous phase during the formation of the double emulsion; the highly hydrophobic chain of the SA anchors the molecule and any associated DNA to the organic phase, or inner aqueous phase.

A high encapsulation efficiency is a desirable property of microsphere formulations for two main reasons, not least the fact that a larger amount of DNA may be delivered *per* mass of microspheres administered *in vivo*. The economics of large-scale production dictates that the efficiency of entrapment make the formulation financially viable, such that the active ingredient, plasmid DNA, is not wasted due to poor efficiencies of incorporation into the proposed formulation. Stearylamine is a cheap excipient which appears to increase the encapsulation efficiency of plasmid DNA into microspheres.

5.3.6 Influence of stearylamine on the release of plasmid from microspheres

The release of the entrapped plasmid was studied, and figure 5.9 shows the release profiles. There was a steady release of the plasmid from microspheres with no SA, and all the plasmid was released by day 28. Addition of SA at 2.5 % suppressed the release, and only ~40 % of the plasmid was released. At 10 % SA concentration, there was a complete inhibition of the entrapped plasmid release.

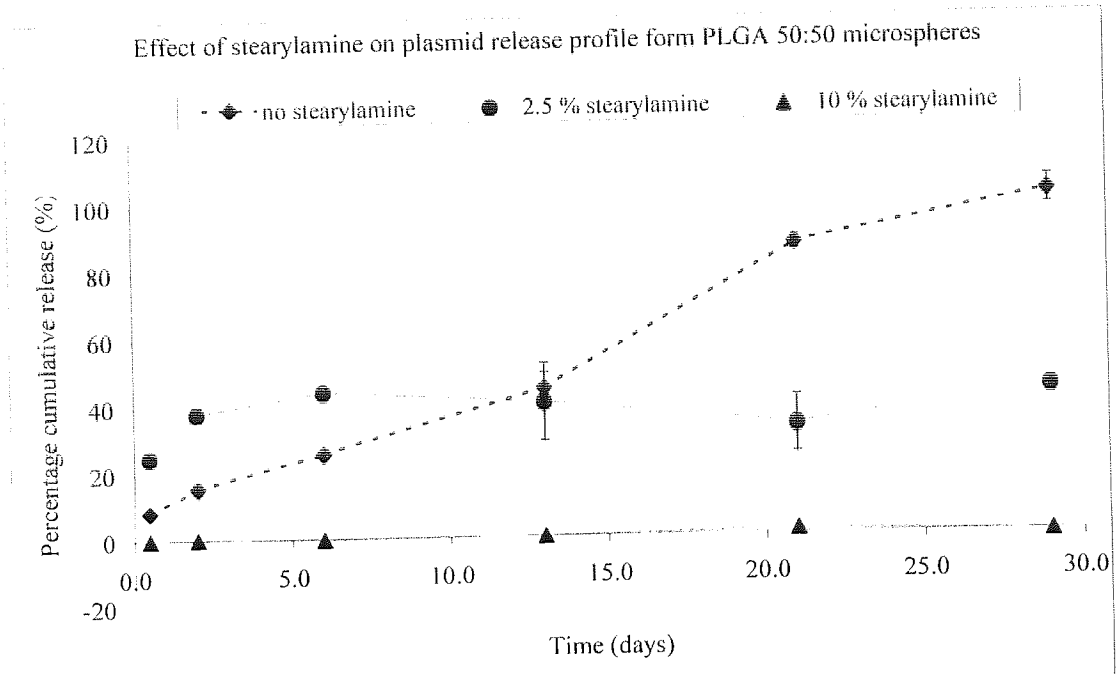


Figure 5.9: Release profile of plasmid DNA from microsphere formulations. The graphs are the mean of triplicate experiments, and the error bars represent the standard error of the mean.

Thus SA progressively reduces the release of plasmid from microspheres, even when present in small concentrations. While the mechanism of this observation is not precisely known at present, a knowledge of the properties of the SA and DNA allows a tentative postulation. The SA molecule has hydrophobic and positive-charge properties, both of which may be responsible for the reduction in the release of plasmid.

An increase in the hydrophobicity of the microspheres, imparted to it by the C_{18} chain of the SA molecule, would reduce the wettability of the microspheres and hinder the penetration of water. This will in turn reduce the rate of degradation of the PLGA polymer, so that less DNA is released in the given time. Coupled with this is the possible close association of the SA with the DNA by a charge interaction. A retention

of the SA in the microspheres will translate into a reduced release of plasmid bound to it. Regardless of the precise mechanism, SA appears to be a useful tool for the control of the release of plasmid from microspheres.

5.3.7 Analysis of plasmid DNA extracted from microspheres formulated with stearylamine

The conformational quality of the plasmid DNA in the microspheres was assessed by agarose gel electrophoresis after extraction from the different formulations. Figure 5.10 shows the agarose gel after a 60 minute electrophoresis run.

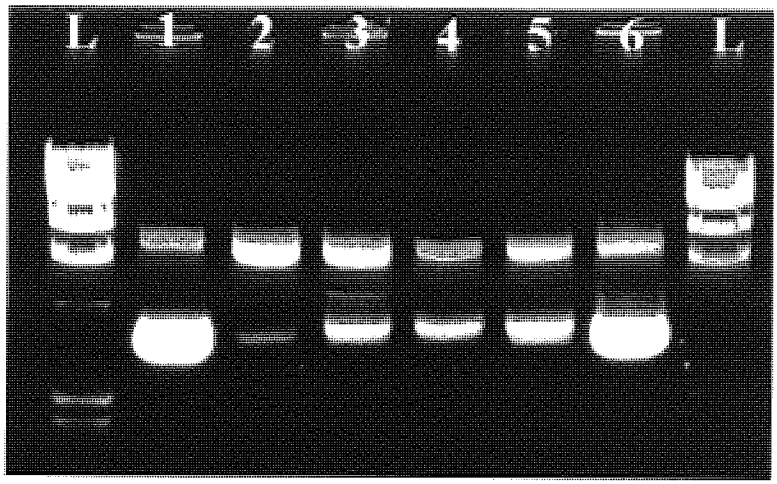


Figure 5.10: Analysis of plasmid DNA extracted from PLGA 50:50 microspheres after lyophilisation. The plasmid samples were obtained from microspheres formulated with no stearylamine(lane 2), 2.5 % stearylamine (lane 3), 5 % stearylamine (lane 4), and 10 % stearylamine (lane 5). Lanes 1 and 6 are control DNA samples (non-formulated), L = λ/Hind III marker. The samples were run for 60 minutes at 80V on a 1 $\%^{m}/_{v}$ agarose gel stained with EtBr.

The gel shows the retention of the supercoiled conformation by the plasmid in all of the formulations, but the inclusion of stearylamine (lanes 3 - 5) resulted in a higher proportion of SC DNA being retained. It is possible that the electrostatic association of SA and DNA is responsible for apparent protection of DNA from the degradative forces of the formulation process.

To evaluate the stability of the formulated plasmid DNA, the microspheres were stored at 4°C, 18°C, and 37°C. At different time points the plasmid DNA was extracted as described in section 4.3.4.4, and analysed by agarose gel electrophoresis. The optical

densities of the bands corresponding to the different conformations were determined as described in section 3.3.1.

The time profiles of the plasmid stability at different temperatures are displayed in figure 5.11a-c. The percentage of supercoiled plasmid remaining after the specified time is presented.

The DNA extracted from 10 %SA microspheres retained some SC conformation after 3 weeks of storage. Plasmid extracted from microspheres with 2.5 % SA retained 23 % of SC DNA, compared with 38 % for plasmid from 10 %SA microspheres, after 1 week. There was a general increase in the loss of the SC conformation with storage at higher temperatures, and only microspheres with 10 % SA were able to preserve the SC DNA of formulated plasmids for any period of time at 37°C. The stabilising effect of the SA on the formulated DNA appears to be concentration dependent, with even 2.5 % SA increasing the retention of SC DNA somewhat, compared with that of microspheres containing no SA at 18°C. Figure 5.11indicate that plasmid DNA incorporated into microspheres with SA were more resistant to the loss of the supercoiled conformation.

The temperature dependence of the loss of the SC conformation suggests that it is driven by a chemical reaction. The introduction of a single break in the DNA backbone will convert SC DNA to the open circular form. Under aqueous conditions, DNA is known to degrade by a two-step process of depurination and β-elimination, which leads to cleavage of the phosphodiester backbone (Evans RK *et. al.*, 2000). Depyrimidation also occurs but at much slower rates. The depurination is acid catalysed and is initiated by protonation of the purine base at the N-7 position, which leads to a cleavage of the n-glycosidic bond to produce an apurinic site. These apurinic sites provide a site for further chemical change in the DNA, leading to strand breakage. The

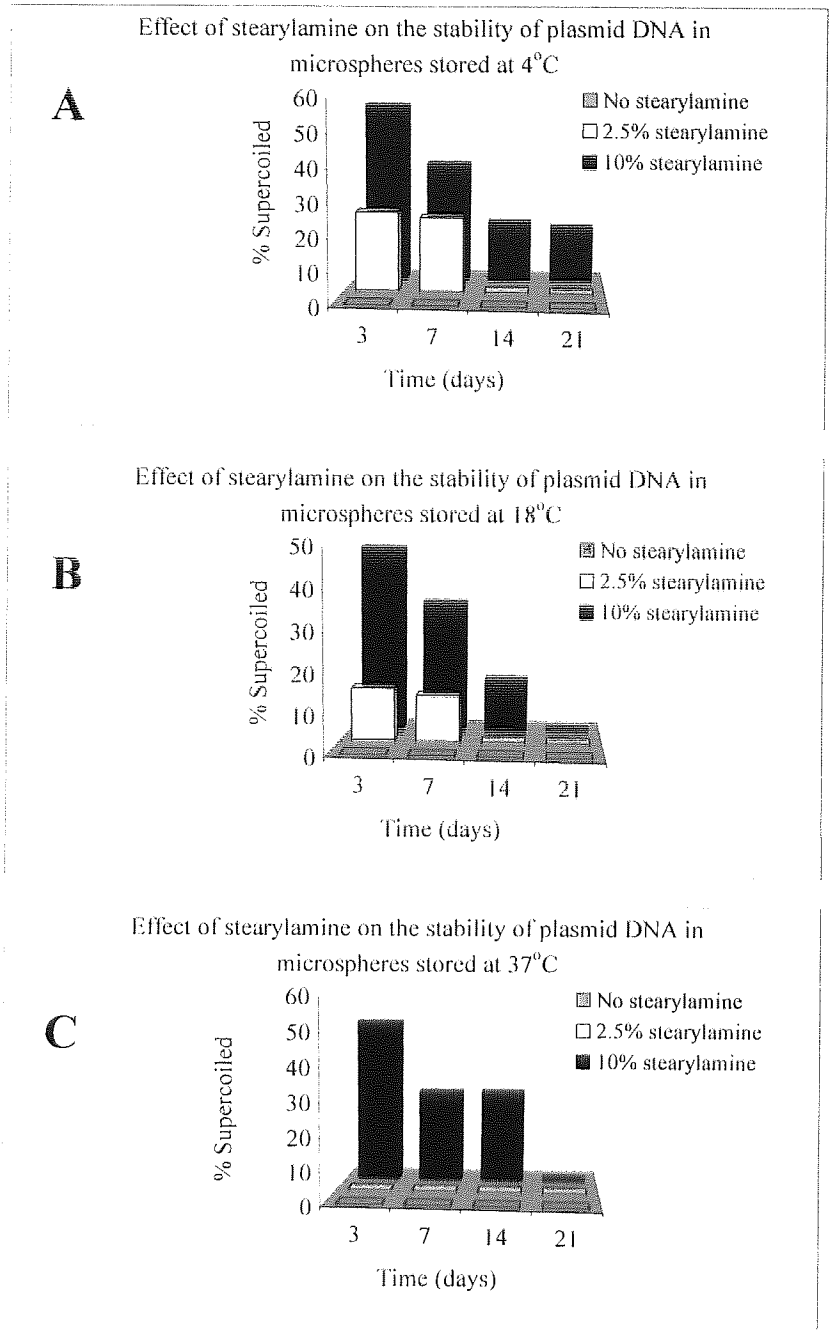


Figure 5.11: Stability of plasmid DNA in microspheres formulated with stearylamine at 2.5 $\%^{m}/_{m}$ and 10 $\%^{m}/_{m}$. All formulations were in triplicate, and the stability was assessed by determining the retained proportion of the plasmid that was in the supercoiled conformation after storage at 4°C (A), 18°C (B) and 37°C (C).

rate of strand breakage may be lower than that of depurination, so that apurinic sites may accumulate in the DNA molecule (Evans RK *et. al.*, 2000; Middaugh CR *et. al.*, 1998). Thus, it is possible that the rapid loss the SC DNA observed in the extracts from

microspheres with no SA are due to the presence of apurinic sites. This is acid catalysed, and the PLGA microspheres have been shown to have acidic interiors (Brunner A *et. al.*, 1999; Fu K *et. al.*, 2000). The presence of the amine groups on the SA molecule could serve to reduce the acid catalysed depurination, however, some amine groups are able to catalyse the β-elimination reaction that cleaves the DNA at apurinic sites (Evans RK *et. al.*, 2000; Middaugh CR *et. al.*, 1998). It is therefore crucial that preparations are entirely devoid of moisture, and that the plasmid DNA to be formulated is of a very pure quality, bearing no apurinic sites.

This study shows that the DNA extracted from the microspheres initially retains a proportion of the SC conformation, and that SA affords a certain amount of protection to the DNA tertiary structure during the formulation process. However, there is rapid loss of the SC DNA on storage at low or elevated temperatures, possibly by the depurination and β-elimination pathways described. SA appears to reduce this chemical degradation. It is hypothesised that the amine group of the SA suppresses the formation of apurinic sites, but further investigations are required to establish this proposed mechanism of protection.

5.3.8 Assessment of *in vitro* uptake of microspheres by RAW cells

The internalisation of the microspheres by a phagocytic cell line, RAW, was studied in order to assess the feasibility of uptake of the DNA vaccine formulation by APCs *in vivo*. The method as described in section 5.2.2 involved placing a suspension of microspheres containing radio-labelled DNA onto the cells. The cells were then washed, so that any subsequent radioactivity detected was ascribed to internalised microspheres.

The washings from the cells were analysed for radioactivity, in order to establish adequacy of the washing method. This is important, because the method of quantifying the uptake does not differentiate between microspheres in the media (i.e. not internalised), or those loosely associated with the cell surface. The graph in figure 5.12a shows the results of the washing procedure, after incubation of the cells with a 10 or 100 μg mass of microspheres. The sensitivity of the scintillation counter was also tested.

The results displayed in figure 5.12b confirms that the limit of detection of the microspheres by the scintillation counter validates the washing procedure. Figure 5.12a shows that a 20-minute count of the microspheres allows the detection of 20 ng of microspheres. Therefore a lack of radioactivity in the washings of the cells is indicative of a lack of microspheres, not an inability to detect microspheres which are present. In figure12b, it can be seen that the there are no particles left after the first wash. There would appear to be no requirement for the second and third wash. Nevertheless, all subsequent experiments were done using three washes of the cells.

The radio-labelled DNA was formulated into microspheres using increasing concentrations of SA. It was anticipated that the increase in positive charge observed will translate into an increase in the microsphere uptake, due to an increased association of the particles with the inherent negative charge of the RAW cells. This was hypothesis was not realised. Microspheres with a SA concentration of more than 2.5 % showed a marked reduction in uptake (Fig 5.13). It has long been established that a net positive charge on particles increases their cellular uptake (Schwendener RA *et. al.*, 1984). Here, it was observed that uptake was actually reduced by the increase in the SA. Whether this is due to some other property of the SA molecule bar its positive charge will require further investigation to establish. Nevertheless, at a SA concentration of 2.5 $\%^{m}/_{m}$, there is no difference in the uptake of the microspheres. It has previously been shown that this SA concentration is consistent with an improvement in microsphere morphology (figure 5.2), and an increase in the encapsulation efficiency of plasmid DNA (figure 5.8).

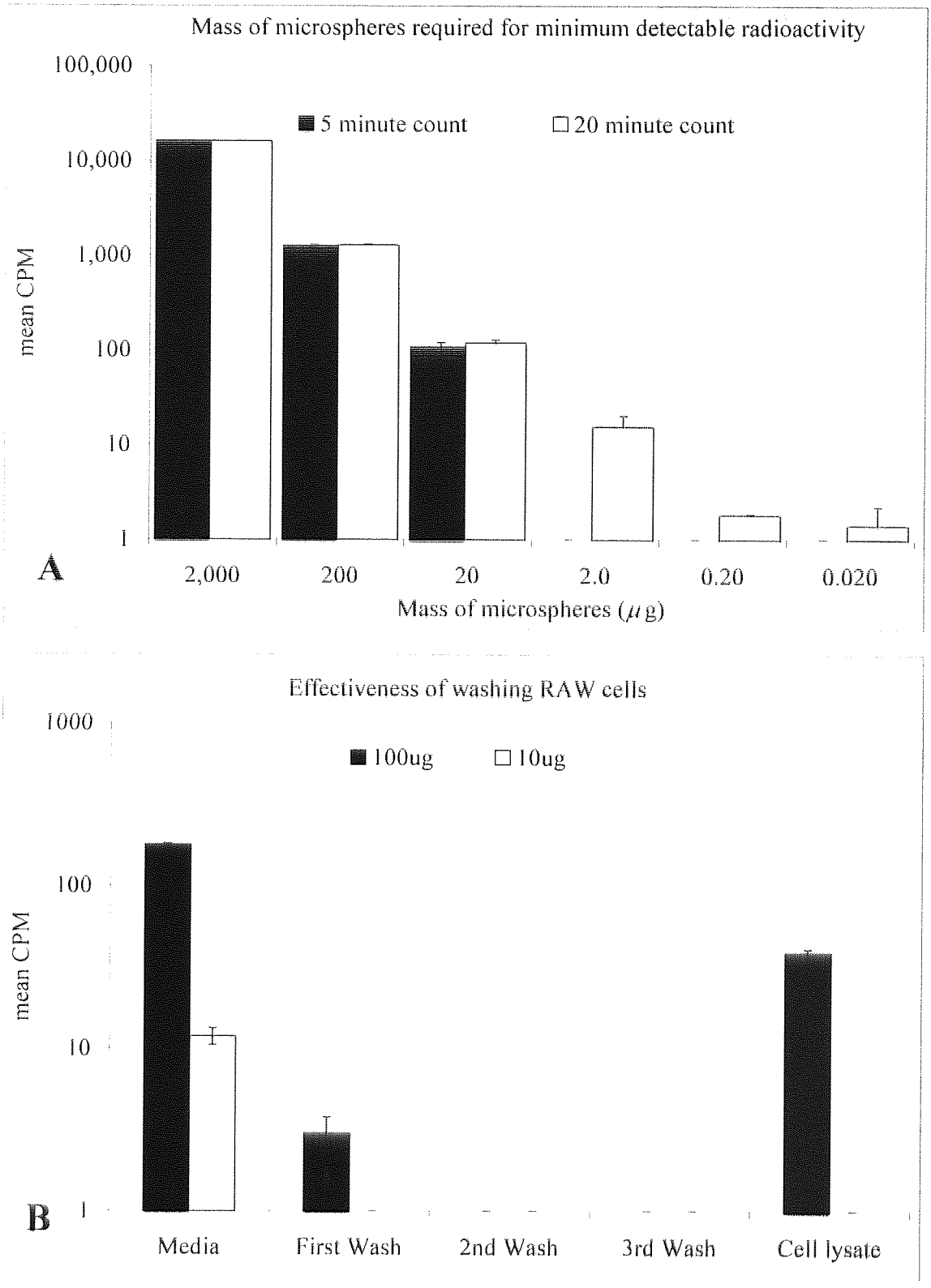


Figure 5.12a and b: The lower limit of detection of microspheres containing radio-labelled DNA by the scintillation counter. Each bar represent the mean of triplicate wells, and the standard error of the mean shown by the error bars. Figure A demonstrates that scintillation counting for 20 minutes is more sensitive at lower masses of microspheres. Figure B confirms the effectiveness of the wash prior to obtaining the cell lysate.

the formulation appears to have a favourable effect on the biological integrity of the plasmid, an observation reflected the in the agarose gel analysis of the structural integrity.

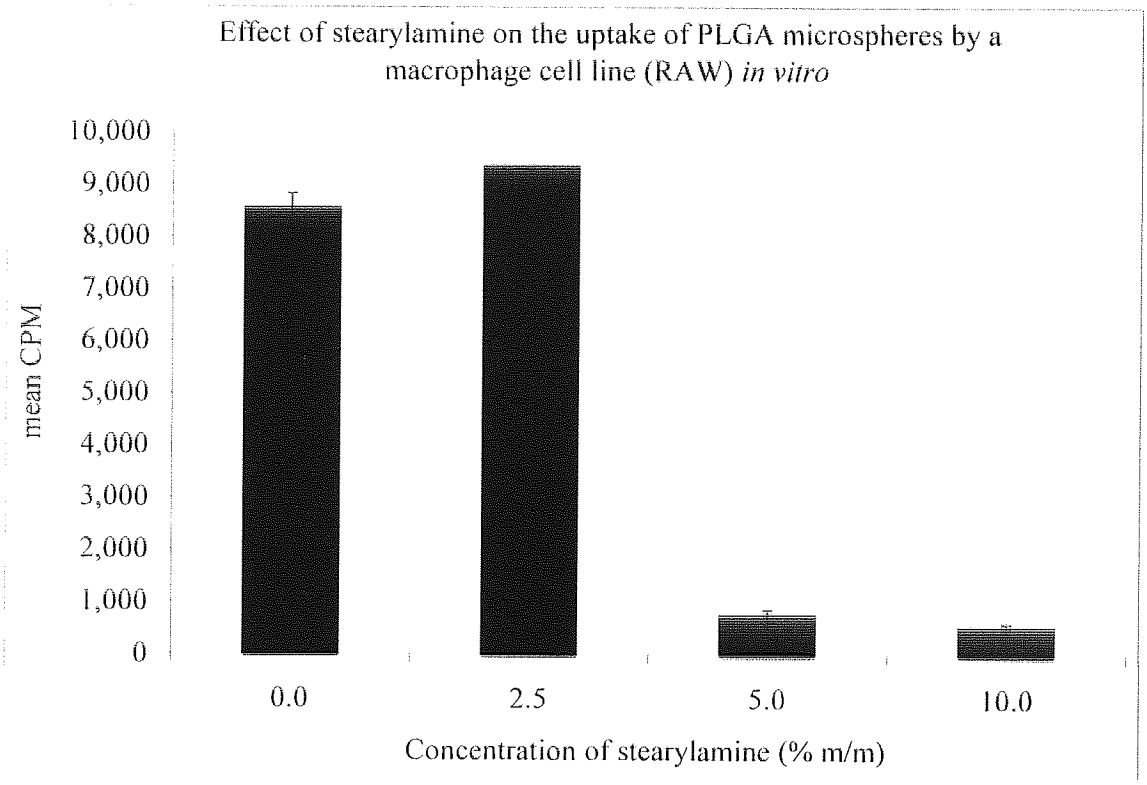


Figure 5.13: Assessment of the *in vitro* uptake of microspheres containing radio-labeled DNA by RAW cells.

5.3.9 Protection of DNA from serum nucleases

Microspheres containing plasmid DNA were incubated with cell culture medium (DMEM) supplemented with 10 % foetal calf serum (FCS), and the extent of protection from nuclease enzymes by microsphere entrapment assessed by agarose gel electrophoresis of the extracted plasmid samples.

Figure 5.14 illustrates the extracted plasmid samples after agarose gel electrophoresis. There is an increased resistance to degradation to the serum nucleases with increasing stearylamine.

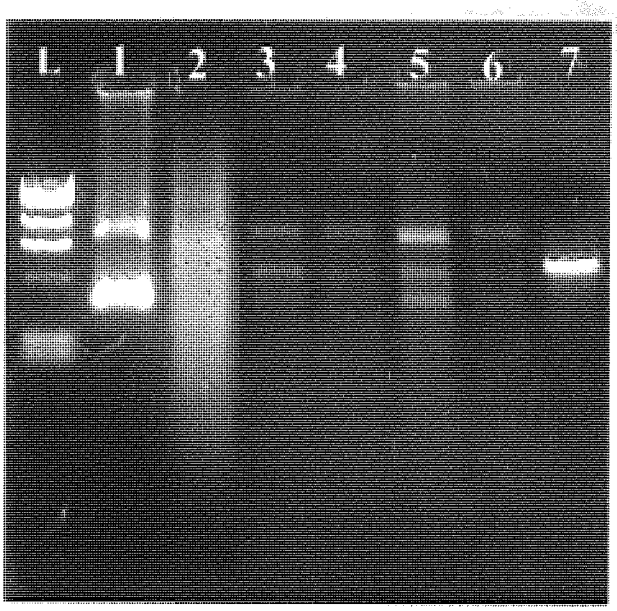


Figure 5.14: Protection of encapsulated plasmid DNA from serum nucleases. Microspheres were incubated in cell culture medium supplemented with 10 $\%^{v}/_{v}$ FCS at 37°C for 4 hours. The DNA was extracted from the microspheres and analysed by 1 $\%^{m}/_{v}$ agarose gel electrophoresis at 80V for 1 hour, using EtBr as the gel stain. The bands represent DNA extracted after incubation from microspheres formulated with stearylamine at concentrations of 0 $\%^{m}/_{m}$ (lane 3); 2.5 $\%^{m}/_{m}$ (lane 4); 5 $\%^{m}/_{v}$ (lane 5); 10 $\%^{m}/_{v}$ (lane 6); L = λ/HindIII size marker, lane 1 is plasmid DNA incubated in media containing no FCS; lane 1 is non-encapsulated plasmid DNA incubated directly in medium with 10 % FCS. Lane 7 is linearised DNA control.

As evidenced by a direct comparison of lanes 2, 3 and 5 in figure 5.14, the microencapsulation of DNA results in protection from enzymatic assault in foetal calf serum for 4 hours at 37°C. The non-encapsulated DNA (lane 2) was totally degraded in this time, while that in microspheres with no SA (lane 3) was protected, and even more protected at 10 % SA (lane 6). Lanes 4 and 6 both show faint DNA bands suggesting protection, and the faintness is most likely due to minute quantities of DNA, and not degradation. All samples show an increased level of linearised DNA; this is indicative of some contact of the DNA with digestive enzymes, and it is proposed that the association of the DNA with the polymer matrix was the main means of protection. The increased level of protection observed in the presence of SA could be a combination of its hydrophobic nature and the electrostatic interaction of the amine with the DNA phosphate backbone, both of which could serve to reduce the accessibility of the DNA to the enzyme.

5.3.10 *In vitro* biological activity of plasmid DNA extracted from PLGA 50:50 microspheres formulated with stearylamine

The formulated plasmid DNA was extracted as described in section 4.3.4.4, and the biological activity assessed by evaluation of the expression of luciferase in a 293 cell line. DNA extracted from microspheres formulated with various concentrations of SA were complexed with SuperFect™ transfection reagent as described in section 2.2.5.3. The luciferase expression levels are shown in figure 5.15.

The expression levels generally follow the trend seen in the structural retention of the SC conformation, shown in figure 5.10. The plasmid encapsulated in microspheres with no SA had visually the least proportion of SC DNA, and showed the lowest level of biological activity. Expression levels generally increased with the concentration of SA in the microsphere formulation. At 10 $\%^{m}/_{v}$ SA there was a reduction in the luciferase expression. This was not expected as there was a high proportion of SC DNA in the extract of plasmid from this formulation.

The experiment shows that plasmid DNA extracted from microspheres formulated with SA by the double emulsion solvent evaporation method is biologically active and capable of expressing the encoded protein and potential antigen. The addition of SA to the formulation appears to have a favourable effect on the biological integrity of the plasmid, an observation reflected in the agarose gel analysis of structural integrity.

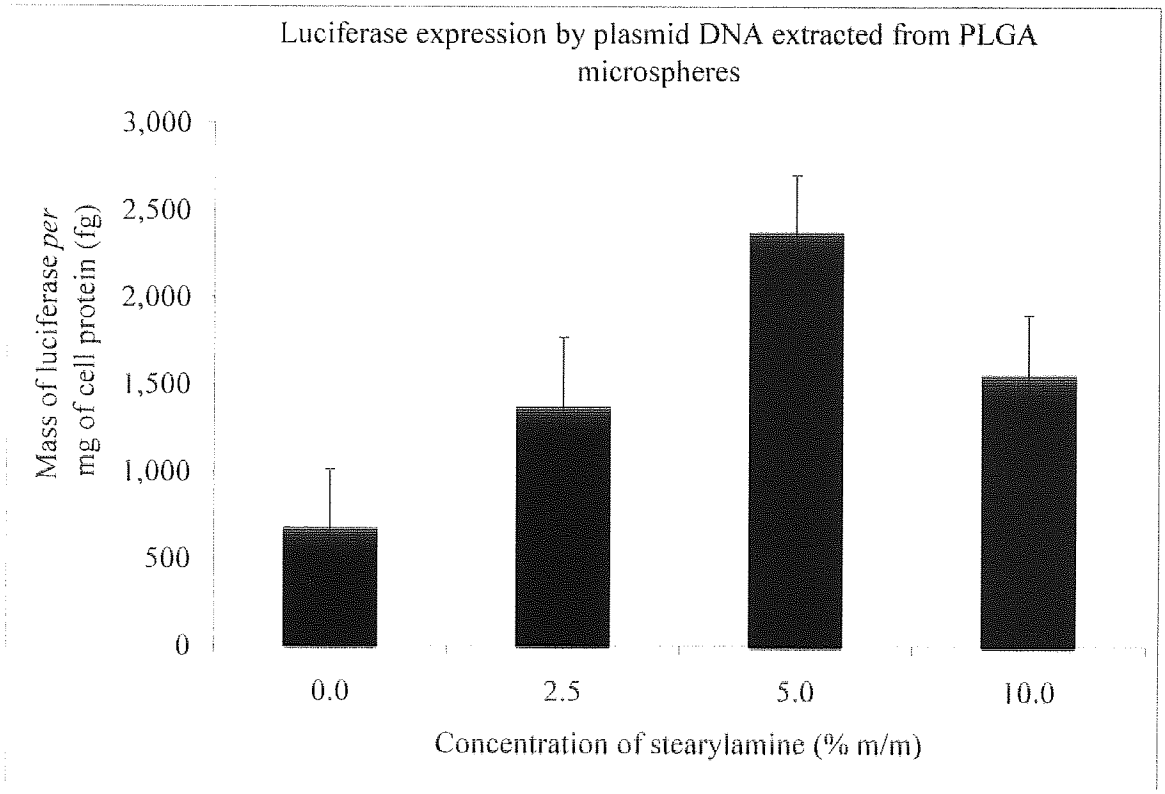


Figure 5.15: Luciferase expression by plasmid DNA extracted from PLGA 50:50 microspheres prepared by the double emulsion solvent evaporation method. The biological activity was evaluated by transfection of an epithelial cell line, 293. Each bar represents the mean mass of luciferase expressed *per* mass of cell protein of three transfection experiments, with the respective standard errors represented by the error bars.

5.4 Conclusions

The use of stearylamine in the formulation of DNA vaccines into microspheres has been investigated, on the premise that the hydrophobic and positive-charge properties of the molecule would present certain desirable features. It was postulated that SA would allow control of the surface charge of the microspheres. A net positive charge on the microspheres increases the cell-surface association, and subsequently increases cellular internalisation. The C_{18} hydrocarbon chain of the molecule could also increase the surface hydrophobicity of the microspheres, further aiding their uptake by cells *in vivo*. This expectation was not borne out, and it would appear that SA actually reduced the uptake of microspheres at high concentrations. However, the uptake of microspheres containing 2.5 $\%^{m}/_{v}$ SA was comparable with microspheres with no SA; a 2.5 $\%^{m}/_{v}$ SA concentration in microspheres was effective at increasing the stability and entrapment

efficiency of the plasmid DNA.

SA increased the efficiency of DNA loading, improved the quality of the encapsulated plasmid DNA in terms of the retention of the SC conformation and also increased the resistance to the degradative action of serum enzymes during a 4 hour incubation. At 10 % SA, the microsphere morphology was disrupted, although an increase in the DNA concentration reversed this trend. Plasmid DNA extracted from the microspheres were shown to be biologically active, and this activity appeared to correlate with the SA concentrations. Another important effect of SA was the increase in the proportion of SC plasmid retained in microspheres. It was found that, generally, the formulated plasmid was not stable in the long term. While it is possible that the initial quality of the DNA is partly responsible for the observed degradation, the formulation process, previously shown to reduce the proportion of the more stable SC plasmid, could have introduced nicks into the formulated plasmid. These act as initiation sites for further chemical degradation of the plasmid.

At this stage the microspheres have not been assayed for their SA content, and all stated concentrations have been nominally assigned to the formulations, depending on the concentration of SA used in the preparation. Further work is needed to determine the exact amount of SA retained within each formulation, and to investigate any interactions between SA, PLGA and DNA.

6. Entrapment of plasmid DNA into microspheres incorporating stearylamine, prepared by a spray-drying method

6.1 Introduction

The formulation of microspheric DNA vaccines by techniques based on the double emulsion solvent evaporation method has been described. One potential disadvantage of this method is the difficulty in scaling-up for large-scale production of formulated vaccines.

Spray-drying is a technique used for large-scale production of dry powders from solutions, suspensions and slurries, simultaneously creating and drying formed particles. The resulting powder characteristics may be controlled and maintained constant throughout the operation. The process allows the production of microspheres in a sterile environment (Masters K, 1991), making it suitable for Good Manufacturing Practice (GMP) production of formulated vaccines.

6.1.1 The spray-drying process

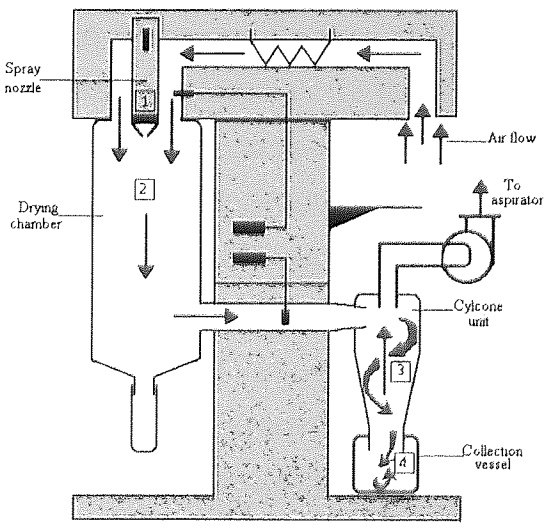


Figure 6.1: Simplified diagram of the Büchi® mini spray-drier. The arrows indicate the direction of heated airflow, provided by the aspirator. The emulsion (w/o) is mixed with high-velocity air which is simultaneously but separately fed into the nozzle (1) to produce atomised droplets. The droplets are sprayed into the drying chamber (2), concurrent with a heated mass of air and rapidly dried to produce particles, which are separated by the cyclone unit (3), and deposited into the collection chamber (4) (Conte U and Giunchedi P, 1995).

Figure 6.1 is a simplified diagrammatic representation of the Büchi® mini spray drier showing the direction of airflow through the system. The product to be spray-dried is fed through the nozzle and atomised by high velocity air (more than 800 NL/h) supplied separately into the nozzle. The resulting spray is instantaneously dried as it is introduced into the heated drying chamber. This inlet drying temperature may be as high as 200°C. It is during this stage of the process that the solvents (both organic and aqueous) are lost by a rapid two-stage evaporation; initially the solvent within the droplet replenishes that lost at the surface by rapid evaporation, this constant diffusion maintains saturated surface conditions, and evaporation occurs at a constant rate. This is the first period of drying. When the rate of solvent loss from the surface of the particle is higher than the rate of replenishing from within the droplet, surface saturation cannot be maintained and a dried shell forms at the droplet surface. At this point the thickness of the dried surface shell increases with time, causing a decrease in the rate of evaporation. This is the second period of drying (Masters K, 1991). By this mechanism of drying the shape of the dried product may by disrupted, depending on the characteristics of the product being spray dried. Figure 6.2 shows a simplified diagram of the possible shapes of dried particles which may either expand, collapse, fracture or disintegrate, and either porous or irregularly shaped particles may form. The product is not subject to the heat of evaporation, and it is outlet temperature which determines the temperature to which the product is transiently exposed during transfer from the drying chamber to the cyclone unit (see Figure 6.1). The main consideration for the formation of spherical particles during formulation of polymers appears to be the choice of polymer, its concentration and the type of organic solvent for the polymer. Conte *et. al.*, found that the best results in terms of microsphere shape were achieved with a 3 % $^{m}/_{v}$ total concentration of polymer and drug (Conte U *et. al.*, 1994), while Walter *et. al.*, used an optimised polymer concentration of $4^{m}/_{v}$ % for DNA formulation (Walter E *et. al.*, 1999). A detailed study by Bodmeier and Chen revealed that the type of polymer, its Mw and degree of viscosity affected the formation of the particles (Bodmeier R and Chen H, 1988). While ethylcellulose solutions led to a spherical product at concentrations as high as 10 $\%^{m}/_{v}$, PLA in DCM resulted in the formation of fibres as low as $1^{m}/_{v}$ %. It was postulated that intermolecular bonds of the polymeric chains can be responsible for very strong chain interaction, and thus a high degree of fibre formation, which stalls the

formation of microspheres.

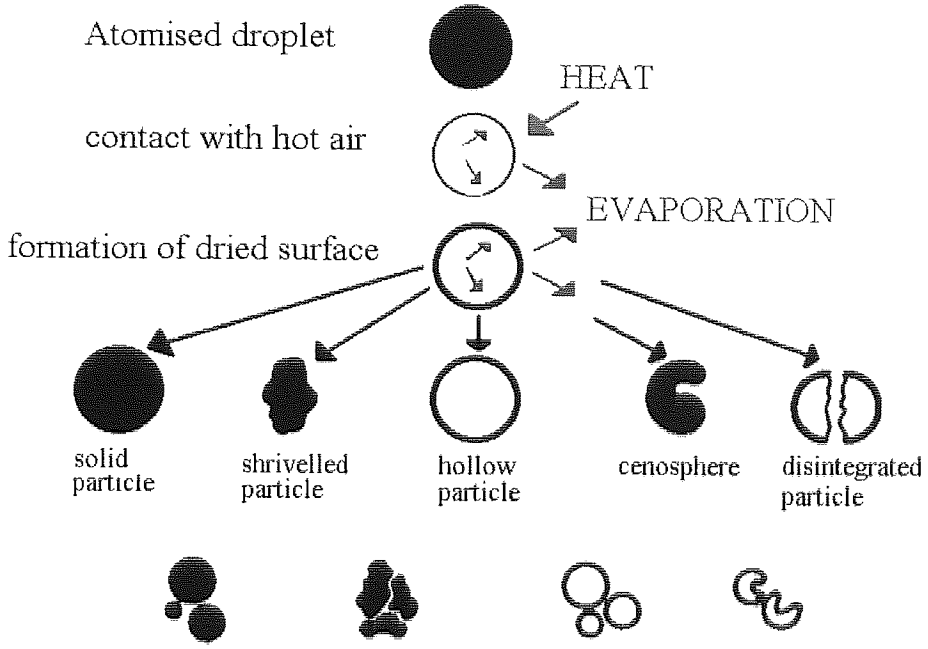


Figure 6.2: Mechanisms of droplet drying illustrated in a simplified manner. The droplets may dry to single, misshapen and agglomerated particles (Masters K, 1991).

The main advantage of the spray-drying process over the double emulsion solvent evaporation method is primarily its potential for scale-up to mass production of the formulated DNA vaccine. This method of production of dry powder is now well established, facilitating the transfer of the technology to industrial-scale manufacture. That the spray-drying process is single-step closed system presents several other advantages; products may be kept sterile, and the manufacturing process parameters can be easily and precisely controlled, monitored and validated to ensure reproducibility. Sterility of the product can be preserved, and Good Manufacturing Practice (GMP) can be strictly adhered to, hence this method of particle formation is pharmaceutically more acceptable.

The heat supplied to the feed is lost mainly as energy to evaporate the solvent, so that heat-labile biomolecules are not exposed high temperatures. Spray-drying also offers a gentler means of droplet formation and there is a reduced contact time between the

DNA and the organic solvent attributable to the rapid evaporation. These qualities translate to a less harsh environment for the DNA being formulated.

There are, however, some drawbacks to the spray-drying method; the process requires large masses of both polymer and plasmid DNA, as the product yield is notoriously low. This a technical drawback in the laboratory-scale, as formulation excipients may either not be readily available, or too costly during this stage of formulation development. The yield may, however, be controlled to some extent by reduced rate of feed (Johansen P *et. al.*, 2000).

In this study, the feasibility of producing a microspheric DNA vaccine formulation by spray-drying was investigated, using PLGA with stearylamine.

6.2 Materials and methods

6.2.1 Spray-drying of PLGA microspheres

Organic solutions of PLGA 50:50 (63 kDa) with various concentrations of stearylamine were made-up to a total polymer concentration of 4 $\%^{m}/_{v}$ in $CHCl_3$ and kept on ice. The volume of the organic solution was 12.5 mL (i.e a total polymer mass of 500mg *per* batch). The organic polymer solution was emulsified with aqueous plasmid DNA solution by homogenising at high speed for 2 minutes, and immediately fed into the 0.5 mm feeder nozzle of the Büchi® Mini Spray-Drier. The emulsion was sprayed at a rate of 1 mL/min, and dried at an inlet temperature of 50°C, with an outlet temperature of 39°C-40°C. The airflow was set at 800 NL/h , and the aspirator at 70%. The dried particles were washed out of the collection vessel and cyclone unit (see diagram) with a 0.1 $\%^{m}/_{v}$ solution of Pluronic F68, filtered through a 0.45 μm-pore cellulose acetate filter, and washed with double-distilled water before desiccation in a vacuum oven for 24 hours.

6.2.2 Characterisation of microspheres

The microspheres obtained were characterised with respect to their size distribution and surface potential, as described in section 4.3.4. The morphology of the particles was

studied by scanning electron microscopy, as described in section 4.3.4.3. The loading and encapsulation efficiency of the DNA was determined by picogreen assay, as described in section 4.3.4.4. The quality of the DNA after formulation and exposure to serum enzymes was also investigated.

6.2.3 Resistance of formulated DNA to serum nucleases

A 10 mg mass of the microsphere preparation was accurately weighed into a sterile microfuge tube, and suspended in 1 mL of cell culture media (DMEM) supplemented with 10 % foetal calf serum. The samples were incubated at 37°C for 4 hours with mild shaking, and then centrifuged at 15,000*g* for 20 min to pellet the microspheres. The medium supernatant was discarded and the pellet washed by re-suspension in sterile PBS. The suspension was centrifuged, the supernatant discarded, and 1 mL of $CHCl_3$ added to dissolve the microspheres. A 100 μL aliquot of sterile TE was added and the mixture left shaking for 20 minutes to allow the DNA to partition into the aqueous phase. A 20 μL sample of the aqueous phase was obtained and analysed for the quality of the DNA by agarose gel electrophoresis.

6.2.4 Release of plasmid DNA from spray dried microspheres

A 10 mg mass of microspheres was accurately weighed into a sterile 2 mL microfuge tube. This mass of microspheres was suspended in 1 mL of sterile PBS with 0.01 $\%^{m}/_{v}$ sodium azide. The suspension was placed at in an orbital shaker at 37°C. The tubes were positioned horizontally to ensure that the mass of microspheres were constantly agitated to prevent stagnation and settling. At set times, a 200 μL aliquot of the suspension was obtained and this was centrifuged at 15,000*g* for 10 minutes to pellet the microspheres. A 50 μL sample of the release medium supernatant was removed replaced with 50 μL of fresh sterile PBS containing 0.01 % sodium azide at 37°C. The 200 μL sample was resuspended and added to the original 2 mL microfuge tube. The 50 μL release medium sample was stored at -20C until analysis for DNA concentration using picogreen assay reagent. All release studies were performed in triplicate.

6.3 Results and discussion

6.3.1 Effect of stearylamine on microsphere morphology

Figure 6.3 below shows the effect of stearylamine on the morphology of particles prepared by spray-drying.

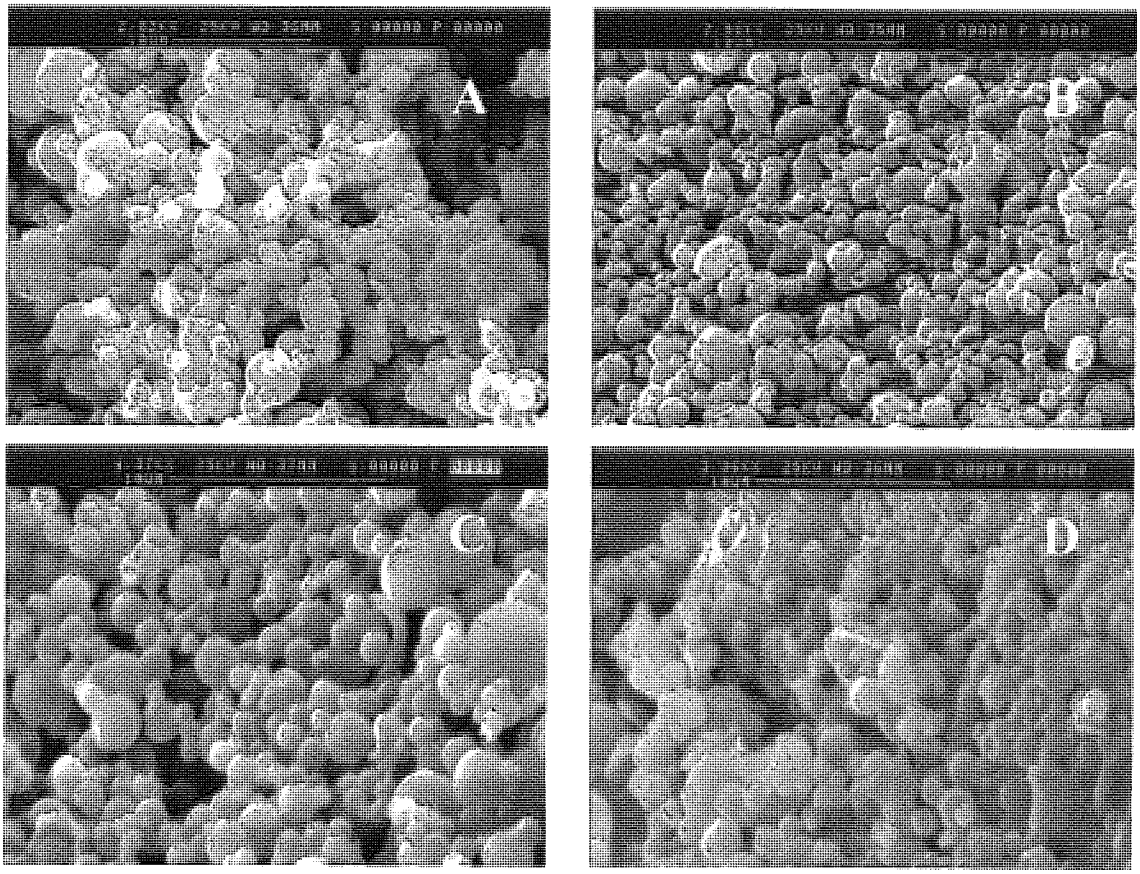


Figure 6.3a-d: Effect of stearylamine on the morphology of particles obtained by the spray-dying of a water-in-oil emulsion of aqueous plasmid DNA and PLGA 50:50 in chloroform, containing stearylamine at 0 $\%^{m}/_{m}$ (a), 5 $\%^{m}/_{m}$ (b), 10 $\%^{m}/_{m}$ (c) and 20 $\%^{m}/_{m}$ (d). There was an improvement in general morphology of the microspheres, regarding formation of discrete particles and reduction in agglomeration, with increasing stearylamine, reaching an optimum at about 10 $\%^{m}/_{m}$ stearylamine concentration. Any further increase in the stearylamine resulted in non-spherical agglomerates of the polymer.

Particles obtained by spray-drying the polymer without stearylamine were typically agglomerated, but distinct microspheres were discernible from the SEM pictures (see fig 6.3a). Addition of stearylamine at a 5 to 10% $^{m}/_{m}$ resulted in more uniformly dispersed microspheres (see fig.6.3 b and c), but any further increase in stearylamine produced malformed, non-spherical agglomerates of the microspheres (see fig.6.3 d).

The morphology of the particles is determined by the formation of discrete droplets during the atomisation stage and then subsequently by the rates of evaporation of the aqueous and organic solvents from the droplet. The emulsion fed into the nozzle is a water-in-oil emulsion and the organic phase, being more volatile, is likely to evaporate at a quicker rate. This will reduce the primary drying phase and a hard polymer shell forms before the internal water phase is formed, increasing the risk of microsphere disruption during the secondary drying phase. The addition of stearylamine might improve the rate of movement of water vapour from the internal structure of the droplet, possibly by intercalating between PLGA molecules. This increase in the ease of water loss from the droplet results in a more uniform secondary drying phase to form microspheres with improved morphology. However, at a stearylamine concentration of more than 10 $\%^{m}/_{m}$, it is possible that the rate of water vapour loss is too rapid, and this disrupts formation of spherical microparticles.

6.3.2 Effect of stearylamine on the encapsulation efficiency of plasmid DNA

That the electrostatic association of the phosphate backbone of DNA molecules with the amine of the stearylamine molecule might lead to increased encapsulation efficiencies was tested by determining the amount of DNA *per* mass of microspheres. The results are shown in figure 6.4 below.

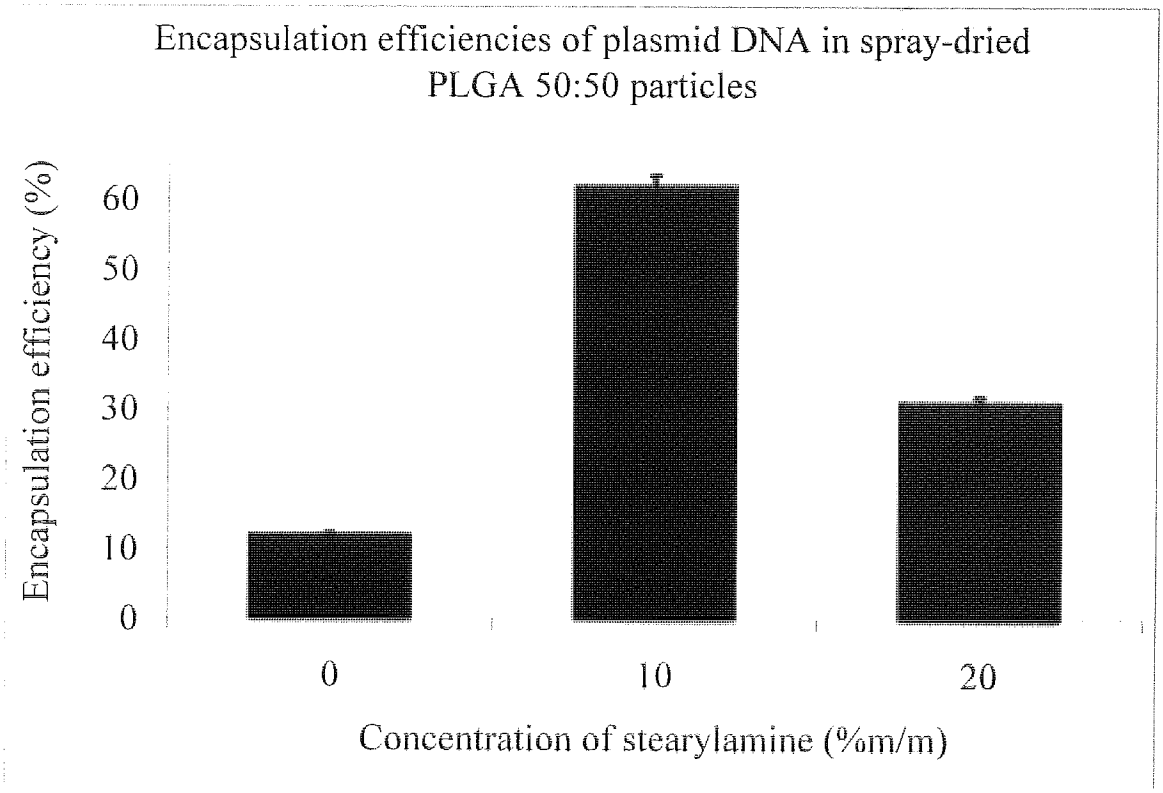


Figure 6.4: Encapsulation efficiencies of plasmid DNA in PLGA 50:50 microspheres formulated with 0, 10 and 20 $\%^{m}/_{m}$ stearylamine by spray-drying. The bars represent the mean value of three experiments and the standard errors represented by the error bars. Inclusion of stearylamine increased the DNA entrapment, but this increase was not so pronounced upon further increase in stearylamine concentration.

The addition of stearylamine to the formulations of microspheres followed the same trend seen with the morphological studies. Microspheres with no stearylamine showed low encapsulation values and at 10 $\%^{m}/_{m}$ stearylamine the encapsulation was increased 5-fold to ~60 %. Increasing the stearylamine concentration in the polymer to 20 $\%^{m}/_{m}$ resulted in a reduction in the encapsulation efficiency to ~30 %. It would appear that the improved morphology of the microspheres obtained with 10 $\%^{m}/_{m}$ SA, also increased the entrapment of DNA. This reinforces the inference that the addition of stearylamine increases the rate of water loss during the secondary drying phase, and that a fine balance between this rate of water loss and retention of the spherical shape of the particle is a crucial factor in the encapsulation efficiency.

6.3.3 Influence of stearylamine on the release of plasmid DNA from microspheres

The effect of SA on the profile of plasmid release is shown in figure 6.5. The values of

the abscissa have been displayed as the $\log_{10}$ to cover the release profiles for 5 % and 10 % SA. There was an initial burst (at 10 hours) of 18 % of the entrapped plasmid, after which little release was observed. By day 38 of the study, about 30 % of the entrapped DNA had been released. Conversely, there was little or no release from microspheres containing SA. At day 28, only 0.18 and 0.07 % of the entrapped DNA had been released from microspheres with 5 $\%^{m}/_{m}$ and 10 $\%^{m}/_{m}$ SA respectively..

There is a marked suppression of the release of plasmid from the SA-microspheres. This is most likely attributable an association between the SA and the DNA, mediated by their respective opposite charges. This is an interesting observation, as it indicates that the SA, originally in the organic phase during the preparation, ultimately interacts with the DNA, originally in the aqueous phase. The significance of this is highlighted when the mechanism of particle formation by spray-drying is considered. As discussed in section 6.1, there is a rapid loss of organic and aqueous solvent from the atomised droplets of the water-in-oil emulsion. It is envisaged that the plasmid DNA is contained within the inner aqueous phase of the droplet, with the polymer (and SA) in the outer organic phase. The release profiles therefore suggest that the DNA is ultimately surface located and this translocation of the aqueous phase must occur during the mass evaporation phase. It is likely that surface-located DNA was released by the microspheres with no SA.

The release profiles may also be explained in terms of the hydrophobicity of the microspheres. PLGA is relatively hydrophilic, and has been shown to degrade within 4 weeks (Chambers M, 2000), (Okada H and Toguchi H, 1995). The addition of SA may have reduced the release of plasmid by increasing the hydrophobicity of the preparation, reducing the extent of water penetration, a factor which directly influences the rate of polymer degradation (Gopferich A, 1996).

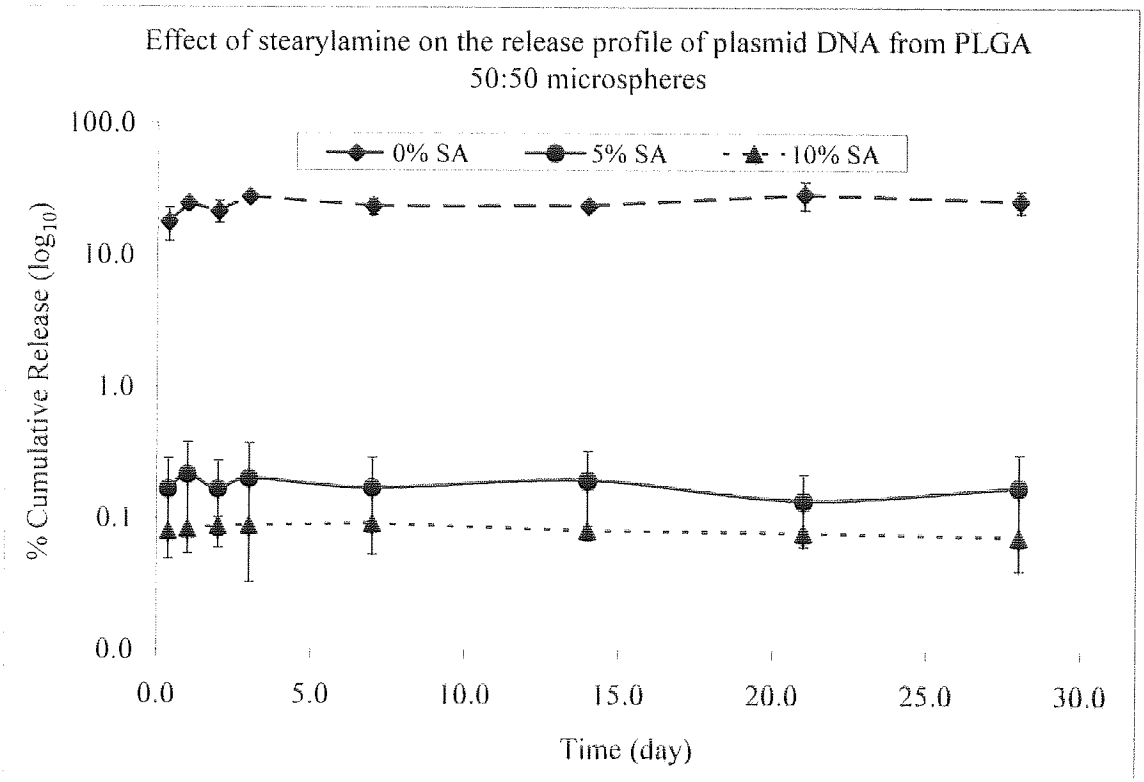


Figure 6.5: Effect of stearylamine on the release of plasmid DNA from PLGA 50:50 microspheres (mean of triplicate experiments showing standard error of the mean).

A reduced rate of release of encapsulated vaccine is desirable for long term immunity after administration. This has been observed with microspheric protein vaccines (Okada H and Toguchi H, 1995). It is thought that the microspheres are sequestered intracellularly, and this location acts as the source of the sustained 'delivery' of the vaccine. Plasmid DNA is, however, more susceptible to intracellular degradation, and correlation of the release rate of plasmid DNA with an immune response remains to be seen. Nevertheless, SA appears to be a useful excipient for controlled DNA vaccine delivery systems.

6.3.4 Effect of stearylamine on the surface charge of microspheres prepared by spray-drying.

PLGA 50:50 microspheres were prepared by spray drying containing increasing concentrations of stearylamine. The zeta potentials were measured as described in section 4.3.4.2 and the results are given in figure 6.6 below.

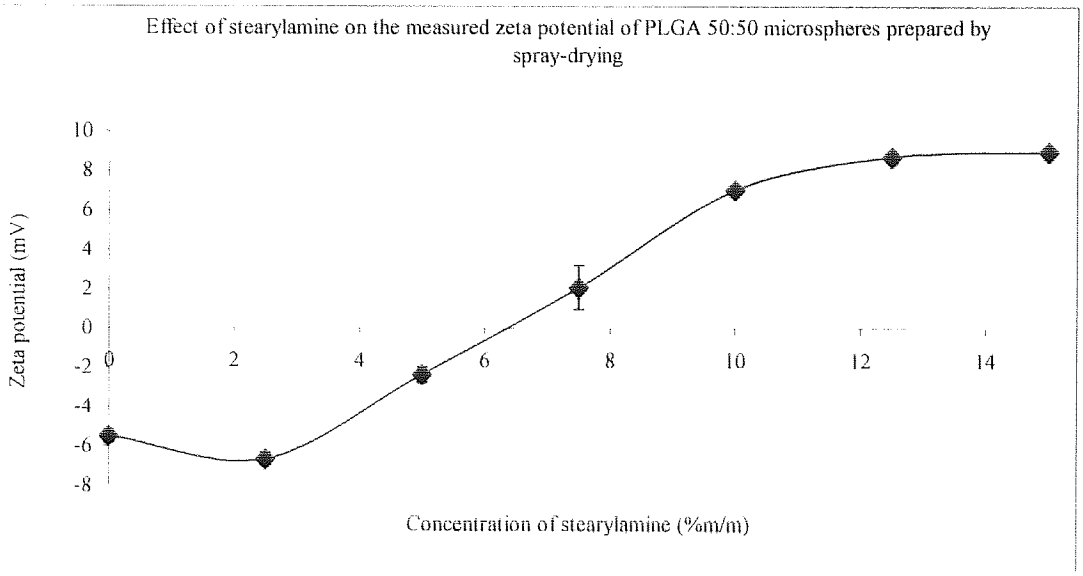


Figure 6.6: Effect of stearylamine on the zeta potential of PLGA 50:50 microspheres prepared by spray-drying. There is a linear increase in surface positive charge with increasing stearylamine concentration up to a saturation point of ~10 $\%^{m}/_{m}$, after which any further increase in SA fails to produce any further increase in zeta potential.

The figure for zeta potentials versus the concentration of stearylamine displays a linear trend between stearylamine concentrations of 2.5 and 10 $\%^{m}/_{m}$. Increasing the stearylamine concentration beyond 10 $\%^{m}/_{m}$ resulted in no further increase in the positive charge. The positive charge is attributable to the amine group on the stearylamine.

It is envisaged that at 10 $\%^{m}/_{m}$ SA concentration, the amine head groups saturate the surface of the particles formed, so that any further addition of the stearylamine molecule cannot result in a corresponding addition of amine head-groups to the microsphere surface.

6.3.5 Effect of stearylamine on the size distribution of PLGA 50:50 microspheres prepared by spray-drying

Size distributions for the spray-dried microspheres were determined by laser diffraction. Figure 6.7 shows the effect of increasing the stearylamine concentration on the median volume diameter. The uniformity is a measure of the absolute deviation from the median size, and indicates the spread of the microsphere size distribution. Stearylamine

reduces the diameter of the microspheres formed by spray-drying, but tends

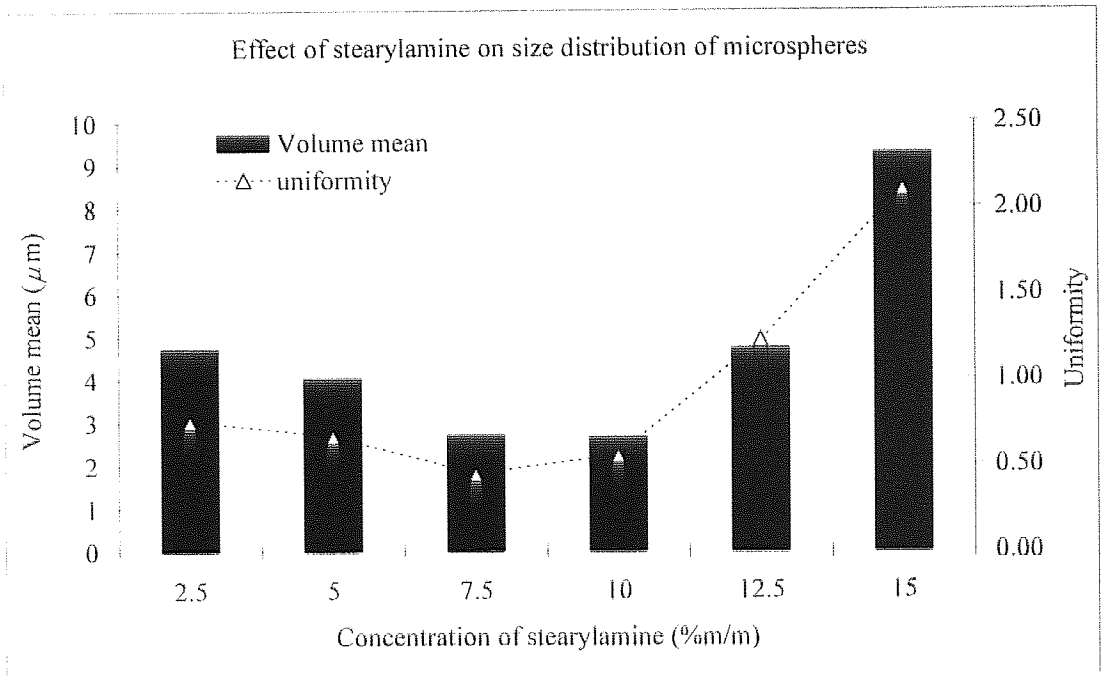


Figure 6.7: Size distributions of microspheres formulated with increasing concentrations of stearylamine.

to cause the formation of large, polydisperse particles at concentrations above 10 $\%^{m}/_{m}$, as confirmed by the morphological studies (see figure 6.7), and indicated by the increase in uniformity and median size. This further confirms that formulation of microspheres with stearylamine, up to a 10 $\%^{m}/_{m}$ concentration, using PLGA 50:50 by spray-drying has significant benefit.

6.3.6 Analysis of plasmid DNA extracted from microspheres formulated with stearylamine by the spray-drying method

Plasmid DNA was extracted from the microspheres as described in section 4.3.4.4, and the structural integrity determined by agarose gel electrophoresis. Figure 6.8 below shows an image of the DNA bands after electrophoresis.

All plasmid samples from microspheres showed a degree of reduction of the SC conformation, compared with the non-encapsulated control (lanes 1 and 9). A broad comparison of the extracted samples reveals that stearylamine has a protective effect on the DNA; the samples in lanes 3 to 8 from microspheres containing increasing

concentrations of stearylamine all show a higher proportion of SC plasmid (lane 4 had very little sample). There is no obvious increase in the proportion of SC plasmid with increasing stearylamine; i.e. the qualitative presence of the amphipathic molecule has a protective effect on the structural integrity of the plasmid during microsphere formation by spray-drying.

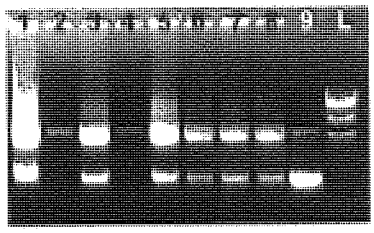


Figure 6.8: Agarose gel electrophoretic analysis of plasmid DNA extracted from PLGA 50:50 microspheres formulated with stearylamine and prepared by spray-drying. Lanes 1 and 9 are non-encapsulated plasmid DNA controls; lanes 2 to 8 are DNA samples extracted from PLGA 50:50 microspheres formulated with stearylamine at concentrations of: 0 $\%^{m}/_{m}$ (lane 2); 2.5 $\%^{m}/_{m}$ (lane 3); 5 $\%^{m}/_{m}$(lane 4); 7.5 $\%^{m}/_{m}$(lane 5); 10 $\%^{m}/_{m}$(lane 6); 12.5 $\%^{m}/_{m}$ (lane 7); 15 $\%^{m}/_{m}$(lane 8). L = λ/hind III size marker. The gel was cast at 1 $\%^{m}/_{v}$, stained with EtBr, and samples were loaded and run at 80V for 1 hour.

The mechanism of this protection is at present unknown, but from knowledge of the physico-chemical characteristics, some tentative postulations may be made; electrostatic interactions between the DNA and SA during the formation of the primary emulsion may afford some protection from the shear forces during homogenisation. Whether this protective property is limited to amphipathic molecules with potential DNA-binding capabilities remains to be elucidated from further work.

6.4 Protection of DNA from serum nucleases

Microspheres encapsulating plasmid DNA were incubated with cell culture media supplemented with 10 % foetal calf serum (FCS), and the protection from nuclease enzymes by microsphere entrapment assessed by agarose gel electrophoresis of the extracted plasmid samples, as described in section 4.3.4.4.

Figure 6.9 below illustrates the extracted plasmid samples after agarose gel electrophoresis. There is an increased resistance to degradation by serum nucleases with increasing stearylamine.

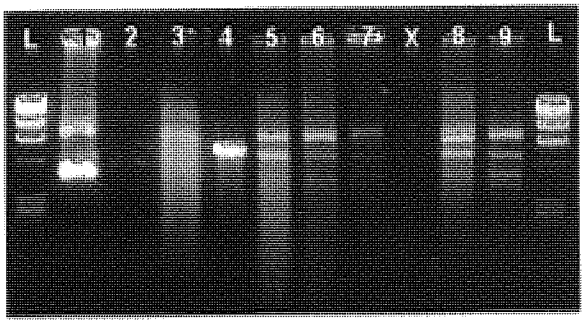


Figure 6.9: Protection of encapsulated plasmid DNA from serum nucleases. Microspheres were incubated in cell culture media supplemented with 10 $\%^{v}/_{v}$ FCS at 37°C for 4 hours. The DNA was extracted from the microspheres and analysed by 1 $\%^{m}/_{v}$ agarose gel electrophoresis at 80V for 1 hour, using EtBr as the gel stain. The bands represent DNA extracted, after incubation, from microspheres formulated with stearylamine at concentrations of : 0 $\%^{m}/_{m}$ (lane 5); 2.5 $\%^{m}/_{m}$ (lane 6); 5 $\%^{m}/_{v}$ (lane 7); 10 $\%^{m}/_{v}$ (lane 8); and 12.5 $\%^{m}/_{v}$ (lane 9). L = λ/HindIII size marker, lane 1 is plasmid DNA incubated in media containing no FCS; lane 3 is non-encapsulated plasmid DNA incubated directly in media with 10 % FCS. Lane 4 is linearised DNA control.

There is some protection of the plasmid DNA from nuclease degradation when plasmid DNA is encapsulated in microspheres. The inclusion of stearylamine in the formulation shows an increased protective tendency, and this is especially so with the higher concentrations of stearylamine in the formulation, as evidenced by the increased intensity SC bands from lanes 6 to lane 9.

The apparent improved protection of DNA from nuclease degradation by inclusion of stearylamine might be due to the hydrophobic nature of the stearylamine. The entrapped plasmid DNA is shielded from the degradative enzymes until the microspheres are penetrated or wetted by the aqueous outer *milieu* containing these enzymes. The hydrophobic nature of stearylamine either prevents or reduces the wettability of the microspheres, resulting in increased protection. The positive charge of the stearylamine might also contribute to its protective tendency; it has been shown that association of plasmid with positively charged lipids protects the plasmid from enzymatic degradation (Zhou X and Huang L, 1994). It is thought that the electrostatic binding of the negative phosphate backbone and positive amine groups changes the structure of the plasmid so that it is not recognised by the active sites of the nuclease enzymes. Any DNA bound to stearylamine will thus be protected from digestion after penetration of the enzymes into the microspheres. Therefore, stearylamine might exert its protective effect by a

combination of its hydrophobic property, imparted to it by the C_{18} aliphatic hydrocarbon chain and the positively charged amine group.

6.5 *In vitro* biological activity of plasmid DNA extracted from PLGA 50:50 microspheres formulated with stearylamine by spray-drying

Formulated microspheres were dissolved in chloroform and the entrapped plasmid DNA partitioned into an aqueous phase consisting of TE buffer. The concentration of the DNA was determined and standardised for all formulations and then used to transfect a 293 cell line to determine biological integrity by assaying for luciferase expression in the cell line.

Figure 6.10 shows levels of luciferase expression by the extracted DNA samples in a 293 cell line. There was at least a 50 % reduction in the transfection efficiencies of plasmid DNA extracted from the microspheres, as determined by the assay of luciferase expression in the 293 cell line. Plasmid DNA extracted from microspheres with stearylamine showed a higher retention of biological activity. The plasmid extracted from microspheres formulated with 5 $\%^{m}/_{m}$ showed a curiously elevated expression of luciferase.

The partial loss in biological activity inferred from the reduction in luciferase expression *in vitro* reflects the loss in the SC conformation observed in (figure 5.12). It has been established that there is a loss of the SC form of the plasmid during the emulsification of the aqueous DNA solution and organic polymer solution (section 3.3.2, figure 3.2), and this is the most likely explanation for the observed loss of structural integrity of the DNA extracted from the microspheres. The effect of the

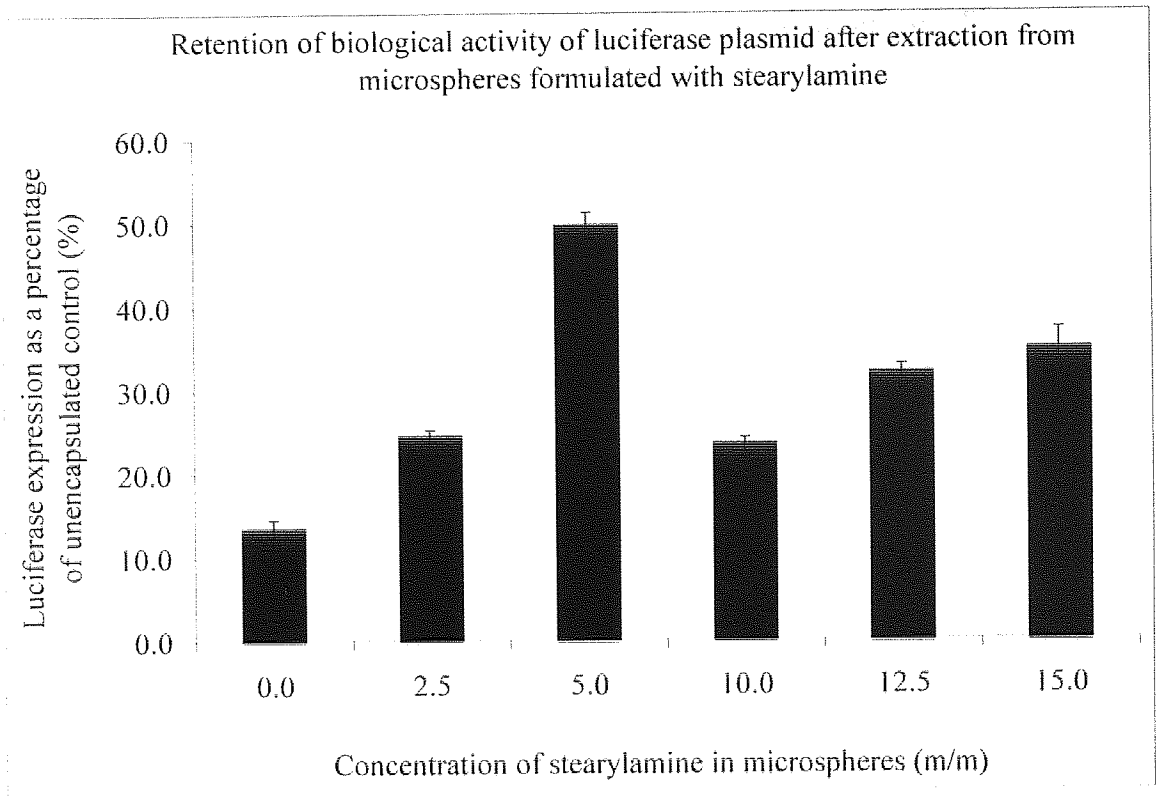


Figure 6.10: Assessment of the biological integrity of luciferase plasmid DNA extracted from PLGA 50:50 microspheres formulated with stearylamine by the spray-drying method. The transfection efficiencies of the extracted plasmid were compared with those of a non-encapsulated control in a 293 cell-line, using SuperFect™ transfection reagent. The bars represent the mean value of three experiments, and the standard errors are represented by the error bars. Luciferase expression was reduced in all preparations, and the preparation containing 5 $\%^{m}/_{m}$ stearylamine showed the highest expression of luciferase.

extraction process on the plasmid has not been investigated. The gentle shaking of the dissolved microspheres and aqueous phase might be of some detriment to the SC form of the plasmid.

6.6 Conclusion

This study has shown that plasmid DNA may be entrapped in PLGA 50:50 microspheres by spray-drying. This method of formulation results in the production of microspheres with size ranges below 10 μm, and makes them suitable for use as vaccines which may be administered by the mucosal route for APC-directed immune elicitation. The addition of stearylamine improved the general morphology of the microspheres prepared by this method. This molecule also improved the resistance of the entrapped DNA to serum nucleases, increased the entrapment efficiency and

reduced the variation in the size distribution. Formulation of the PLGA 50:50 microsphere with stearylamine results in the improvement in all the above factors at an optimal concentration of about 10 $\%^{m}/_{m}$. This concentration is at present a nominal one, as the precise concentration of stearylamine actually retained in the microspheres has yet to be determined. However, the increase in surface potential of microspheres with increasing stearylamine concentration infers a quantitative increase in stearylamine retained in the formulated microspheres.

The biological activity of plasmid DNA extracted from the microspheres prepared by spray-drying was generally less than that of plasmid alone. This reduction in transfective ability is attributable to the loss of the SC form of the plasmid during the early stages of homogenisation required for the emulsification of the aqueous DNA and organic polymer phase during fabrication. The particulate nature of the formulated DNA vaccine might offset this reduction in transfection activity by increasing uptake of the DNA by APCs which drain into the lymph nodes, directing a cell mediated immune response. The encapsulation of the DNA also protects it from serum nucleases, a property of the microspheres which is enhanced by formulation with stearylamine.

The inclusion of stearylamine beyond a concentration of 10 $\%^{m}/_{m}$ in the microspheres is detrimental to the morphology, size distribution, and entrapment efficiency of DNA.

The use of stearylamine has been shown to be advantageous in the formulation of DNA vaccines by the spray-drying method. The fact that the advantageous properties of the molecule are attributable to its hydrophobic and charged nature begs further investigation and trials of other molecules with these properties for microsphere formulation of DNA vaccines.

7. *In vivo* evaluation of microspheric DNA vaccine formulations using a Hepatitis B plasmid

7.1 Introduction

7.1.1 Hepatitis B: the disease

The first recorded epidemic of Hepatitis B (Hep B) occurred during the last century in 1883. The outbreak was described by 191 cases of jaundice among 1295 people after inoculation with a smallpox vaccine that contained human lymph (Krugman S and Stevens CE, 1994). It is estimated that 300 million people world-wide are chronic carriers of the hepatitis B virus (HBV), with a prevalence rate of 10 to 20 % in China, Southeast Asia and subsaharan Africa. The virus is transmitted mainly by percutaneous or sexual contact. Each year approximately 4,000 deaths due to cirrhosis and 800 deaths due to hepatocellular carcinoma that are related to chronic hepatitis B infection are estimated to occur.

Most subjects with clinically apparent Hep B infections present hepatocellular necrosis, which is usually followed by elimination of the hepatitis B virus (HBV) and subsequent recovery. Viral replication, however, may persist in about 2 to 5 % of otherwise healthy patients. The HBV does not have a direct cytopathic effect on hepatocytes; liver cell necrosis is probably due to CTL mediated destruction of virally infected hepatocytes (Krugman S and Stevens CE, 1994).

7.1.2 Hepatitis B virus

The HBV is a 42-nm enveloped virus and belongs to the Hepadanvirus class of viruses. The genome is a molecule of partially double-stranded circular, DNA. The core of the HBV also contains enzymes, a DNA-dependent DNA polymerase which reproduces the DNA during viral replication. The virion has a major outer surface glycoprotein (HBsAg), and an inner core protein, (HBcAg). The major component of HBsAg is the 226-amino acid S antigen which is inserted in the lipid envelope. There are two additional HBsAg glycoprotiens designated pre-S1 and pre-S2 because of their location in the HBV genome, but these are present to a lesser extent. In addition to whole

secreted virus particles, infected individuals have in their serum, incomplete spherical and tubular subviral particles (22 nm) consisting of the HBV S antigen with variable amounts of pre-S1 and pre-S2 and in some cases, a soluble antigen, HBeAg.

The HBsAg has multiple antigenic specificities; a shared group-specific determinant, designated *a*, and at least two mutually exclusive subdeterminants, *d* or *y* and *w* or *r*. Thus four principal phenotypes are recognised: *adw*, *adr*, *ayw*, and *ayr*. These subtypes vary in their distribution throughout the world and have been valuable epidemiological markers of infection. Immunological protection against infection appears to be conferred primarily by antibody against the *a* specificity.

7.1.3 Hepatitis B vaccine

In 1968, Prince identified an antigen in the serum of patients with post-transfusion Hep B (Prince AM, 1968). This discovery led to the development of sensitive and specific markers of Hep B virus infection, and the eventual development of a vaccine. It was demonstrated that heat-inactivated serum containing HBV and HBsAg was not infectious, but was partially protective against subsequent exposure to HBV. The detection of antibodies against the virus in recipients of the heat-inactivated preparation indicated that the non-infectious HBsAg particle was the immunising antigen needed for vaccine production.

Currently licensed inactivated Hep B vaccines are predominantly the purified 22-nm particles of the S antigen of HBsAg that is derived from the plasma of chronic carriers, or recombinant cells of organisms that produce HBsAg. Some vaccines also include varying amounts of pre-S1 or pre-S2, and the need for their inclusion is debatable (Krugman S and Stevens CE, 1994).

Recombinant technology for Hep B vaccine involves the insertion of segments of the HBV genome that encode HBsAg into plasmids, which in yeast (*Saccharomyces Cerevisiae*) or cultured mammalian cells (Chinese hamster ovary, or CHO cells) then express HBsAg. The recombinant cells synthesise a linear HBV-encoded polypeptide which adopts a globular form structurally and immunologically identical to the plasma-

derived protein, except for the absence of glycosylation, which in this instance does not compromise the efficacy of the vaccine (Dertzbaugh MT, 1998). The recombinant vaccine consists of the purified antigen adsorbed onto the surface of aluminium hydroxide as an adjuvant (Mehta DK, 2000).

7.1.4 Hepatitis B DNA vaccine

A DNA-based vaccine involves the transfer of a gene, by direct or indirect means, such that the protein subsequently produced acts as an antigen and induces a humoral and/or cellular-mediated immunological response as discussed in chapter 1 (page 32).

The Hep B plasmid, shown in figure 7.1 expresses the *ayw* subtype of the surface S protein, whose expression is driven by a CMV promoter. It has an ampicillin resistance gene for antibiotic selection.

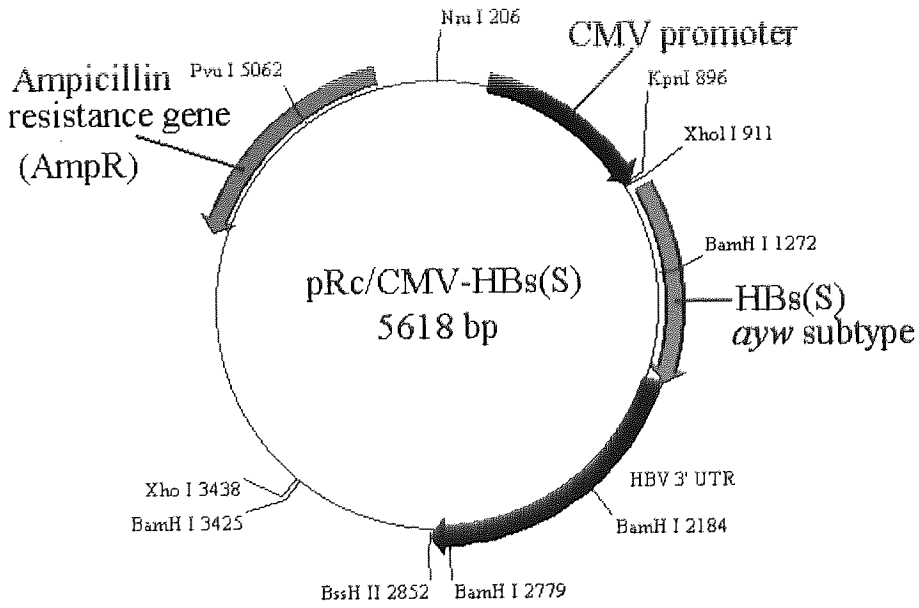


Figure 7.1: Plasmid map of HBsAg-expressing plasmid, derived from pRc/CMV (Clonetech, UK).

Figure 7.2 shows the transmembrane location of the HBV envelope protein. The *ayw* subtype of the S protein consists of a 226-amino acid polypeptide which spans the membrane of the endoplasmic reticulum (ER). Two other forms of the envelope protein, the middle (M) and large (L) polypeptides, are N-terminal extensions of 55 (pre-S2) and

165 (pre-S1) amino acids, respectively, to the S protein.

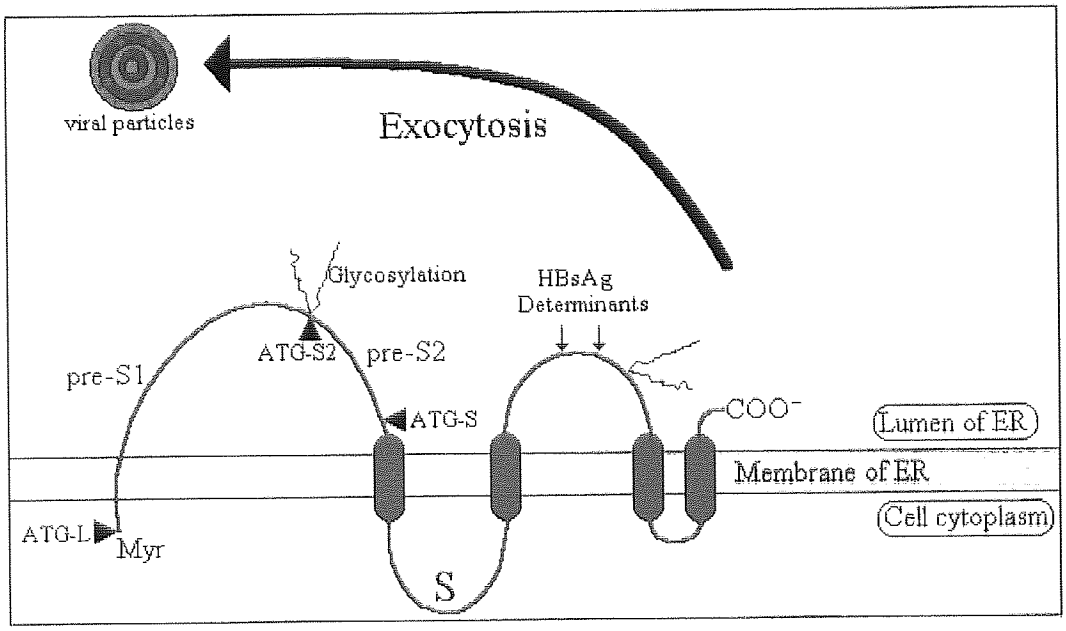


Figure 7.2: Transmembrane orientation and various features of the HBV envelope protein, adapted from (Whalen RG and Davis HL, 1995).

The HBV envelope polypeptide is characterised by four transmembrane domains that are contained within the 226-amino acid major form (S). The N-terminal extremity of the S polypeptide (black triangle labelled ATG-S) projects into the lumen of ER. The pre-S2 sequences, which are found in the M polypeptide, are also lumenal; the N-terminus is marked by a black triangle labelled ATG-S2. The pre-S1 sequences present in the L polypeptide either adopt a conformation with a part of the sequence in the ER membrane as shown in figure 7.2, or it may cause the pre-S2 and the first transmembrane domain to remain on the cytoplasmic side of the ER membrane. The N-terminus of the L polypeptide (black triangle labeled ATG-L) is myristylated, and this may also contribute to the disposition of the pre-S1 sequences. Glycosylation sites are present near the N-terminus of the pre-S2 sequences, and on the lumenal loop of the S polypeptide. Linear antibody-binding epitopes are located in the pre-S2 sequences, while the conformation epitopes of the HBsAg are found in the S sequences and are located on the lumenal loop. After insertion of the polypeptide into the ER membrane, the polypeptide forms dimers and aggregation among dimers occurs within the plane of the ER memebrane. Subsequently, the aggregated dimers bud into the lumen of the ER

and form particles in which the pre-S1 and pre-S2 sequences, as well as the HBsAg determinant, are exposed on the outside. The particles are secreted *via* a constitutive exocytotic mechanism (Whalen RG and Davis HL, 1995).

7.1.5 Immunological responses to Hepatitis B DNA vaccines

Davis *et. al.*, first demonstrated that direct i.m. injection of 200 μg of a plasmid vector encoding the HBsAg leads to secretion of the viral surface protein into the circulation (Davis HL *et. al.*, 1993). The resulting immune response produced antibody levels in mice that were greater than those obtained after injection of naturally occurring or recombinant antigen. This was the first clear demonstration of the secretion of a foreign protein by muscle tissue after synthesis from a cloned gene introduced by direct transfer of a pure recombinant plasmid DNA. The authors detected antibody titres 10 days after the single administration, and this increased for at least two months. It was concluded that the antigen entered the circulation by active secretion, rather than passive release from damaged cells. This is in contrast to a report by Ulmer *et. al.*, who showed a protective response against influenza A following injection of a plasmid DNA which expressed the viral nucleoprotein. This was thought to result not from secretion of the antigen, but the intracellular processing of the antigen and surface presentation *via* MHC class I molecules (Ulmer JB *et. al.*, 1993).

In a later study, Whalen *et. al.*, evaluated the cytokine profile of the T cell responses induced by plasmid DNA injection (Whalen RG *et. al.*, 1995). The profiles suggested an exclusive and strong Th-1 type response to the injected DNA, with high levels of IFN-γ and IL-2 and background levels of Th-2 type cytokines IL-4, IL-5 and IL-10. The authors also stated that antibody subclass characterisation revealed correspondingly high serum IgG_{2a} levels. Thus, the administration of the HBsAg plasmid by i.m. injection results in a strong Th-1 type response to the expressed antigen.

The administration of microparticulated plasmid DNA has been investigated by Alpar *et. al.*, who first observed that OVA plasmid DNA absorbed onto the surface of cationic microparticles resulted in the high serum anti-OVA IgG after nasal administration (Alpar HO *et. al.*, 1997). Several other groups have reported the potential of

microparticulated plasmid DNA (see table 1.4, page 54).

In this study, the immune response of mice to Hep B plasmid formulated with stearylamine-PLGA microparticles was investigated.

7.2 Materials and methods

7.2.1 Experimental animals used in the study

All subjects used were 4 to 6 week old Balb/c female mice, unless stated otherwise, and all handling procedures were performed strictly as dictated by the Animals (Scientific Procedures) Act 1986.

7.2.2 Intramuscular administration of plasmid DNA formulations

The quadriceps of the mice were injected with 50 μg of the formulated or naked (free) plasmid DNA in 50 μL of sterile PBS using a sterile 26G needle and 1 mL syringe. Animals were dosed three times at weekly intervals, so that each animal received a total plasmid dose of 150 μg. Blood was obtained by a 100 μL tail vein bleed *per* mouse, days 4, 11, 34 (week 5) and 62 (week 9) after the last boost. On day 137, the mice were injected with 10 μg each of the Hep B subunit protein, and bled as before on day 144 (week 20). Blood samples were collected into microfuge tubes and coagulated at 4°C overnight. The samples were then centrifuged at 15,000g for 20 minutes and the serum collected, pooled for each group, and placed at -70°C until analysed for antibody titres.

7.2.3 Quantitative assay for immunoglobulin titres in murine blood sera using enzyme-linked immunosorbent assay (ELISA)

The wells of a 96-well microtitre plate were coated with 60 μL of a 3 μg/mL solution of the Hep B antigen (Aldevron LLC, USA). The plates were incubated at 4°C for 12 - 18 hours. The wells were washed once with PBST, and blocked with 100 μL of a 4% $^{m}/_{v}$ BSA solution, to prevent any non-specific binding to the walls of the well. After a 1 hour incubation at 37°C, the wells were washed three times with PBST, and 50 μL of serially diluted blood sera added sequentially to the coated wells. The plate was

incubated a 37°C for one hour, washed three times with PBST, and 50 μL of anti-idiotypic antibodies to the antigen added to each of the wells. After a further one-hour incubation at 37°C the wells were washed three times with PBST and 50 μL of ABTS substrate in citrate buffer added to each well. The absorbance of the solution in each of the wells was determined photometrically at 405 nm with a microplate reader.

7.3 Results and discussion

7.3.1 Immune response to Hep B plasmid formulated with PLGA 50:50 microspheres containing stearylamine

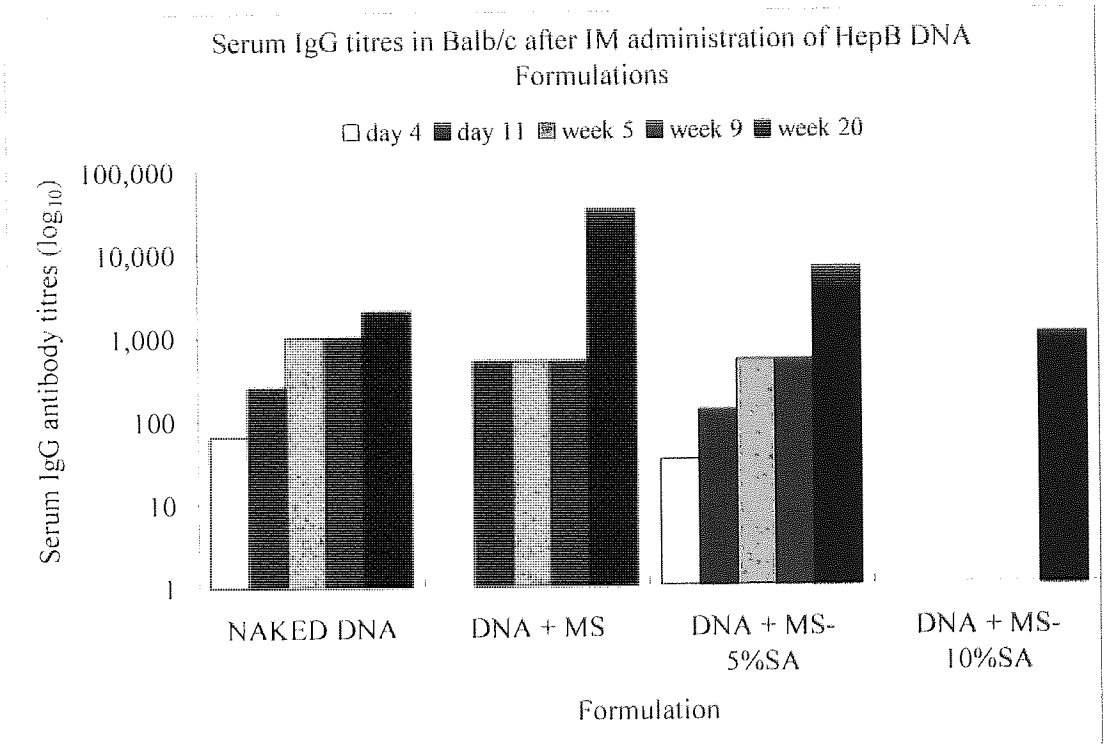


Figure 7.3: Serum anti-HBsAg IgG titres in mice immunised with Hep B plasmid formulated with PLGA 50:50 microspheres containing no stearylamine (DNA+MS), 5 % stearylamine (DNA+MS-5 %SA) and 10 $\%^{m}/_{m}$ stearylamine (DNA+MS-10 %SA). The mice were also administered naked or unformulated plasmid DNA as a comparison. Each bar represents the end-point antibody titres. The blood sera of each group of 5 mice were pooled prior to each assay.

Figure 7.3 shows the serum anti-HBsAg IgG in mice serum after i.m. immunisation with the formulated Hep B plasmid. The antibody responses were further characterised with respect the IgG_{2a} and IgG_1 subclasses. Figure 7.4 shows the IgG_{2a} subclass titres;

there was a complete lack of IgG_1 in the mice sera.

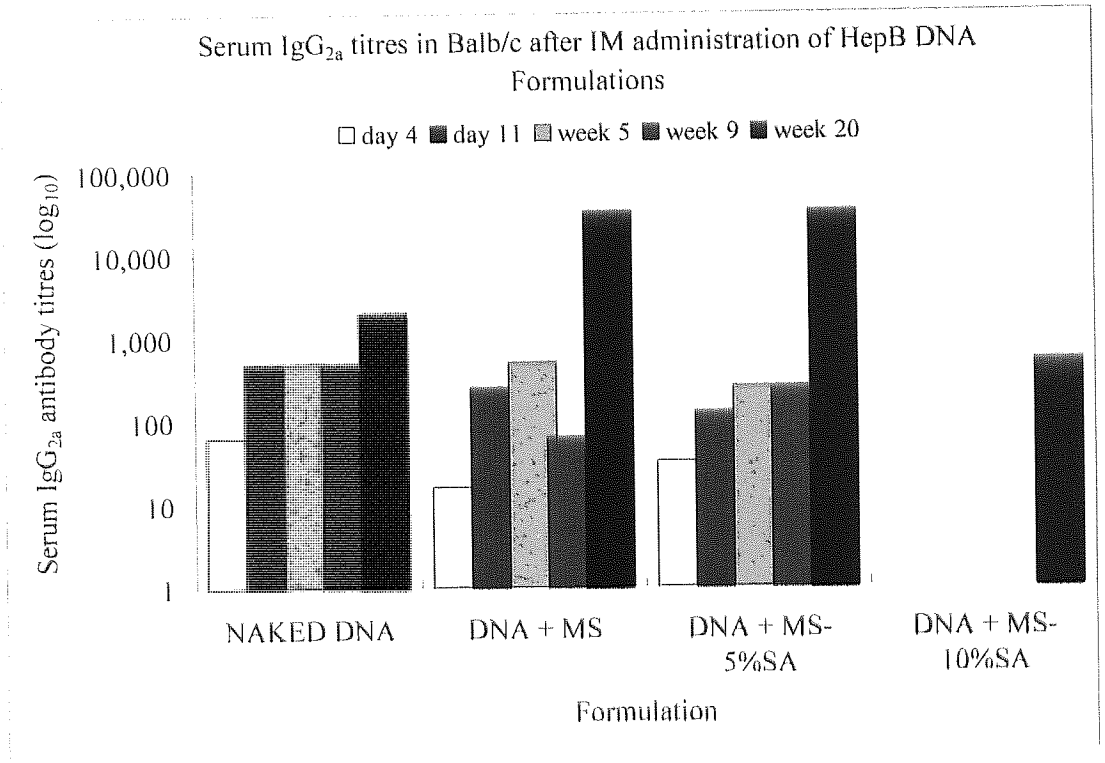


Figure 7.4: Serum anti-HBsAg IgG_{2a} subclass titres in mice immunised with Hep B plasmid formulated with PLGA 50:50 microspheres containing no stearylamine (DNA+MS), 5 % stearylamine (DNA+MS-5 %SA). The mice were also administered naked or unformulated plasmid DNA as a comparison. Each bar represents the end-point antibody titres. The blood sera of each group of 5 mice were pooled prior to each assay.

The earliest antibody responses were observed by naked DNA and MS-5 %SA formulations, 4 days after the last boost (see figure 7.4). By day 11 all formulations except MS-10 %SA had produced serum antibodies, with MS-5 %SA displaying the highest levels. However, by week 5 the highest serum antibody levels were produced by naked DNA. Formulation MS-5 %SA had the lowest rate of antibody-level increase, and all groups showed a steady level after week 5. It is important to note that by week 9 (i.e. prior to boosting with the subunit protein) MS-10 %SA showed no serum antibodies.

The mice were further boosted with the Hep B protein on day 137, and the serum antibody levels determined on week 9. The IgG response followed a trend; increasing the SA concentration reduced the humoral response. In fact, IgG levels in mice

immunised with plain PLGA-formulated DNA were more than 15 times higher than those of naked DNA. Addition of 5 % SA reduced this to about 3 times the level of naked DNA. Antibodies were detected in the serum of mice immunised with MS-10 %SA only after the subunit protein boost, and these levels were comparable to those of the naked DNA.

The antibody responses were further characterised for the subclasses, IgG_1 and IgG_{2a}. The antibody titres in the serum of mice dosed with 5 % SA microspheres, and that of those dosed with naked DNA showed similar trends with respect to time, and were not different from the IgG titres (see figure 7.4). The 0 % SA microspheres on the other hand showed a decrease in the IgG_{2a} titres on week 9. There were no detectable IgG_1 antibodies at any of the time points, suggesting an exclusively Th-1 type immune response for all the formulations. A formulation consisting of 10 % SA was also tested *in vivo*, but no antibody titres were detected by week 9. The IgG_{2a} titres after the subunit boosts followed the same trend as the IgG titres.

The plasmid DNA was formulated with SA microspheres to evaluate the effect of the microsphere-DNA association on the immune response, and to compare that with the response to naked DNA, and DNA co-administered with microspheres with no SA. It is interesting to note that the formulation with no SA, and therefore no association or interaction between the microspheres and DNA gave the highest initial responses, which was sustained for the duration of the experiment, and the increase in the SA reduced the response. Intramuscularly administered DNA bound to microsphere surfaces by electrostatic interaction has been shown to elicit superior humoral and cellular immune responses in mice (Singh M *et. al.*, 2000). These results suggest the opposite effect.

That the naked DNA showed a lower rate of increase of the antibody titre implies that the presence of the microspheres, in conjunction with the plasmid DNA, had an effect on the ultimate immunological presentation of the expressed antigen. The precise mechanism by which i.m. administration of plasmid DNA results in the presentation of the encoded antigen has not been fully elucidated; however current thinking favours the following theory:

Muscle fibres are directly transfected by the injection with the plasmid. The antigen is synthesised *in situ* and accumulates in the myocytes. These cells present the relevant antigenic epitopes by MHC class I molecules and the cell surface. Recognition of the antigen presented by these molecules by CD8+ T cells results in generation of the Th-1 type immune response, and could account for high IgG_{2a} antibody titres observed. However, myocytes are not professional APCs, in that they do not possess the associated co-stimulatory molecules thought to be necessary for effective elicitation of the cell-mediated immune response (Cruse JM and Lewis RE, 1999). The antigen, if secreted, as in the case of HBsAg, may be engulfed by professional APCs present in the area surrounding the site of injection. Digestion and presentation of the antigen fragments will normally result in MHC class II presentation, but some proteins may be shunted into the MHC class I presentation pathway. The third proposal of the mechanism by which i.m. injection leads to cell mediated immunity is the direct uptake of the plasmid by APCs (Whalen RG *et. al.*, 1995). Current scientific opinion favours a combination of these three possible mechanisms that leads to the observed effect, as the existence of any one of the three cannot be firmly disproved.

It is therefore likely that the uptake of microspheres by APCs leads to the simultaneous internalisation of the plasmid DNA, by APCs, or that the presence of the microspheres stimulated the recruitment of APCs to the surrounding area, so that expressed and secreted HBsAg particles were more rapidly internalised, leading to a more rapid antibody response. The kinetics of the immune response to the plasmid DNA co-administered with 5 %SA microspheres was observed to be similar to that of the naked DNA, but when co-administered with 10 %SA-microspheres, there was antibody response detectable by week 9. Introduction of the viral subunit to the mice *via* the intramuscular route enhanced the antibody titres, and increasing the SA content in the formulation reduced this effect. This observation suggests that there is a strong memory response. Further experiments are required to verify the precise immunological events at the cellular level.

7.4 Conclusions

It has been shown that the formulation of Hep B plasmid DNA with PLGA 50:50 microspheres affects not only the magnitude of the humoral immune response, but

increases the rate of increase of *in vivo* antibody titres up to a maximal level equalling that observed after naked DNA administration. Formulation with 5 % SA microspheres appears to result in humoral kinetics and magnitude similar to that of naked DNA, and increasing the SA level to 10 % suppresses the antibody production. There appears to be a strong immunological memory stimulated by the formulations, and SA again suppresses this effect, which is strongest with plain PLGA microspheres. The mechanism by which SA causes this effect is as yet not known, but it is likely that retention of the DNA on the particle surface by strong amine-phosphate backbone electrostatic interaction plays a role.

The immune response to these formulations requires further characterisation with respect to cell mediated immunity, including cytokine profiles and splenic T cell stimulation indices.

8. Overall summary and conclusions

The initial studies confirmed the activity and integrity of the plasmid after successful amplification and harvesting from transformed *E. coli*. The plasmid thus obtained was formulated into microspheres using two methods of preparation; the solvent evaporation and the spray-drying methods. The merits of each of the preparations were demonstrated in relation to the stability of the formulated plasmid DNA. A comparison of these two processes revealed the spray-drying method to be less degradative to the DNA, as assessed by agarose gel electrophoresis and transfection efficiencies. Plasmid from spray-dried particles expressed higher levels of luciferase *in vitro*, compared with that extracted from microspheres prepared by the solvent evaporation. From studies on the effect of the formulation processes on plasmid DNA, it can be concluded that the homogenisation process is responsible for the loss of structural and biological function of the plasmid. Thus the spray-drying method appears to be a more viable formulation method for microspheric DNA vaccines.

Loading efficiencies were generally higher in the PLGA microspheres prepared by solvent evaporation (*circa* 50 % compared with 10 % for spray-dried microspheres). The addition of SA as a formulation agent presented an interesting effect. Microspheres with increasing SA concentrations, prepared by spray-drying were more uniform in shape and diameter. Size distribution analysis showed a reduction in the mean diameter on addition of SA, up to 10 % concentration, after which there was an increase, and a disruption of the smooth uniform morphology, observed by SEM. A similar effect was noted for microspheres produced by solvent evaporation, but the disruptive effect of SA occurred at a lower concentration. SA effected an increase in the loading efficiency in both methods, and at a 10 % concentration, preserved the retention of the SC conformation for longer, compared with either 0 or 2.5 % SA. Production of microspheres by the spray-drying is a more viable option for large-scale production. By this method, GMP conditions may be adhered to; spray-drying is a more established procedure in the pharmaceutical manufacturing industry. Parameters like temperature and rate of feed can be more easily monitored and controlled. The major drawback with the spray-drying method is the low yield of product. This is of major concern when working at the at the laboratory scale, when batch sizes of less than a gram are used. It

is anticipated that at higher batch sizes, the proportion of polymer material lost due to dead volumes and impaction on the vessel walls will represent a less significant percentage of the total polymer mass.

The release profiles of plasmid DNA from the microspheres showed that SA suppressed the release of DNA molecules, and this effect was more pronounced with increasing SA concentrations. It was not determined whether this was due either to the hydrophobic character of the SA molecule, or the interaction between the DNA and the SA, or both. This may be determined by a study of the release characteristics of an electro-neutral molecule from SA-PLGA microspheres.

Thus SA has potential value in microspheric DNA vaccine formulations. It appears that its effects are largely due to the combination of the positive charge and hydrophobic hydrocarbon chain. This property has been exploited in liposomes to impart a positive charge (Wang D *et. al.*, 1996). While this was observed in the microsphere formulations, it was also noted that SA had a protected the DNA from physical degradation during the fabrication process. The effect of physico-chemically similar molecules like dioctadecylamidoglycylspermie (DOGS) or dimethyldioctadecyl ammonium bromide (DDAB) as well as uncharged 'lipid' molecules like cholesterol may also be investigated for their effects on microsphere formulations of plasmid DNA, using both spray-drying and solvent evaporation.

Microspheres were also prepared to entrap DNA-PEI polyplexes. This approach to DNA microparticulate formulation increased the loading efficiency and there was no effect noted on either the surface charge, size distribution or morphology of the microspheres. The particles thus prepared were shown to be internalised by macrophage cells *in vitro*, and this was further confirmed by confocal microscopy. There was some retention of the SC DNA when formulated by this method, but this remains to be quantified. The complexation of the plasmid DNA with PEI was not to the detriment of the plasmid's physical or biological integrity. Thus, it is most likely that the process of the double emulsion solvent evaporation accounted for the loss of SC plasmid conformation. The formulation of plasmid DNA by encapsulation of polyplexes

requires further investigation, specifically regarding the stability and release of the polyplexes from the microspheres. The use of different polymers may also allow a further increase in the encapsulation efficiency.

The aim of this work was the fabrication of plasmid DNA into polymeric microparticles. The increased uptake of the particulated DNA vaccine by APCs has been shown to enhanced its effect. Plasmid DNA was produced, and the effect of the microencapsulation process noted. The most favourable of these processes was then used to formulate the plasmid DNA into microspheres. The use of positively-charged agents improved on the formulation in terms of the loading efficiencies and stability.

The *in vivo* activity of the plasmid DNA administered with plain and SA-blended PLGA microspheres was assessed. There was initially no humoral advantage to this formulation, as antibody titres were highest in mice immunised with naked or unformulated DNA, and higher SA concentrations prevented the formation of serum anti-HBsAg IgG. The administration of the protein stimulated the highest antibody response in mice dosed with plasmid DNA formulated with plain PLGA microspheres and inclusion of SA reduced this effect. It was interesting to note that the formulation which previously showed no antibody response, raised serum IgG titres comparable with that of naked DNA after boosting with the protein. This suggests a cellular phenomenon which required characterisation in terms of splenocyte proliferation and cytokine profiles. That the anti-HBsAg IgG titres were greater in mice immunised with formulated DNA, compared with the naked DNA is not insignificant.

While the aims and objectives were largely met, there is the potential for further work to improve on certain aspects of the microencapsulation of plasmid DNA, in terms of the range of polymers available for microsphere formation and pharmaceutical excipients for plasmid stabilisation.

9. Future work

The scope of continuation of the work presented in this thesis is outlined below:

Plasmid purification by the method described yielded about 10 to 15 mg from a 2.5 L culture of *E. coli*; the plasmid has a high copy number, and the origin of replication allows for increased plasmid numbers *per* cell by use of Chloramphenicol amplification. This technique inhibits the *E. coli*, but not plasmid replication, resulting in higher densities of plasmid *per E. coli* cell. The resulting increase in plasmid:cell mass ratio could increase the plasmid yield.

The uptake of the microspheres containing plasmid DNA and DNA:PEI polyplexes by macrophages *in vitro* was confirmed by fluorescence and confocal microscopy. While there was no qualitative effect of the different formulations on the uptake, this needs further study by quantitative analysis of the microsphere uptake. The use of either primary macrophages or dendritic cells will be a more realistic simulation of the *in vivo* situation, and this may be further pursued by a study of the biodistribution of the microparticles after administration *via* different routes. The effects size distribution, polymer, SA content, DNA loading and location (encapsulated or surface-adsorbed) surface charge and hydrophobicity have on *in vivo* uptake may all be established.

The release profiles of encapsulated DNA showed that a significant proportion of DNA was retained in the PLGA-SA microspheres after 30 days. The biological integrity of the released and retained DNA needs to be determined by transfection studies. This will establish the rate at which formulated DNA should be released after administration *in vivo*; if the retained DNA is degraded after a few days in the release media then it is likely that a more rapidly degrading microsphere formulation is more viable for encapsulated DNA. The stability results obtained thus far suggest that the SA may afford some protection to the plasmid from chemical degradation, whether this will be seen in the aqueous environment *in vitro* or *in vivo* remains to be seen. Nevertheless, there is also the possibility of including buffering agents to the microspheres during fabrication to effect a protection from the low pH thought to be responsible for initiating the chemical degradation of the DNA.

The potential of the encapsulated polyplexes needs further investigation to be fully realised; it is anticipated that the released polyplexes in the biological milieu will be taken-up by APCs, so that there will be little loss of activity and dose by enzymatic degradation *in vivo*. The PEI also has a buffering effect in the lysosomes of macrophages, and this further protection of the plasmid can only serve to enhance the immune response. The release of the polyplexes from the microspheres can be assayed by the addition of a strong polyanion to the release media prior to assay to ensure decomplexation of the polyplexes. An increase in the free DNA concentration after decomplexation is confirmation of its presence as PEI:DNA complexes, and the structural integrity of the released DNA can be checked by agarose gel electrophoresis. Another issue with the encapsulated polyplexes is the formulation of the DNA:PEI complexes; the parameters required to form stable and discrete complexes prior to encapsulation needs to be established. However, the proof of the principle of encapsulating polyplexes in polymeric microspheres has been accomplished.

The immunological response to the DNA formulated with plain PLGA and SA-PLGA microspheres presented an interesting effect; increasing the SA reduced the humoral response to the Hep B antigen. The precise mechanism by which SA causes this suppression is yet to be determined. A plausible theory is the strong electrostatic retention of the plasmid by the SA, reducing the total mass of DNA available for translation and ultimate presentation, effectively reducing the dose of plasmid. The amount of free DNA in the microsphere-DNA mixture may be quantified to verify the retention of the plasmid on the microspheres surface with increasing SA concentration. A larger range of SA concentrations may also be tried with the same protocol to further confirm an SA dose dependency on the magnitude of the immune response. The increase in the serum anti-HBsAg IgG antigen after boosting with antigen was highest when DNA was administered as plain microspheres. The mechanism by which the microspheres cause this effect also needs clarification. An *in vitro* transfection of macrophages and epithelial cells in the presence of microspheres may shed some light on this phenomenon. Alternatively, the injection site after i.m. administration of naked and formulated DNA, may be excised and assayed for the levels of expressed protein. This will highlight the role of the microspheres, in terms of uptake and expression of the

protein at the injection site. A splenic or draining lymph node - T cell proliferation assay will be useful for determining the importance of the translocation of the plasmid or expressed protein to either the spleen or the lymph nodes, and this results of such a study will offer a possible explanation of the apparent strong immunological memory response observed.

The work presented in this thesis offers a foundation for further development of novel DNA vaccine formulations. Through the exploitation of such technologies, it is anticipated that the full immunological benefits of DNA vaccines will be realised.

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11. Appendix

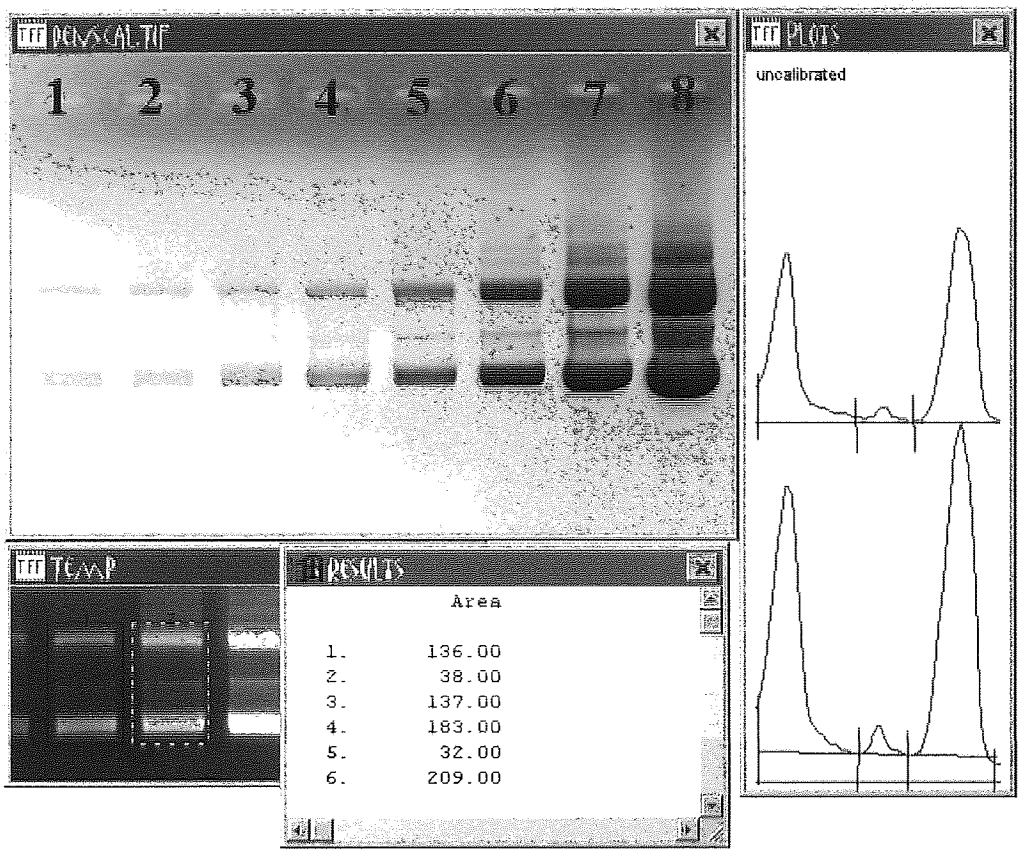

Figure 11.1: Sample of gel analysis by the Scion™ Image Software. A linear relationship was established between the total peak area and mass of plasmid DNA *per* well.