Liposome-mediated antigen delivery: formulation and optimisation

Sarah Elizabeth McNeil

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Aston University

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Abstract

Live conventional vaccines are generally effective at provoking protective immunity against the infectious agent. However, there are many disadvantages regarding their adverse side effects and overall safety profile. Alternative vaccine strategies such as subunit and plasmid-based vaccines, using recombinant technology, are much safer, yet less effective. Therefore, the immunogenicity of such vaccines could be enhanced by utilising delivery and/or adjuvant systems, to provoke the appropriate immune responses.

The role of liposomal systems within plasmid-based delivery was examined, looking at the effects of varying liposomal composition and method of preparation on the physical characteristics, transfection efficiency *in vitro* and immunogenicity *in vivo*. Compared to naked DNA alone, entrapment of plasmid DNA within liposomal vesicles results in complete protection from degradation by intracellular enzymes, with the DNA maintaining full structure and function. For liposome-mediated gene delivery small cationic lipids have been shown to be potent candidates, acquiring a net positive charge, which effectively interact with the anionic charges of the DNA to generate high incorporation values. In contrast, neutral liposomes are much larger aggregated structures with lower incorporation of DNA. The method of preparation was shown to effect the spatial location of plasmid DNA to liposomal systems. The dehydration-rehydration procedure (DRV) carried out in the presence of DNA effectively entraps the plasmid with little effect on liposome size and surface charge. Alternatively, upon addition of plasmid DNA the measured vesicle size of small unilamellar vesicles (SUV) or 'empty' (water containing) DRV increases due to the formation of SUV-DNA or DRV-DNA complexes, with the majority of the DNA localised on the surface of the liposomes. When applied *in vitro*, transfection efficiency of SUV-DNA complexes was greater than DRV(DNA). Transfection efficiency of SUV-DNA complexes varied depending on the cationic lipid present within the lipid bilayer, with DC-Chol showing most efficiency. Furthermore, these DC-Chol cationic liposomes were formulated in combination with two different 'helper' lipids, the fusogenic lipid dioleoyl phosphatidylethanolamine (DOPE) or the stabilising lipid Chol. The manner in which complexes form, the resultant structure and their transfection efficiency *in vitro* varied depending on the combining effects of both the type of 'helper' lipid incorporated within the lipid bilayer and total lipid to DNA charge ratio, with the overall structural size playing a significant role in promoting transfection. Transfection efficiency *in vitro* was significantly reduced when complexes were stabilised by the inclusion of phosphatidylcholines, with both the phospholipid head group and the alkyl-chain length influencing transfection efficiency. The production of DRV vesicles incorporating DNA were also produced in the range of 100-200nm by the addition of a disaccharide (i.e. sucrose), prior to freeze-drying during the dehydration-rehydration procedure. In this instance, with an increase in sucrose/lipid mass ratio, the z-average diameter of liposomes decreased, while the percentage plasmid DNA, pRc/CMV HBS, entrapment remained relatively high (92%). Despite this, these small DRV(DNA) were found to be poor transfecting agents *in vitro*.

After an initial screening process in vitro, a select few liposomal systems were subcutaneously administered *in vivo*. For all the liposomal formulations tested there was no induction of a humoral immune response, as no antibody titres were detected against the encoded antigen. However, SUV-DNA complexes composed of PC:Chol:DC-Chol (16:8:4 μmole/ml) and the production of small modified DRV(DNA) by the addition of sucrose generated sufficiently high levels of cell-mediated immunity.

With regard to protein antigen delivery and adjuvanticity, the association with liposomal systems significantly enhances the immunogenicity of the fusion protein, Ag85B-ESAT-6, a promising tuberculosis vaccine antigen. Several factors were shown to influence adjuvanticity of these liposomal systems. For example, the inclusion of the immunomodulator, TDB, effectively enhanced immunity against tuberculosis by increasing the adjuvanticity of these liposomal systems. Such immune responses were prolonged and most effective when these liposomal systems were either neutral or possessed a net positive charge rather than a negative charge and when the protein antigen was entrapped within these vesicles rather than surface complexed. Therefore, the overall protection against infection by tuberculosis was enhanced, presumably as a result of these liposomes forming depots, whereby the protein antigen is released slowly and at a controlled rate, maintaining therapeutic levels of the antigen *in vivo* to exert its therapeutic effect.

Keywords: Plasmid DNA, protein, transfection efficiency, tuberculosis, adjuvant, liposome, sub-unit vaccine.

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'The time has come to close the book on infective diseases.
We have basically wiped out infection in the US.'
(William Stewart, US Surgeon-General, 1960s)

Abbreviations

APC	Antigen presenting cells
Chol	Cholesterol
CMI	Cell mediated immunity
DC-Chol	3β-[N-(N′,N′-Dimethylaminoethane)-carbamoyl]Cholesterol
DDA	Dimethyldioctadecylammonium bromide
DMPC	Dimyritoyl phosphotidylcholine
DMPE	Dimyritoyl phosphatidylethanolamine
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DOPC	Dioleoyl phosphatidylcholine
DOPE	Dioleoyl phosphatidylethanolamine
DOTAP	Dioleoyl trimethylammonium-propane
DPTAP	Dipalmitoyl trimethylammonium-propane
DRV	Dehydration-rehydration vesicles
DSPC	Distearoyl phosphotidylcholine
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ESEM	Environmental scanning electron microscopy
HI	Humoral immunity
LUV	Large unilamellar vesicles
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MLV	Multilamellar vesicles
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline containing 0.025% Tween-20
PC	Egg phosphotidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PS	Phosphatidylserine
SA	Stearylamine
SUV	Small unilamellar vesicles
TDB	Trehalose dibehenate
TDM	Trehalose dimycolate
TEM	Transmission electron microscopy

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1. Introduction

1.1 Vaccines

The need and demand for vaccines against infectious diseases is becoming increasing crucial. The spread of infectious diseases is still a major and serious problem, as approximately one-third of the estimated 52 million worldwide annual deaths are attributed to infectious diseases (Madigan *et al.*, 2002). Some of the most prevalent diseases include; Tuberculosis, acquired immunodeficiency syndrome (AIDS), malaria and hepatitis (all types). The widespread of such diseases could be prevented by the application of vaccines.

The immune system works in such a complex way in which to protect us against infectious diseases. There are two basic types of immunity; 'innate' which consists of ready-made antibodies that are quickly supplied and induced serum components and 'adaptive' immunity in which the body produces its own specific immune responses after exposure to a pathogen. After natural exposure to viruses or bacteria, the 'adaptive' immune system is able to 'learn' and therefore react more rapidly upon further exposure to the pathogen or disease causing agent (Leserman, 2004), thus eliminating the pathogen, by stimulating T and B lymphocyte responses before it can initiate another infection (Spack and Sorgi, 2001). This active immune response can be the natural result of an exposure to an infection or it is possible to induce an 'adaptive' immune response by immunisation with the appropriate antigen, whereby immunity is conferred to infectious disease by artificial methods to expand the protein antigens specific clones. By immunising an individual against a specific infectious disease, the injected vaccine candidate would be recognised as foreign within the patient and subsequently subjected to the similar immune pathways as the antigens of the specific pathogen. Therefore, the individual is able to actively produce the appropriate and specific antibodies against the pathogen, therefore developing immunity against subsequent challenges, without ever actually developing and suffering from the disease itself.

1.2 What are our options for active immunity?

1.2.1 Conventional vaccines

The overall objective of vaccines is to exploit the hosts' natural defence system thereby promoting long-term immunological protection against the establishment of an infection. Developments in genetic vaccination over the past two or three decades, has provided us with a promising and convenient method of generating protective immunity against the

injected antigen-encoded protein and plasmid DNA (O'Hagan *et al.*, 2001; Gregoriadis, 1998). However, before our present understanding of recombinant DNA technology and gene therapy, conventional vaccines are the dominant vaccines used to induce the necessary immune responses against viruses, bacteria and parasites. These forms of vaccines are either live attenuated vaccines or inactivated, which are both very efficient and fully capable of activating immune pathways. However, these vaccines can be potentially unstable and are plagued by safety concerns (Verma and Somia, 1997). During replication, the attenuated organism is capable of reverting back to its pathogenic or even into a more pathogenic state (Gregoriadis, 1998).

1.2.1.1 Live attenuated vaccines

Such vaccines are usually derived from attenuated strains of the specific isolated bacteria or virus. For example, the vaccine Bacille Calmette-Guérin (BCG) is an attenuated strain of *Mycobacterium bovis* and is one of the most used vaccines worldwide used to vaccinate and protect patients against infection from tuberculosis. Tuberculosis is caused by an infection with *Mycobacterium tuberculosis* and is considered as one of the world's most infectious diseases, as it still remains as one of the major global health problems and to date with at least one third of the world's population having been infected. Although, immunisation with BCG is one of the most widely used vaccines and has shown to effectively initiate high levels of primary immune responses within the patient, it appears that this vaccine has not had a huge impact on the epidemiological state of tuberculosis, as stated by Advisory Council for the Elimination of Tuberculosis (ACET). It seems that the efficacy of protection from tuberculosis after immunisation with the BCG vaccine varies from 0 to 80% within the immunised population (Fine, 1995).

Another example of a live attenuated vaccine is the live (oral) polio vaccine, where the infectious part of the virus is attenuated. This vaccine is know as the Sabin vaccine, since it was developed by Albert Bruce Sabin and became available for human use in 1963 (www.accessexcellence.org/AE/AEC/CC/polio.html). However, in addition to the attenuated vaccine, there is another type of poliomyelitis vaccine in which the infectious part of the organism is inactivated by the chemical formalin. This vaccine was developed by Dr Jonas Salk.

1.2.1.2 Inactivated vaccines

Various other vaccines against infectious diseases can currently be prepared by inactivation. The influenza vaccine Fluarix® (GSK) and Enzira® (Chiron Vaccines) are a suspension of formaldehyde-inactivated influenza virus which is administered as a single dose, injected intramusculary. However, immunisation against influenza is only recommended to those persons at high risk, including those that suffer with chronic respiratory, heart and liver diseases and all persons over 65 years (www.bnf.org).

The associated adverse side effects of these viral or bacterial vaccines, whether it is a vaccine based on live or inactivated organisms, range from a simple headache and nausea to neurological complications, vaccine associated disease and in some severe cases even death (Huang *et al.*, 2004). Since the severe outbreak of AIDS in 1981 (Madigan *et al.*, 2002), and the complications related to immune suppressed individuals, there has been an increased awareness of the safety concerns associated with various vaccine candidates (Gregoriadis, 1990). The associated toxicity concerns and adverse side effects are an unacceptable problem, which therefore suggests alternative strategies need to be considered and assessed. Accordingly, alternative vaccine strategies emerged, with the development of non-viral systems that work at the molecular level to elicit specific immune responses and overall are considered safe (Gregoriadis, 1998; Spack and Sorgi, 2001). These vaccines are derived from extracts of or detoxified exotoxins produced by a micro organism (www.bnf.org). These new generation vaccine systems include;

(1) Plasmid DNA
(2) Recombinant subunit proteins
(3) Synthetic peptides
(4) Protein polysaccharide conjugates

Current examples of these vaccines include; diphtheria vaccine, tetanus vaccine, hepatitis B vaccines. Diphtheria vaccines are prepared from the toxin of *Corynebacterium diphtheriae* and adsorption onto an adjuvant system, such as aluminium hydroxide or aluminium phosphate, improves the antigenicity (www.bnf.org). However, this vaccine is given in combination with various other vaccine systems, such as the tetanus vaccine (toxoid), the acellular form of pertussis and the inactivated form of poliomyelitis vaccine components adsorbed on a mineral carrier (www.bnf.org). These combination vaccines are mainly

administered in childhood at 2, 3 and 4 months and subsequently 3-5 and 16-18 years. Current licenced hepatitis B vaccines include, Engerix B® (GSK), HBvaxPRO® (Aventis Pasteur) and RECOMBIVAX HB® (Merck) which consist of inactivated hepatitis B virus surface antigen (HbsAg) prepared biosynthetically within yeast cells by recombinant DNA technique, and subsequently adsorbed onto aluminium based systems in order to enhance the antigens immunogenicity.

However, although these non-viral vaccines are considered as weak immunogens in comparison to viruses, research into non-viral gene transfer has intensified over the past few years, since these new generation vaccines offer highly improved safety advantages over viral vaccines.

1.3 Viral vectors

The use of retroviral transfer as viral vaccines has previously proven to be highly efficient although only within actively dividing cells, thus, the use of retroviruses for gene transfer is relatively limited. In addition, the safety of this viral vector as a vaccine is a major area of concern, due the possibility of triggering new associated infections by spontaneous reactivation of the integrated retroviral DNA (Smith, 2003). Gene transfer by this method is random, so there is no control over the exact location in which the gene becomes inserted, which can have severe detrimental effects by inhibiting normal cellular functioning and even activate oncogenes (Patil *et al.*, 2005). Indeed, these safety concerns became apparent when two patients developed leukaemia during a clinic trial for the treatment of X-linked severe combined immunodeficiency disease (SCID-X) using retrovirus as the vector (Buckley, 2002; Kaiser, 2003). Adenovirus is an alternative viral vector that is capable of infecting both non-dividing and dividing cells, therefore is more efficient for use within gene therapy compared to retroviruses. Gendicine is an adenovirus encoding the tumour suppressor p53 gene and is a Chinese-FDA approved gene therapy treatment (Patil *et al.*, 2005). However, once again the severe toxicity problems associated with these viral systems immediately emerged with the death of Jesse Gelsinger when his immune response went out of control after being injected with an adenovirus vector used to deliver the corrective gene (Ferber, 2001). This was the first reported death within human clinic trails that was directly attributed to gene therapy (Zallen, 2000; Smith, 2003) and as a consequence all gene therapy trials

were temporary suspended within the US. Although, the majority of reported adverse side effects are mild, in some cases they are extremely serious, as those cases detailed previously. Therefore, the overall safety profile of vaccines largely became public concern (Duclos, 2004) partly instigating the launch of an Immunization Safety Priority Project (ISPP) by the World Health Organization (WHO). Ever since, the WHO have devoted significant energy and resources to defining international quality standards to ensure the safety of all immunisations (Duclods and Hofmann, 2001) and have worked and consulted with vaccine manufacturers to enhance their compliance with these standards and manufacturing practices.

1.4 Immunisation with subunit proteins

Subunit vaccines only use a small part of the organism in order to initiate a strong immune response. A gene or genes that code for specific subunits from the organisms genome are identified and isolated. Research into the immune mechanisms of infectious diseases and the mechanisms involved in the hosts' immune response has immensely intensified over the past decade, providing essential information for the design of promising vaccine candidates (Kaufmann, 2005). These continuous research efforts into identifying and characterising the potential protective epitopes of the disease causing agents, are crucial in order to produce potentially effective subunit vaccines, that are much safer than viral vaccines yet still just as effective after immunisation. Indeed, with respect to tuberculosis, such research has resulted in mapping of the complete genome of *M. tuberculosis* (Cole, 1996; Cole *et al.*, 1998). This extensive research strategy has enabled the ongoing identification of key protective epitopes and protein antigens expressed during infection with *M. tuberculosis* (Bramwell and Perrie, 2005). Various protective epitopes and protein antigens have been discovered and isolated within the secreted or surface proteins of mycobacterial short term culture filtrates (Andersen, 1994; Harboe *et al.*, 1998).

Immunisation with protein antigens alone is insufficient as a candidate vaccine, as proteins are unable to initiate adequate levels of immune responses, as a result of their low immunogenicity (Gregoriadis, 1994; Leserman, 2004). Therefore, the need for an effective adjuvant or particulate delivery system is well recognised, which will hopefully enhance and promote antigen presentation, to elicit specific protective immune responses against the

associated antigen. Thereby, the development of successful subunit vaccines through delivery systems could provide an enhanced immunisation system to replace the current BCG vaccine.

1.5 Immunisation with plasmid DNA

The objective of using plasmid DNA as a vaccine system is to mimic the viral infection, which is reported to be an advantage over conventional subunit vaccines (Gregoriadis *et al.*, 1998), by eliciting both humoral and cellular immune responses, without producing any associated symptoms of the disease. Plasmid double stranded DNA is a high molecular weight polymer of nucleotides containing transgenes, which encode for specific protein antigens (Patil *et al.*, 2005). Plasmid DNA has several advantages for use within gene transfer. Firstly, DNA is easy and inexpensive to manufacture, by simply amplifying the plasmid constructs using bacteria, such as *Escherichia coli* (*E. coli*) (Gregoriadis, 1998). Plasmids are more temperature stable than live vaccines and have been shown to have a very good safety profile (Gregoriadis, 1998). However, the ability of naked plasmid DNA to elicit effective levels of immune responses remains controversial, with discrepancies between studies regarding efficacy of gene expression and antibody responses (Deck *et al.*, 1997; Johnson *et al.*, 2000). However, it seems that after intramuscular injection of the naked plasmid DNA, encoding the targeted antigen results in gene expression (Wolff *et al.*, 1990) and leads to both protective humoral (i.e. B-lymphocytes) and cell-mediated (i.e. T-lymphocytes and cytokines) immune responses (Gregoriadis, 1998; Chattergoon *et al.*, 1997). In a Phase I clinical study, it was observed that following intramuscular injection of the plasmid DNA encoding a gene for *Plasmodium falciparum*, cytotoxic-T-lymphocyte (CTL) responses against the antigen were detected within patients (Wang *et al.*, 1998). Following this route of administration, is seems that a small amount of the plasmid DNA is internalised by myocytes, although, only a small fraction of muscle cells participate in sufficient DNA uptake (Davis *et al.*, 1993) and as these cells lack co-stimulatory molecules they are unable to act as professional antigen-presenting cells (APC) (Perrie *et al.*, 2000; Davis *et al.*, 1993). Further reports have also identified that the injected plasmid DNA can transfect APC, such as dendritic cells, directly at the site of injection, enabling efficient antigen presentation of the processed antigen (Gregoriadis, 1998; Perrie *et al.*, 2001).

As well as direct intramuscular injection of naked plasmid DNA, there are various other techniques in which plasmid DNA can be delivered in order to promote and enhance efficient immune responses to the encoded antigen. The various techniques utilised to facilitate DNA uptake into cells, include; microinjection (McAllister *et al.*, 2000), electroporation (Regnier *et al.*, 2000) and particle bombardment (Luo and Saltzman, 2000). Particle bombardment involves the transfer of plasmid DNA-coated with gold microparticles by immunisation with the use of gene gun. This method of administration has proved to generate efficient transfection directly into the cytosol of dendritic cells localised within the skin, *in vivo* (Fynan *et al.*, 1993; Weiss *et al.*, 2000), thus inducing protective levels of immune responses even with only small doses of DNA (Condon *et al.*, 1996). However, immunisation by gene gun requires direct injection to the target tissue meaning that the administration of DNA is restricted to local expression within the dermis, muscle and mucosal tissues (Patil *et al.*, 2005). Therefore, when the naked DNA is administered by many of the other routes the plasmid is rapidly degraded, leading to less efficient gene transfer and reduced expression (Spack and Sorgi, 2001).

The use of naked DNA for vaccination has also shown to possess several disadvantages. For instance, the cellular uptake of the plasmid DNA molecule is impeded by several inherent factors such as; size, instability and charge (Patil *et al.*, 2005). Moreover, when naked DNA is injected *in vivo*, the plasmid is readily exposed to and degraded by deoxyribonuclease present within the interstitial fluid (Davis *et al.*, 1993; Manickan *et al.*, 1997; Gregoriadis *et al.*, 1998), meaning that, large quantities of DNA need to be injected with the possibility of requiring multiple injections, in order to elicit the appropriate levels of protective immune responses (Manickan *et al.*, 1997; Gregoriadis, 1998). Despite these problems, work in the laboratory has continued, in order to try to improve the uptake and expression of DNA vaccines, to induce an enhanced level of protective immunity within the host. The application of naked DNA is effective and viable when the DNA is applied in close proximity to the site of pathology and away from any degradable components within the plasma. However, if the DNA is required to reach anatomically inaccessible sites, naked DNA will be ineffective (Brown *et al.*, 2001).

Gregoriadis *et al.*, (1997) proposed that direct transfer to the APC, which are recognised as the cells of the immune response (Lewis and Babiuk, 1999), might be the preferred target for

the plasmid DNA to transfect, in order to enhance cell-mediated and humoral immune responses. Therefore a vaccine carrier that is safe and efficient is the ideal candidate for the delivery of the antigen-encoded plasmid DNA to the APC. This led to the development of non-viral vectors, also known as particulate delivery systems, with the idea that these systems will not only protect the plasmid from rapid degradation by extracellular enzymes but will also act as effective transport systems for the introduction of DNA into cells other than APC infiltrating the site of injection, thus enabling specific targeting to immunologically relevant cells and optimising immune responses to the encoded antigen (Gregoriadis, 1998).

1.6 Particulate delivery systems (non-viral)

Since subunit, peptide and plasmid DNA vaccines are unable to elicit sufficient levels of immune response, the application of adjuvants have proven to be successful in inducing increased levels of immunity for a wide variety of antigens (Alison and Gregoriadis, 1974; Gupta and Siber, 1995; Brandt *et al.*, 2002). An adjuvant is any substance that is able to enhance a specific immune response, increasing both cellular and humoral immunity, to an antigen compared to the antigen alone (Gupta and Siber, 1995; O'Hagan *et al.*, 2001). The ideal adjuvant would act by selectively modulating and enhancing the immune responses by; increasing the immunogenicity of the weak antigen to elicit a protective immune response, while the adjuvant itself remains stable in terms of cellular toxicity. Also as the immunogenicity of the vaccine is increased, compared to injection of antigen alone, the dose of antigen within the vaccine will decrease, therefore also reducing overall costs (Gupta and Siber, 1995).

So far, the mechanisms in which such adjuvant systems enhance immune responses has not been fully detailed, thus the development of new generation adjuvants is often made more difficult. It is initially thought that adjuvants may act in different ways; e,g, acting as a delivery system, transporting the associated antigen to the desired site (e.g. regional lymph nodes, APC) or the adjuvant may act as a depot, where the injected adjuvant remains locally at the site of injection, releasing the incorporated antigen at a slow and steady rate. Therefore, therapeutic levels of antigen are maintained for prolonged periods of time and thus enabling effective antigen presentation by the APC, while additionally this may allow

for a decrease in the frequency of vaccine administration (Gupta and Siber, 1995; Storm and Crommelin, 1998).

Aluminum-based systems were the first licensed adjuvants, which have since been utilised as successful components within many human and veterinary vaccines (Gupta and Siber, 1995; Gupta, 1998) and remain the only FDA licensed adjuvant. The way in which these aluminum systems work to enhance the immune response is to form a depot at the site of injection. Recent developments against cervical cancer and the human papillomarvirus (HPV) have led to the development of two different vaccine candidates, in which aluminum systems are utilised as an adjuvant (Cohen, 2005). They are both now in phase III efficacy trials in more than 50,000 people in several countries in order to improve their safety and efficiency (Cohen, 2005). Firstly, Merck & Co. of Rathway, New Jersey have developed a vaccine which is made in yeast and consists of many genotypes that allow immunisation against HPV for both male and females (Cohen, 2005). Aluminum is utilised as the adjuvant for delivery and immune-boosting for this Merck vaccine. In contrast, GSK's vaccine is made in baculovirus and is only available for immunisation for women. The adjuvant utilised for this vaccine candidate was ASO4, which contains a mixture of aluminum and a bacterial lipid (Cohen, 2005). In phase II clinical trials both vaccine candidates demonstrated 100% protection against HPV, however, research is still ongoing with the projects due to and 2007-2010 (Cohan, 2005).

One aluminum based licensed vaccine is RECOMBIVAX HB®, which was developed within the Merck research laboratories, is a subunit viral vaccine that has been derived from the hepatitis B surface antigen. It is harvested and purified within yeast cells (*Saccharomyces cerevisiae*), so is easy to produce and the resultant purified subunit vaccine is coprecipitated with an alum-based system to form an adjuvanted vaccine system. However, although this subunit vaccine is effective at decreasing the burden of the disease, the vaccine requires three doses over a period of about 8 months. This high dose regime means that it is more difficult to deliver the vaccine within some parts of the world and in some cases the vaccination may not even be completed. Therefore, researchers are constantly striving to develop new and improved vaccine candidates, which will not only reduce the vaccine regime and dose, but will also reduce production and manufacturing costs, making it available to a larger percentage of the world's population. Therefore, the application of

particulate delivery systems for subunit and plasmid delivery is an attractive vaccine candidate.

By utilising delivery systems, they could increase the immunogenicity of the antigen and enhance the associated immune response, thereby reduce the required dose of the vaccine and subsequently lower the overall costs. As well as enhancing the immunogenicity of the response, these delivery systems could also enable manipulation of the type of immune response that is generated. The development of such new delivery systems may allow effective utilisation of subunit and plasmid antigens which have previously ineffective at inducing the effective and appropriate responses as well as improving the responses to existing vaccines (Lofthouse, 2002).

Aluminium-based adjuvant systems have shown to elicit a Th2 dominant immune response, however these systems were found to be poor at inducing cell-mediated immunity (Audibert and Lise, 1993; Gupta and Siber, 1995). Also, although aluminium based adjuvants have demonstrated a good safety profile over many decades (Bramwell and Perrie, 2005), in recent years there has been some concern over the safety of this adjuvant system, as it has been associated with severe local reactions and allergic reactions, such as erythema and subcutaneous nodules (Baylor *et al.*, 2002; Bramwell and Perrie, 2005). As a consequence of these reported possible adverse effects to aluminium based systems, research efforts have now focused on the development of alternative adjuvants and/or delivery systems. Once again, it seems that the main problem associated with various adjuvant systems is mainly due to their toxicity and adverse side effects, which should be monitored (Gupta and Siber, 1995). However, in comparison to viral vectors, very few delivery systems generate severe adverse side effects.

Adjuvant system	Description	Applications and studies
Cells and cytokines	Cytokines stimulate cells of the immune system	Dendritic cells are currently being tested for their adjuvant potential. It seems mouse dendritic cells assist in the migration of natural killer cells to the lymph nodes, to elicit immunity (Pashine *et al.*, 2005). However, they are difficult to make with high costs.
Solid particles	Primarily composed of biodegradable and biocompatible polyesters, such as polylactide-co-glycolides (PLGA)	Have been extensively used as sutures, bone implants, (Matsusue *et al.*, 1995) and also as supporting scaffolds in tissue engineering studies (Langer, 1997). However, solid particles have also shown to be relatively potent adjuvant systems for both protein antigens and plasmid DNA (O'Hagan *et al.*, 2001) and even in some studies they have shown much potential for the delivery as a combination vaccines, such as enhancing protection against toxins after immunisation with the combination vaccine of toxin and the synthetic peptide of *Plasmodium falciparum* (Peyre *et al.*, 2004).
Emulsions	Either prepared as an oil in water or water in oil emulsion	Freund's adjuvants (FA) are formulated as water in mineral oil. Freund's Complete adjuvant (FCA) also contains heat-killed *M. tuberculosis* organisms or various other strains (Freund, 1951) that enhances its adjuvanticity, therefore, FCA is extremely potent but is also very toxic. Incomplete FA is a safer adjuvant with the absence of the mycobacteria. Both these adjuvants are good at inducing high levels of CMI and HI, but have severe toxicity concerns because the mineral oil cannot be easily metabolised and the mycobacterial elements can elicit severe granulomatous reactions. Licensed adjuvant, MF59, a squalene oil in water emulsion with no additional immunostimulatory adjuvants (Ott *et al.*, 1995). Clinical trials of MF59 as an adjuvant for influenza vaccine showed its adjuvanticity and safety profile and has been approved as a licensed vaccine product within Europe (FLUAD™) (DeDonato *et al.*, 1999).
Liposomes	Lamellar vesicles formulated with phospholipids	Antigens and adjuvants can be entrapped inside or coated onto the surface of these spherical vesicles. Discussed further in section 1.7
Niosomes	Non-ionic surfactants	Niosomes lack toxicity and have been reported to be relatively stable structures (Rogerson *et al.*, 1988; Yoshioka *et al.*, 1994). Studies have shown that antigens can be entrapped within gels consisting of niosomes contained within a tubular network. It is within these niosomes that the antigen is entrapped. When applied *in vivo*, the gel breaks down releasing the niosomes entrapping antigen, generating an effective depot effect (Murdan *et al.*, 1999; Murdan *et al.*, 1999).

Table 1.1 Various types of adjuvant and particulate delivery systems

As shown in Table 1.1, within subunit and plasmid vaccine delivery, the application of several particulate delivery systems, including; colloidal phospholipid lipid and surfactant-

based vesicles (i.e. liposomes) (Aramaki *et al.*, 1994), non-ionic surfactant vesicles (i.e. niosomes) (Brewer & Alexander, 1992) as well as polymeric solid particles and nano-particles (O'Hagan *et al.*, 1993; Florence *et al.*, 1995) have proved successful. Indeed, a report released in October 2005 by Chiron discussed the possible beneficial effects of applying the o/w emulsion adjuvant, MF59, as an adjuvant vaccine against H9N2 avian influenza strain (www.chiron.com/pipeline/vaccinespipe/avian.html). MF59 is already an approved and licensed adjuvant vaccine for influenza. In a 96 patient study, it was reported that formulations with MF59 resulted in high immunogenicity, which induced antibody levels believed to confer protection against the influenza strain. The addition of the MF59 adjuvant also decreased the dose of antigen to 3.75 μg, which is a quarter of the normal influenza dose required to induce protective immunity. This emphasises the beneficial effect and potential application of adjuvant systems, not only for current infectious diseases, but also for future diseases and possible outbreaks. Most of these delivery systems have been exploited for their inherent associated adjuvanticity, as well as their ability to augment the protective immune responses to poorly immunogenic antigens. These particulate delivery systems protect the antigen from *in vivo* degradation and thus allow vaccine administration by different routes. These promising vaccine candidates are additionally advantageous, as they possess increased safety profiles in comparison to live vaccines.

Although, compared to viral vaccines, these non-viral delivery systems possess lower levels of immune responses, which has been attributed to poor cellular uptake *in vivo*, these vaccines posses several advantages over viral vaccines. Firstly, these particulate delivery systems can be altered and manipulated to meet the requirements of the vaccine candidate, with opportunities to formulate and optimise the delivery systems chemical and physical properties. Additionally, from a pharmaceutical perspective, these synthetic systems can be produced on a large scale, with effective reproducibility and stability. With regard to the lower transfection efficiency than viral vectors, its seems that the potency of such non-viral vaccines can be greatly improved and rendered more efficient by association with immunological adjuvants, however, the safety of these co-adjuvants needs to be considered and evaluated.

1.7 Liposomes

The origin of liposomes dates back to 1965, when Bangham discovered the potential use of swollen phospholipid lamella, (i.e. liposomes), as an efficient model for biomembranes and since then, liposomes have assisted in our current understanding of the biological membranes structure and function (Bangham *et al.*, 1965; Bangham, 1983). However, it wasn't till the early 1970's, that the potential role of liposomes as a delivery system, for a multitude of drugs and antigens, was discovered and developed (Gregoriadis and Ryman, 1971). It is now well recognised that once administered *in vivo*, liposomes are rapidly taken up by macrophages, such as in the liver and spleen, where they are gradually degraded in lysosomal vacuoles, thus delivering and releasing the incorporated solutes efficiently (Segal *et al.*, 1974). This observation emphasises the potential use of liposomal vesicles as a protective and effective delivery system for an immense range of solutes.

Liposomes are vesicles containing an assembly of lipid molecules within a lamellar arrangement, with each single lipid molecule (i.e. amphiphile) consisting of both polar (i.e. hydrophilic) and non-polar regions (i.e. hydrophobic) (Figure 1.1). When these lipid molecules are dispersed within a polar solvent, such as water, these amphiphile molecules rearrange themselves in such a way that the non-polar regions are brought into close proximity with each other and thereby masked from the aqueous environment by the adjacent polar regions (Figure 1.1). As the liposomes form, the aqueous environment in which these vesicles are dispersed, is passively entrapped within the vesicle structure, therefore lipid bilayers form within a lamellar arrangement, where the non-polar regions are 'sandwiched' between the polar regions of the phospholipids, in order to conceal the non-polar regions from the bulk of the internal polar solvent (Figure 1.1). During the formation of these closed lipid bilayers, the solutes, such as drugs, proteins and nucleic acids, contained within the polar solvent will also become entrapped within these liposomes, thus the entrapped material is protected and can be effectively delivered within these closed vesicles (Gregoriadis, 2002). Overall, liposomes can be defined as 'vesicles consisting of one or more concentric bilayers alternating with aqueous compartments' (Gregoriadis *et al.*, 1999).

1.7.1 Liposome morphology

The number of lipid bilayers present within the liposomal vesicle is dependent on the method of preparation. There are numerous ways to generate liposomes, in which each preparation

meets widely different requirements. Multilamellar vesicles (MLV) are the simplest liposomal vesicles to make. MLV liposomes are prepared by the established film method (Bangham *et al.*, 1965), involving mixing of the selected lipid components within a solvent (i.e. chloroform/methanol) mixture, which is then evaporated by rotary evaporation. Once the solvent is completely removed, a dry lipid film forms and as the aqueous solution (i.e. water) is added to hydrate the dry film, the lipid components begin to break away gradually forming large spherical liposomes, with each consisting of numerous concentric lipid bilayers entrapping water (Figure 1.1). As these vesicles form, the lipid components arrange themselves in a random manner, therefore the size of MLV liposomes cannot be controlled and therefore within the liposomal solution there are heterogeneous population of MLV sizes, ranging from as small as 100nm to a few microns in size (Gregoriadis, 2002).

After preparation of MLV liposomes, these large liposomal structures can be dramatically reduced in size and their morphology can be completely altered by sonication of the MLV suspension, generating small unilamellar vesicles (SUV). In comparison to MLV, as illustrated in Figure 1.1, these SUV liposomes only possess one lipid bilayer and are homogeneous in size. Vesicle sizes of SUV are approximately <100 nm, although the lower limit of the size of these liposomes is predetermined by the maximum curvature that the lipid bilayer can achieve (Gregoriadis, 1990). Due to their relatively small vesicle size, these SUV liposomes have an added advantage, as they are able to remain within the systemic circulation for longer periods of time, in comparison to the larger MLV. It has previously been shown that MLV liposomes, when injected intravenously, were rapidly cleared from circulation, whereas in contrast SUV liposomes, with an average vesicle diameter of < 200 nm, were retained for longer (Juliano and Stamp, 1975).

In a bid to enhance the efficiency of drug incorporation within liposomes Kirby and Gregoriadis (1984) developed a method of producing liposomes, which is known as the dehydration-rehydration procedure. This method produces vesicles that undergo various transformations in physical characterisation, in order to generate the ideal completed liposomes. The procedure involves mixing the material to be entrapped, together with the SUV before freeze-drying. In this way, all of the lipid can be brought into contact with all of the solute in the anhydrous state. During the dehydration-rehydration procedure the concentration of the vesicles combined with the reduction in hydrophobic forces causes loss

of vesicle stability thereby promoting fusion of these destabilised SUV into larger multilamellar vesicles entrapping solute as they form (Deamer and Barchfield, 1982) into dehydration-rehydration vesicles (DRV). This procedure has been utilised in numerous studies and is a well-established method that produces liposome vesicles that entrap a high quantity of plasmid DNA.

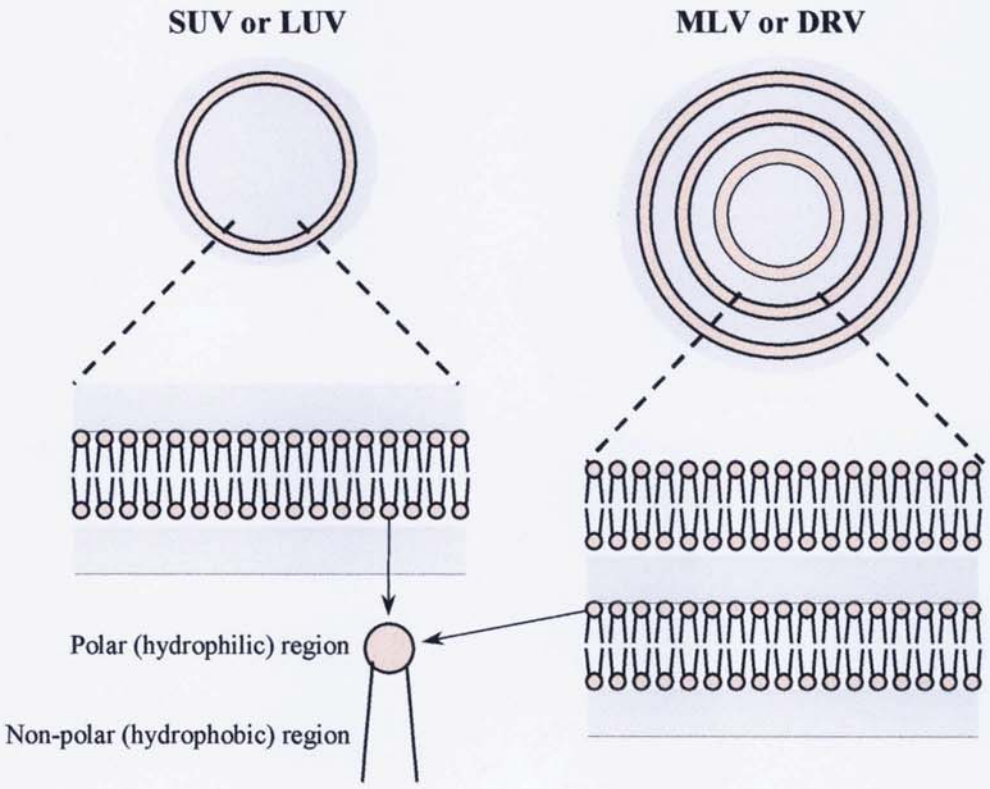


Figure 1.1 A diagrammatic illustration of various liposome systems. The grey areas represent the aqueous environment either surrounding the liposome vesicle or entrapped within and between the lipid bilayers. It is within this aqueous environment in which the appropriate and specific solutes can become entrapped (i.e. hydrophilic solutes). Hydrophobic solutes become entrapped within the lipid bilayers.

1.7.2 Liposome composition

When formulating liposomes, there is an immense diversity with regard to the various lipid components that can be used to manufacture these vesicles. Each lipid component varies to the degree of head-group size, alkyl chain length and extent of chain saturation (Felnerova *et al.*, 2004). The characteristics of the vesicle, such as the surface charge, size and lipid composition, could promote the transfection of liposome incorporated DNA or uptake of protein antigen to the APC (Gregoriadis *et al.*, 1998). Phospholtidylcholine (PC) and its

derivatives, have surfaced as the main lipid excipient of liposome products due to its non-toxic biodegradable profile (Storm and Crommelin, 1998; Mohammed *et al.*, 2004). Depending on the nature of the phospholipid component, the lipid bilayer of liposomal vesicles can subsist within a fluid or rigid state at ambient temperature. The state and characteristics of amphiphiles is dependent on the lipids gel-liquid crystalline phase transition temperature (T_c). This means that the liposome bilayers can be in fluid or rigid state, in which the transition temperature is below or above ambient temperature, respectively (Gregoriadis *et al.*, 1998). The phase transition temperature of phospholipids is influenced by numerous factors including the lipids alkyl hydrocarbon chain length. As the alkyl chain increases in length, the van der Waal interactions between the lipid chains becomes stronger, thereby in order to disrupt the stringent and rigid lipid packing of the liposome bilayer, more energy needs to be applied, thus increases the phase transition temperature. For example, DMPC has a hydrocarbon chain length of C_{14} and a phase transition temperature of 23°C, whereas DSPC has a hydrocarbon chain length of C_{18}, resulting in a higher phase transition temperature of 55°C, as more energy is required to disrupt the ordered packing and to subsist in a fluid state. To that end, DSPC is classed as a more rigid lipid than DMPC forming more stable bilayers for liposomes.

It is well established that the inclusion of cholesterol (Chol) within the liposomal formulation stabilises lipid by increasing the orientation, ordering and consequently the packing of the hydrophobic chains of the phospholipids (Bhattacharya and Halder, 1996). Chol is said to act a space-filling molecule, in which Chol accommodates within the molecular cavities of the bilayer membrane, thus decreasing the permeability of the membrane (Demel *et al.*, 1972). This increased stability was found to enhance the half-life of the liposome vesicles and the residence time of liposomes within circulation (Senior and Gregoriadis, 1982). With a more rigid and highly packed bilayer, such liposomes are less likely to be attacked and broken down by blood proteins, such as high density lipoproteins (HDL), therefore, will retain their entrapped solute content for longer periods of time, enhancing the ability of these injected liposomes in exerting their therapeutic effect. The same applies for the inclusion of high transition phospholipids, such as DSPC. In contrast, liposomes enriched with the fusogenic lipid DOPE, are les stable, however, liposomes enriched with DOPE have the ability to form an inverted hexagonal phase due to the lipids molecular cone shape, enabling efficient membrane fusion to allow entry into the target cell

and membrane destabilisation for effective release of the incorporated solute (Litzinger and Huang, 1992).

It is now well established that the type of immune response required can be generated *in vivo* by modulating the adjuvant and/or delivery system used for immunisation (Audibert and Lise, 1993; Gupta and Siber, 1995). With respect to liposomes, this can be achieved by selection of the appropriate lipid excipients within the bilayer and the suitable method of preparation, liposome morphology and the manner of antigen association.

1.7.3 Liposomes: delivery of protein antigens

When the protein antigen is administered *in vivo*, the injected antigen can interact with two different major histocompatibility complex (MHC) molecules, well recognised as class I or class II. These MHC molecules are cell surface proteins that can be found on professional APC (i.e. macrophages and dendritic cells), although MHC I protein molecules can be found on the cell surface of all nucleated cells. The major function of these MHC molecules is to interact with and bind to the processed foreign protein antigens, which are then transported through the cytosol to be expressed on the surface of the APC where the peptide-MHC complex will interact with T cell receptors (Madigan *et al.*, 2002). However, the type of MHC molecule that binds to and presents the antigen is dependent on the source of the protein antigen. In the case of subunit protein antigens they are primarily processed as exogenous antigens by the immune system. Therefore, although they are fully capable of inducing a predominant humoral response by presentation by the MHC II molecules to the Th1 pathway by $CD4^+$ T lymphocytes, these introduced antigens are unable to be presented by the MHC I molecules due to their inability to gain access into the cytosol of the target cell (Spack and Sorgi, 2001). Therefore when these antigens are injected alone they are unable to prime a MHC class I-restricted cytotoxic T lymphocyte (CTL) immune response. However, the MHC I antigen presentation of exogenous antigens can be induced by delivering and artificially introducing these antigens into the cytoplasm of the target cell by utilising liposomal systems (Rao and Alving, 2000), which will also provoke the release of Th1 cytokines that will additionally stimulate strong CTL responses (Spack and Sorgi, 2001).

The immunological role of liposomes for efficient adjuvant for protein delivery was first established when negatively charged liposomes were found to be effective against diphtheria

toxoid (Allison and Gregoriadis, 1974). Ever since, liposomes have been utilised as effective adjuvants and/or delivery systems for many protein antigens as they are rapidly taken up by macrophages, thus are fully capable of enhancing specific immune responses. Indeed, by the incorporation of haemagglutinin (HA), the major surface antigen of the influenza virus, into liposomal vesicles the immunogenicity of the antigen was enhanced, thus inducing the production of HA-specific serum and mucosal antibodies, such as IgG and IgA, which was established to be of crucial importance for the protection against influenza infection (Renegar and Small, 1991). Various reports indicate that for immunisation against influenza new vaccines strategies should aim at the induction of an IgA response at the respiratory tract, resulting in long term production of IgG and IgA antibodies and protection against infection with the influenza virus. However, current vaccine strategies administer free subunit preparations via the intramuscular route. By the application of liposomes as an adjuvant system, the subunit vaccine could be successfully administered via the intranasal route avoiding the need for intramuscular injection. Liposomes enhance the immunogenicity of antigens proving to be more effective at promoting protective immunity then free antigen, such that intranasal administration of liposome-incorporated antigen has been shown to induce higher values of protection against influenza than when the mice were immunised intramuscularly with the free subunit vaccine (de Haan *et al.*, 1995).

The concept of antigen delivery is further complicated by the type of immune response that is required from the antigen, in order to combat the disease target, as different antigens will require different responses (Lima *et al.*, 2004). Indeed, toxins require a dominant humoral response whereas the induction of a more cell-mediated immunity, with the production of a Th1 response, is required for most intracellular bacteria (Lima *et al.*, 2004). Thus, due to the broad spectrum and diversity between different protein antigens, different antigens require different properties from the adjuvant systems. Therefore, these liposomal vesicles have to be correctly formulated in order to fulfil their role in eliciting the appropriate immune responses to the associated antigen (Lima *et al.*, 2004), however it seems that the adjuvanticity of liposomes is not associated to any definitive formulation, regarding any specific lipid composition or structure (Gregoriadis, 1990; Bramwell and Perrie, 2005).

There is no definitive understanding of the role liposomes offer as an effective adjuvant system. As detailed previously, liposomes can either act as a delivery system transporting the

incorporated antigen to the target cells thus mobilising the antigen from the area of injection to the regional draining lymph nodes, where they interact with antigen-presenting cells, while simultaneously protecting the antigen from the harsh extracellular conditions within the biological milieu. Alternatively (or in addition) these vesicles may form a depot at the site of injection, resulting in sustained release of the incorporated antigen (Gupta and Siber, 1995; Cox and Coulter, 1997; Storm and Crommelin, 1998), as shown with the aluminum-based systems. Previous reports propose that it is this depot effect that is the fundamental factor that determines the level of immunity against the antigen (Iezzi *et al.*, 1998; Zinkernagel, 2000), and is highly dependent on the amount of time in which the antigen has to interact with the target cells, such as the regional lymph nodes. However, the role of the delivery system is dependent on the vaccine antigen. Therefore, currently, the application of liposomal vesicles as a particulate delivery system for subunit vaccines offers great potential.

The efficacy of liposomes as efficient adjuvant systems has been demonstrated within an immense number of studies for various subunit antigens. For example, various studies have demonstrated the efficacy of liposome vesicles that were formulated as a delivery system against infection with plague. The fraction 1 (F1) antigen is a major protein component of the pathogenic agent that causes plague, *Yersinia pestis*, which contains subunits that possess anti-phagocytic properties (Sabhnani *et al.*, 2003). Therefore, this F1 antigen has been utilised as a potential vaccine antigen against infection with plague. Nonetheless, as with the majority of protein antigens, this F1 antigen is a weak immunogenic molecule and is rapidly degraded once applied *in vivo*, therefore several studies have reported the use of liposomes as an effective adjuvant system to enhance the immunogenicity of this antigen. Sabhnani *et al.* (2003) found that liposome delivery systems, DRV composed of PC:Chol:PG, entrapping the F1 antigen were more immunogenic than alum-based systems, generating a mixture of both high levels of Th1 and Th2 type responses. In a separate study, the F1 antigen was surface adsorbed on to DRV liposomes composed of DSPC:Chol:DPPC (Reddin *et al.*, 1998). Results from this study showed that after immunisation sufficient levels of IgG were detected within serum and after subsequent challenges survival rates were 80% in the mice injected by the intraperitoneal route (Reddin *et al.*, 1998). This experimental evidence suggests that the delivery of subunit antigens via liposomes can promote immunological responses and seems promising for use of liposomes as an effective delivery vector against infectious diseases.

Delivery of antigens in liposome systems can induce a variety of responses. Liposomal vesicle encapsulating a variety of allergens initiates a Th1 dominant response characterised by the production of cytokines such as, IFN-γ and IL-2 and high antibody titres of IgG2 (Sehra *et al.*, 1998), whereas other studies have shown that a predominant production of IgG1 antibodies indicating a Th2 dominant response after immunisation with *Leishmania* antigens entrapped within liposomes (Afrin *et al.*, 2000). Liposomal charge, lipid composition, method of preparation and number of layers (morphology) all appear to influence the adjuvant activity, depending on the protein antigen.

1.7.3.1 Liposome composition

The lipid composition of liposomes can be extensively modified and optimised, which is one of the great advantages of liposomes as an effective delivery system. Various studies have reported the effect of different lipid compositions for a variety of protein antigens. One such study, examined the effect of MLV liposomal surface charge on the adjuvant action of these systems when incorporating the model protein antigen chicken egg albumin (OVA) (Nakanishi *et al.*, 1997). In this context, it was found that the positively charged liposomal antigen delivery systems were preferentially taken up by macrophages and subsequently delivered to the cytosol of APC for antigen presentation to the MHC class I molecules. Overall, the authors results demonstrated that these positively charged stearylamine (SA) liposomes composed of PC:Chol:SA, were more efficient as an immunoadjuvant system, as negative L-α-dimyristoyl phosphatidic acid (PA) liposomes PC:Chol:PA, or neutral liposomes PC:Chol, were barely taken up by the macrophages. In addition, the immune response for these positively charged liposomes was prolonged for an extended length of time compared to neutral and negative counterparts and it was even shown that the antibody titres of IgG1 were higher than OVA contained within Alum (Nakanishi *et al.*1997).

It seems that for protein antigen delivery, liposome adjuvants that are formulated with stable lipids, such as; DMPC and DSPC, or are further stabilised with the addition of cholesterol (Chol), which is know to increase bilayer rigidity and stability (Gregoriadis and Davis, 1979; Gregoriadis, 1993), is advantageous for the elicitation of immune responses against the subunit vaccines. Indeed, studies using the antigens of *Leishmania major* have shown that DRV liposome consisting of DSPC were potentially useful adjuvant delivery systems within the mouse model of the disease (Kahl *et al.*, 1989). It is well established that stable

liposomes consisting of either high melting phospholipids and/or Chol remain within circulation for longer periods of time, as these rigid liposomes are relatively resistant to attack from plasma high density lipoproteins present within serum (Gregoriadis, 1990). Therefore, these intact liposomal systems will retain the incorporated protein antigen for longer periods of time, either for successful antigen delivery to the APC, thus artificially introducing the exogenous antigen into the cytoplasm of the cell to induce the preferential immunogenic response or additionally remain intact locally at the site of injection, releasing the incorporated antigen at a controlled and steady rate, therefore maintaining therapeutic levels of antigen within the host to enable more effective antigen presentation to the APC. However, although within many studies the incorporation of high transition temperature phospholipids was advantageous, in initial studies it was shown that DSPC liposomes lacked the adjuvant activity required for initiating the appropriate immune responses (Kirby and Gregoriadis, 1984). Thus, this emphasises, that there is an apparent antigen specificity of liposome formulations for the promotion of immune responses against the specific associated protein antigen, highlighting that there is no definitive liposome formulation for subunit vaccines (Bramwell and Perrie, 2005). These stable liposomes are able to effectively protect and deliver the antigen within circulation, and although they are considered poor immunogens, their immunogenicity can easily be enhanced by the addition of immunomodulators or co-adjuvants.

Liposomal systems can be modified in order to enhance specific immune responses. The immune responses to the incorporated protein antigen have been shown to increase upon the addition of; cationic lipids within the liposomes lipid bilayer (e.g. Tan and Gregoriadis, 1991; Davidsen *et al.*, 2005), incorporation of ligands (Gregoriadis *et al.*, 1993) and also the incorporation of co-adjuvants within the liposome formulation. One strategy that has shown possible potential is the use of adjuvants and/or immunomodulating molecules (e.g. cytokines) in combination with a particulate delivery system such as liposomes (Rao *et al.*, 1999; Rao *et al.*, 2002). To that end, some of the new generation adjuvants incorporate a chemical variant, such as monophosphoryl lipid A (MPL) or other "detoxified" cell wall constituents of bacteria (Rudbach *et al.*, 1988).

Several studies have shown that in order to initiate the required levels of immunity to the subunit vaccine, the liposomal adjuvant system may need to be modified with the inclusion

of immunomodulators as co-adjuvants within the delivery system (Olsen *et al.*, 2001). Within this cited study and referring back to the identified protective epitopes and/or protein antigens found within mycobacterial culture filtrates, the authors found that although they offer an attractive source for eliciting protective immune responses, it was shown that these identified proteins possess low inherent immunogenicity when injected alone (Andersen, 1994; Harboe *et al.*, 1998). Further studies revealed that when these isolated protein antigens were incorporated within liposomal vesicles composed of dimethyldioctadecylammonium (DDA), a cationic lipid that self assembles into closed bilayers when hydrated within an aqueous environment, to some extent increased and induced protective immunity against infection with tuberculosis (Olsen *et al.*, 2001). It was concluded that these DDA delivery systems form depots at the site of injection when administered subcutaneously, which can remain locally where it maintains the sustained and slow release of the associated protein antigen, as immunity remained high for extended periods of time with responses even still showing at day 210 (Olsen *et al.*, 2001; Holten-Andersen *et al.*, 2004). Although, DDA alone was able to enhance immune responses, it has been shown to be poor at inducing a Th1 response therefore, by the addition of immunomodulators, the immune response was enhanced even further and essentially induced high levels of protective immunity against tuberculosis. As the adjuvant forms a depot, Holten-Andersen and co-workers concluded that the possible role of DDA within these liposomal adjuvant systems could be to assist and enhance in the uptake of antigen and immunomodulator together into APC. This emphasises the requirement of an effective adjuvant delivery system that will enhance the immunogenicity of these specific antigen and direct the immune response to develop the required cell-mediated immunity (CMI) (Olsen *et al.*, 2001; Lima *et al.*, 2004). Such immunomodulators, which are effective at enhancing the immunogenicity of liposomal systems against tuberculosis and other infectious organisms, are detailed in Table 1.2.

More recent studies found that the subunit vaccine prepared by the fusion of two different immunodominant protein antigens, Ag85B and ESAT-6, (i.e. Ag85B-ESAT-6) incorporated and delivered within liposome systems was more effective than the fusion protein injected alone. Interestingly, this fusion molecule was also shown to be more effective at inducing protection against tuberculosis, than when these two different antigens were incorporated and delivered within liposomes separately (Andersen *et al.*, 1995; Brandt *et al.*, 1996; Olsen *et al.*, 2001).

Immunomodulator	Description
MPL (monophosphoryl lipid A)	Induces the synthesis and secretion of various cytokines (Ulrich and Myers, 1995) and is effective at potentiating mucosal and systemic immune responses to the incorporated antigen (Childers *et al.*, 2000). This adjuvant has no observed side effects, other than minor irritation at the injection site (Thoelen *et al.*, 1998).
MDP (muramyl dipeptide)	Derived from bacterial cell walls and activates the macrophages thus regulates the immune system (Murata *et al.*, 1997).
TDM (trehalose 6,6'-dimycolate)	Cord factor, which is a glycolipid present on the cell membrane surface of *M. tuberculosis*. Activates macrophages and synthesis of cytokines, to drive a Th1 immune response. It is extremely toxic as it induces hypersensitivity granulomas, complex inflammatory events and apoptosis (Yamagami *et al.*, 2001).
TDB (trehalose 6,6'-dibehenate)	An analogue of TDM but consists of shorter fatty acid chains therefore is considered to be less toxic (Davidsen *et al.*, 2005). Very immunogenic as a co-adjuvant for eliciting protective immunity against tuberculosis (Holten-Andersen *et al.* 2004; Davidsen *et al.*, 2005)

Table 1.2 A selection of immunomodulators, which could enhance the immunogenicity of liposome systems.

1.7.3.2 Liposome morphology

The method of preparing liposomes may affect the way in which these vesicles are processed once administered *in vivo*. Liposomes are said to be able to incorporate a huge concentration of protein antigen, even with regard to the smaller liposomes that posses only a vesicle diameter of 100-200 nm (Lima, 2004). Therefore, irrespective of preparation method, liposomal systems have the capacity to entrap large quantities of protein antigen, however, preparation, morphology and location of protein antigen on the vesicles will possibly influence the manner in which these liposomes are handled by the immune system consequently leading to activation of distinct immune pathways (Thérien and Shahum, 1996).

The physical association and location of the antigen within delivery systems has been shown to influence, not only the type of immune response but also the strength of the response against certain antigens (Shahum and Thérien, 1988; Fortin *et al.*, 1996). The manner in which the antigen is associated to the liposomal delivery system, whether it be encapsulated or surface bound, may determine the route in which these different preparations proceed within professional APC and thereby induce the activation of specific cell populations and

elicit different immunogenic responses (Fortin *et al.*, 1996). In contrast, an early study showed that for toxoid delivery via liposomal formulation, antibody responses to the immunopurified tetanus toxoid was no different between toxoid linked to the surface of MLV and toxoid entrapped within DRV, implying that the physical location of the antigen has no influential effect on induction of specific immune responses (Davis and Gregoriadis, 1987). However, it should be dually noted that the manner in which the antigen is associated is likely to be antigen specific, as various studies have shown the beneficial effect of both encapsulated and surface linked (Harding *et al.*, 1991; Fortin *et al.*, 1996). The high entrapment of protein antigens since the development of the DRV procedure (Kirby and Gregoriadis, 1984) has shown to be immunogenic for a variety of antigens, including; e.g Factor VIII protein (Kirby and Gregoriadis, 1984) and tetanus toxoid (Davis and Gregoriadis, 1987). Indeed it has been implied that the physical association between the liposome and antigen is a mandatory factor for the liposomes to exert their adjuvant, yet de Haan and co-workers (1995) found that high levels of immune stimulation and antibody production still arose following intranasal immunisation with empty liposomes 24 hours prior to intranasal administration of the free subunit antigen.

1.7.4 Liposomes: delivery of plasmid-based antigens

Efficient delivery of DNA into mammalian cells using liposome-mediated gene transfer systems has many advantages over viral vectors. Firstly, they are non-immunogenic, thus it is highly unlikely that there will be adverse side effects once injected within the patient and secondly, they are easy to produce and manufacture thereby overall costs will be low. However, their ability to transfect cells is still highly inefficient in comparison and needs to be improved. When applying liposomes as delivery vehicles for plasmid DNA, it is well recognised that liposomes possess several significant advantages over immunisation with plasmid DNA alone. Although, immunisation with naked DNA is able to induce both humoral and cell-mediated immune responses to the encoded antigen within animal models, when applied within humans, immunisation with plasmid DNA alone is less effective (Ulmer, 1999), with a large variation of efficacy. In addition, when naked plasmid DNA is administered by a different route other than intramuscular, such as subcutaneous or intravenous, gene transfer and expression is low and inefficient. Injection with the DNA alone relies on the ability of myocytes to readily endocytose the DNA to initiate an immune response. However, as myocytes are not professional antigen presenting cells, they lack the

MHC II co-stimulatory molecules and cytokines, so they are not completely efficient at eliciting the appropriate immune response (Gregoriadis *et al.*, 2002). Therefore direct uptake by the APC might be the preferred target for the plasmid DNA, in order to enhance cell-mediated and humoral immune responses to the specific plasmid administered (Gregoriadis *et al.*, (1997). To that end, the potency of DNA vaccines needs to be enhanced, via formulation and association with a delivery system. The incorporation of plasmid DNA within liposome systems has been shown to enhance and promote immune responses, as the plasmid associated with liposomes is avidly internalised by the APC infiltrating the site of injection or migrates towards the regional draining lymph nodes (Turner *et al.*, 1983; Velinova *et al.*, 1996), where it is readily taken up, thus avoiding the use of myocytes. This effective mechanism of delivery has shown to efficiently induce and promote both cell mediated and humoral immunity against the encoded antigen in many studies (Gregoriadis *et al.*, 1997; Perrie *et al.*, 2001; Perrie *et al.*, 2003).

As well as acting as an efficient delivery system, liposomes also function as an efficient protective barrier for the entrapped plasmid. Whereas, naked plasmids can be readily degraded into smaller nucleotides thereby losing its original structure and function, these liposomal vesicles effectively form sealed structures, in which the lipid bilayers segregate the entrapped solute from the harsh external environment of the hosts' biological milieu (Perrie *et al.*, 2003). Therefore, this lipid bilayer acts as a protective barrier prohibiting degrading intracellular enzymes from gaining access and consequently maintaining DNA structure and function. Wong *et al.* (2001) established that intranasal immunisation of liposome entrapped pCI plasmid encoding HA (pCI-HA10) against the influenza virus, provided enhanced and stronger mucosal immunity, compared to when it was administered naked. In addition, intramuscular immunisation using the hepatitis B plasmid (pRc/CMV HBS) entrapped within liposomes composed of PC:DOPE:DOTAP, generated higher levels of antibody responses than when the plasmid was injected alone (Gregoriadis *et al.*, 1996). Both reports concluded that the liposomes were effectively obstructing nuclease degradation of the plasmid DNA within the lungs, thus enhancing body retention times, plasmid delivery and thus increasing immunological responses. Indeed, reports have shown that when plasmid DNA was administered in the form of stabilised plasmid DNA-lipid particles, the half-life of the plasmid was increased to more than 6 hours (Tam *et al.*, 2000).

The structure of the lipids used and the ratio of lipid to DNA have previously been shown to determine the level of transfection efficiency *in vivo* (Song *et al.*, 1997). Therefore, using the appropriate components to manufacture liposomes, the immunological adjuvanticity of these vesicles can be optimised.

1.7.4.1 Vesicle surface charge

The application of conventional liposomes (Patil *et al.*, 2004), which were prepared with the addition of either a neutral zwitterionic lipid or an anionic lipid, were inefficient at entrapping high quantities of plasmid DNA and were also ineffective at mediating liposome-cell interactions, due to the electrostatic charge repulsions between the liposomes and anionic cell membrane (Mannino *et al.*, 1979; Perrie and Gregoriadis, 2000). Therefore, attempts to formulate more efficient liposome systems to enhance plasmid entrapment and cellular uptake of plasmid DNA, led to the development of positively charged liposomes (i.e. cationic liposomes). The first synthesised cationic lipid, DOTMA, was established by Felgner *et al.* (1987). It was established that when these cationic liposomes were simply mixed with the plasmid, the positive surface charge of liposomes containing DOTMA, spontaneously interacted with and became bound to the negative charges of the DNA strand via strong electrostatic interactions, forming cationic liposome-DNA complexes, in which the cationic liposomes surrounds the strand of plasmid DNA and neutralises the plasmids negative charges (Felgner *et al.*, 1987). The use of cationic liposomes as an efficient non-viral carrier system for the delivery of DNA to a wide range of mammalian cells has been extensively reported for both *in vitro* and *in vivo* (e.g. Zabner, 1997; Gregoriadis *et al.*, 2002; Perrie *et al.*, 2003). Cationic liposomes are the preferred systems to utilise, that provide clinically effective gene delivery, than any other chemical non-viral system (Lemoine, 1999). Although, currently cationic liposomes are less effective than viral vectors, they are rapidly becoming the more preferred gene delivery system due to the toxicity problems associated with viral systems (Zallen, 2000).

It is well established that the use of cationic liposomes is the most widespread non-viral method to deliver genes in therapeutic and vaccine applications due to their ease of formulation and production (Ghosh et al, 2000; Patil *et al.*, 2005). They can be non-immunogenic in nature and they possess relatively low toxicity concerns, in which the liposome composition can be formulated in order to meet the standard requirements.

Through continued optimisation of current successful cationic liposomes, these non-viral carrier systems will provide us with a much safer alternative.

Cationic lipids have been shown to be potent candidates for liposomes-mediated gene delivery, however efficiency can vary between different cationic lipids. Since the pioneering discovery of the cationic lipid DOTMA and its high *in vitro* transfection efficiency in combination with the helper lipid DOPE, the liposome formulation also known as Lipofectin® discovered by Felgner *et al.* (1987), there have been a multitude of various other synthesised cationic lipids. Generally, these amphiphillic cationic molecules consist of a positively charged polar head group linked to a hydrophobic domain, known as the alkyl chains. There are a number of variations between different cationic lipids, which makes them structurally different and therefore liposomal structures vary depending on the cationic used, such variations include; the number of alkyl chains within the hydrophobic domain, the length of the alkyl chains and the degree of nonsaturation (Elouahabi and Ruysschaert, 2004). Further approaches to improve the transfection efficiency of cationic liposomes lead to the development of the cholesterol based cationic lipid DC-Chol (Goa and Huang, 1991).

Despite extensive investigations into cationic liposomes as carrier systems for DNA, the relationship between liposome structure and transfection efficiency is yet to be fully understood (Escriou *et al.*, 1998; Zuidam & Barenholz, 1998; Birchall *et al.*, 1999; Almofti *et al.*, 2003). Efficient delivery of DNA by liposomes is known to be dependent on numerous factors including: type of cationic lipid used, lipid to DNA ratio, cell type and the type of neutral lipid used (Zabner, 1997). All these factors contribute, not only to the formation of liposome-DNA complexes, but also to liposome-DNA complex cell interactions. Prior to the addition of DNA, these cationic liposomes are relatively small in size, typically < 100 nm. However, after mixing with a solution of DNA, complexes form through the spontaneous electrostatic interactions between the cationic lipids and DNA. This spontaneous association leads to a diverse array of liposome structures, which can form complexes prepared at different lipid to DNA ratios, and even for those prepared at the same ratio. Additionally, as these cationic liposome-DNA complexes form, the binding reactions could also result in vesicle aggregation as a result of liposome-DNA-liposome cross linking, producing a mass of aggregated liposomes (Bally *et al.*, 1999). Therefore, as visualised microscopically, by freeze-fracture, transmission electron microscopy and various other

techniques, a very heterogeneous population of vesicles is produced with size possibly ranging from as little as 200 nm to larger structures of approximately 1-2 μm (Sternberg *et al.*, 1994; Eastman *et al.*, 1997). It seems that only a fraction of these complexes are responsible for efficient transfection (Faneca et al, 2002; Zhang et al, 2004), making it difficult to distinguish between the 'active' and 'non-active' structures. By identifying the most effective structures, this would provide gene research with a very important advance into understanding and improving gene transfer accordingly, however this has proved to be more difficult than it seems.

When developing liposomes as an effective delivery system for plasmid DNA, each lipid used to formulate the liposomal vesicle will have an effect on the resultant structure and function when applied *in vitro* and *in vivo*. Therefore, there have been an immense number of studies investigating the effect of neutral 'helper' lipid as well as the cationic lipid within gene delivery.

1.7.4.2 Cellular pathways and plasmid delivery

Efficient liposomal systems need to combat a number of systematic and cellular barriers in order to effectively deliver and transfer the associated plasmid DNA. When applied *in vivo*, liposomes efficiently protect the DNA from degradation, as detailed previously. However, there are other factors to consider, such as serum proteins present within the blood. The majority of *in vitro* transfection studies with various liposomes incorporating plasmid DNA are carried out within serum-free conditions, therefore do no take into account the effects of serum proteins, present within the blood plasma, when these liposome-DNA systems are applied *in vivo*. Studies that have been carried out within serum conditions have noted the detrimental effect of these anionic proteins, in which the presence of serum during complexes formation inhibited the aggregation process between cationic SUV liposomes and the plasmid DNA (Escriou *et al.*, 1998). Alternatively the anionic components within the serum could displace the complexed DNA thereby generating naked DNA and reducing the DNA load of the liposomes. Yet, some reports state that the effect of serum is dose dependent in which serum can have positive or negative effects depending on cationic lipid to DNA charge ratios (Tros de Ilarduya and Dűzgűnes, 2000).

The stages and events of liposome-DNA complex entry into the cell and subsequent gene expression within the nucleus and protein production are illustrated within Figure 1.2, providing a simplistic outlook of each stage leading up to gene expression, *in vitro* or *in vivo*.

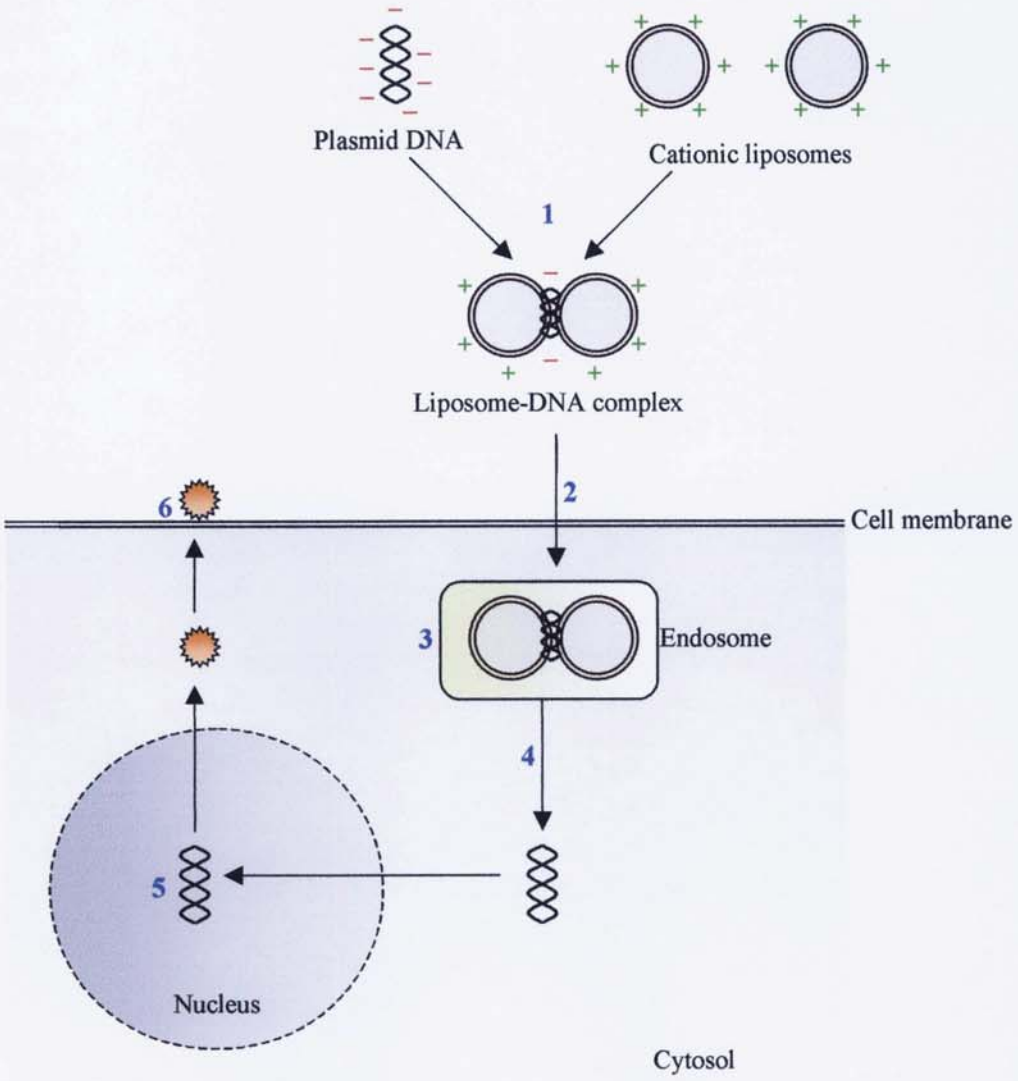


Figure 1.2 A simplistic schematic illustration of the cellular pathways of liposome-DNA complex transfection. Firstly, (1) the plasmid DNA and cationic liposomes are mixed at the required concentrations and charge ratios forming liposome-DNA complexes, due to the electrostatic interactions between the negative (–) and positive (+) charges of the plasmid DNA and cationic liposomes, respectively, condensing and protecting the plasmid DNA. (2) the liposome-DNA complex then enters the cell primarily by endocytosis. (3) within the endosome the liposomal lipid bilayers are broken down and (4) the plasmid DNA alone is then released from the endosomal compartment into the cytosol of the cell. (5) the plasmid DNA is then reported to enter the nucleus where it under goes transcription. (6) the transcribed and translated protein antigen is then released from the nucleus and presented by the cell.

Upon reaching the target cell, the rate at which the liposome system enters the cell via the cell membrane is highly dependent on the lipid to DNA charge ratio, as well as the influence of additional lipids within the bilayer (Tseng and Huang, 1998). Entry into the cell is said to occur primarily by endocytosis, with the liposome system internalised within an endosomal compartment (Zhou and Huang, 1994). Although the mode of entry is still under debate, as some reports demonstrate the role of membrane fusion in liposome-DNA complex uptake (Felgner *et al.*, 1987). Alternatively, other reports have concluded an interaction of both methods of internalisation. In this cited study, both endocytosis inhibitors (1 μg/ml of Antimycin A, 10 Mm NaF and 0.1% (w/v) sodium azide) and fusion inhibitors (100 μM Z-Phe-Phe-Gly) reduced the efficiency of gene transfection of A431 cells (a human epidermoid carcinoma cell line), indicating the contribution of both systems, where membrane fusion facilitates endocytosis of larger complexes that are not able to fuse with the cell membrane (Almofti *et al.*, 2003). However, endocytosis is considered to be the main route of plasmid DNA entry (Xu and Szoka, 1996).

Once inside the cell, the plasmid DNA needs to enter the cytsol before it fuses with the lysosomes, as the lysosome enzymes will digest the exogenous plasmid, and finally the nucleus for gene expression to occur. Previous reports have suggested that the plasmid DNA within liposome-DNA complexes, escapes the endosome, before reaching the lysosome, via the flip-flop mechanism (Szoka *et al.*, 1996; Zelphati and Szoka, 1998). This mechanism involves the contribution of both cationic lipid and the fusogenic lipid DOPE, the destabilising lipid that disrupts the endosomes. The cationic lipids within the liposome-DNA complex interact with the anionic lipids from the cytoplasm-facing endosomal monolayer resulting in lateral diffusion of these endosomal anionic lipids and the release of the plasmid DNA into the cytoplasm. *In vitro* models demonstrate this, in which negatively charged liposomes, which mimic the endosomal environment, interact and mix with the cationic lipids, eventually fusing together leading to destabilisation of the complex thus releasing the incorporated DNA into the cytoplasm in the process (Xu and Szoka, 1996; Harvie *et al.*, 1998; Tseng and Huang, 1998), as determined by measuring DNA intercalating dye binding, the amount of lipid mixing between cationic and anionic liposomes and the increase sensitivity of DNA to DNase I digestion.

The last and final barrier for effective gene expression is entry into the nucleus. It has been proposed that transfection *in vitro* would be more efficient in rapidly dividing cells (Bally *et al.*, 1999). However, the mechanisms of plasmid entry into the nucleus remain poorly defined and understood. Various studies suggest different ideas, stating that the DNA enters as naked DNA (Zelphati and Szoka, 1996) or alternatively lipid assists in nuclear uptake by fusing with the nuclear pores, as lipid present in an adjacent position to the nuclear pore complex and within the nucleus (Labat-Moleur *et al.*, 1996). During each of these stages throughout gene transfer and gene expression, the liposome composition is highly likely to play an effective role, thus affecting the rate and overall efficiency of internalisation and dissociation of the complexed plasmid DNA.

1.7.4.3 Addition of neutral lipids

As each lipid component within the liposomal system contributes to the gene transfer *in vitro* and *in vivo*, this indicates that the liposomes primary function can be optimised by utilising the most appropriate lipids for optimising the delivery of plasmid DNA (Gregoriadis, 1995). In addition to a cationic lipid, the inclusion of a helper lipid is required, not only to stabilise the cationic liposome, as pure cationic lipids repel each other (Davidson *et al.*, 2005), but also to induce high levels of transfection, mediating cellular interactions, fusion and entry into the cytoplasm (Perrie *et al.*, 2004). Therefore, the helper lipid must provide stability yet also enhance the fusogenicity of the liposomes to promote cellular uptake and gene expression (Spack and Sorgi, 2001). However, efficiency varies depending on the type of helper lipid incorporated within the liposome bilayer, therefore the selection of the correct helper lipid may be essential in determining the appropriate immune response.

PC has surfaced as the main lipid component of liposomes (Storm *et al.*, 1998). Recent studies carried out by Perrie *et al.*, (2002) revealed that vaccination of the liposome entrapped DNA (i.e. DRV(DNA)) could be provided by the oral route. The liposomes contained within simulated gastric and intestinal fluids, exhibited minimal loss of the DNA entrapped within the liposomes. In addition, during the duration of the experiment the vesicles encapsulating the DNA produced significantly higher IgA responses against the encoded antigen HBsAg than naked DNA and the expression of the gene was significantly higher for the entrapped DNA compared to the naked DNA. This suggests that administration of the entrapped DNA by the oral route is immensely effective in promoting

and providing the host with the appropriate immune response to the encoded antigen, performing better than naked DNA. This study also demonstrates that the most effective liposomal formulations included DSPC, which Perrie *et al.*, (2002) concluded was probably due to its transition temperature being higher than the physiological temperatures, causing the liposomal structure to become more rigid.

Helper lipids are said to influence the way the lipid self-assembles and additionally affect the electrostatics between the cationic liposomes and DNA within the complex (Hirsh-Lerner *et al.*, 2005). The morphology of the liposome-DNA complexes can vary depending on the helper lipid employed and as previous studies have revealed there is an existence of two different phases; a multilamellar structure (L_{α}^{C}) with the DNA confined between two lipid bilayers and columnar inverted hexagonal (H_{II}^{C}) liquid-crystalline structure where the lipid surrounds the DNA and arranges itself into a hexagonal lattice (Congiu *et al.*, 2004). Complexes containing dioleoyl phosphatidylethanolamine (DOPE), which forms H_{II}^{C} structures, in equal combination with a cationic lipid exhibit higher levels of transfection *in vitro* than those containing any other helper lipid, such as cholesterol (Chol), which is considered to be better *in vivo* (Templeton *et al.*, 1997; Sternberg *et al.*, 1998; Hirsch-Lerner *et al.*, 2005). However, *in vitro* Chol as a helper lipid has not been investigated in any great detail.

The majority of studies carried out on liposome-DNA complexes for *in vitro* transfection, incorporate DOPE as the helper within the liposomal formulation. Phosphatidylethanolamines (PE), such as DOPE, prefer the packing geometry of a H_{II}^{C} (i.e. an inverted cone shape) as this phospholipids consist of a relatively small head group region and two bulky fatty alkyl chains. The primary function of these PE lipids is to enhance membrane fusion and destabilisation, facilitating the disruption of endosomes and the release of the incorporated plasmid DNA into the cytoplasm of the cell, as these lipids are pH-sensitive and undergo structural changes within acidic conditions (Ropert, 1999). The concept of utilising the fusogenic lipid, DOPE, within liposomal systems is to mimic the role of viruses, which fuse with the cell membrane primarily by endocytosis and thus delivering their genetic material to the cytosol before reaching the lysosomes. Indeed, studies have shown that cationic liposome-DNA complexes consisting of the fusogenic lipid DOPE, existing within a H_{II}^{C} configuration, interact and readily fuse with anionic vesicle. In

contrast, Chol is utilised as a helper lipid within liposomal formulations that is known to stabilise the cationic liposomes and render the lipid bilayers more rigid. These physiochemical characteristics of Chol, when present within the liposomal formulations, have demonstrated a prolonged half-life of liposome-DNA complexes, when in circulation (Zemlińska *et al.*, 2002). DOPE is a common helper lipid used *in vitro* and has proved to be highly efficient, in contrast, Chol has shown to be less effective *in vitro*, but *in vivo* this helper lipid has shown to promote higher and more sustained transgenic expression (Liu *et al.*, 1997; Tseng and Huang, 1998).

1.7.4.4 Morphology and vesicle characteristics

As well as lipid composition, the method used to prepare the liposomal vesicles may also dramatically influence transfection efficiency of the plasmid DNA. The cited reports and gene transfer studies carried out *in vitro*, previously detailed in section 1.7.4.1 and 1.7.4.3, mainly utilise SUV liposomes complexed to the plasmid DNA. However, there is a range of different ways to incorporate plasmid DNA and to prepare the final liposomal delivery system, each different with regard to morphology and the physical location of the plasmid.

As well as the morphology and physical association and location of the plasmid DNA, it has also previously been shown that liposomal size can influence the liposomes delivery efficiency of the plasmid DNA and also its ability to transfect cells *in vitro* and *in vivo*. Vesicles of a small size have shown to prolong the presence of liposomes *in vivo* and accordingly deliver the plasmid DNA directly and efficiently to the APC (Zadi and Gregoriadis, 2000). It was discovered that that after intravenous injection, those vesicles that possess a larger size than 200nm, were cleared from the blood circulation very rapidly and digested by enzymes, when they are redirected towards the reticuloendothelial system (Gregoriadis, 2003). In light of this, the ideal liposomal size that can achieve the optimum performance and exhibit prolonged circulation times *in vivo*, with the plasmid DNA being delivered efficiently to the disease sites, needs to be produced. There are various ways of producing small vesicles, including extraction, microfluidization and sonication. However, these procedures are carried out in the presence of the entrapped material and therefore, due to the disruptive effects of these methods, are only reliable for certain solutes. Plasmid DNA is very susceptible to the destructive and detrimental effects of these procedures, and are more likely to shear and cleave under these conditions. In 2000, Zadi and Gregoriadis

developed a novel and non-destructive method of producing small liposomal vesicles, which are still able to retain a high percentage of entrapped solutes. It is a modification of the DRV procedure, where a disaccharide is utilised as a cryoprotectant. The method is an extension of Crowe and Clegg's (1973) suggestion that disaccharides added to the vesicles could replace the water that normally surrounds the polar groups of the lipids. The addition of cyroprotectants is a hypothesis known as the 'water replacement hypothesis' as proposed by Oliver *et al.*, (1998). When liposomes are subjected to the DRV procedure, the freeze-drying process removes the water content of the vesicles. With water removal, the vesicles fuse due to the physical changes in the bilayer structure, with a transition from the gel to liquid phase, creating larger liposomes than SUV liposomes prior to the process. The disaccharides interact with the polar groups by hydrogen bonds and as a consequence stabilise the vesicles during freeze-drying (Crowe *et al.*, 1988).

The DRV procedure has also been utilised within DNA delivery systems and employs exactly the same methods as those used for the entrapment of protein antigens. Previous studies have demonstrated that entrapment of DNA within DRV liposomes (DRV(DNA)) generates high values of entrapment for a wide variety of different plasmids and as expected incorporation values were higher when a cationic lipid was included within the formulation (Gregoriadis, 1998; Gregoriadis *et al.*, 1998). Within these studies, it was confirmed by gel electrophoresis that the DNA was indeed entrapped within the liposome vesicles when prepared by the DRV procedure. Additionally, further studies showed that the entrapped plasmid DNA could possibly be less susceptible to degradation by extracellular nucleases due to the inability of these digestive enzymes gaining access to the entrapped and protected DNA (Gregoriadis *et al.*, 1996). However, although within DRV(DNA) the entrapped DNA is inaccessible to enzymes, it was also shown that liposome-complexed DNA also exhibited significant resistance to degradation by the extracellular enzymes. Although, liposome-DNA complexes do not actively entrap the DNA, the complex and aggregation process appears to significantly condense the plasmid DNA strand and it is possible that the highly condensed state of the plasmid DNA strand within the complex prevents the enzymes from gaining access to the cleaving sites present within the plasmid strand (Felgner and Rhodes, 1991).

It has been previously been postulated that the plasmid DNA within SUV-DNA complexes is able to effectively transfect cells by the flip-flop mechanism, as detailed above. However,

for transfection to efficiently occur in the case of DRV(DNA), the entrapped plasmid must effectively escape the liposomal vesicles and thus degradation within the lysosome. It has been suggested that this may occur in a similar fashion to that seen with the liposome-DNA complexes but this question still remains to be answered (Gregoriadis *et al.*, 1997; Gregoriadis *et al.*, 1998). Indeed, entrapment of plasmid DNA within cationic liposomes has previously been shown to be successful at inducing both humoral and cell-mediated immunity when immunised by various routes, including intramuscular and subcutaneous, with various different plasmid DNA, such as the plasmid encoding the S region of the hepatitis B surface antigen and the plasmid encoding the influenza virus nucleoprotein (NP), respectively (Gregoriadis *et al.*, 1997; Gregoriadis *et al.*, 1998; Perrie *et al.*, 2001).

1.8 Conclusion

Overall, liposomes exhibit great potential as an effective delivery system for alternative recombinant vaccines by efficiently delivering the material and thus enhancing their immunogenicity and specific immune responses *in vivo*. The toxicity of liposome systems is relatively low (Hug and Sleight, 1991; Paukku *et al.*, 1997), with a minimal level of adverse side effects, therefore are more advantageous for human use than viral vectors. However, there is not one formulation that 'fits all', meaning that liposome systems need to be prepared and manufactured to meet the requirements of the specific disease target. Also, the full extent of the mechanism and functioning of liposomes is still relatively unknown and research in which to develop further understanding is ongoing.

1.9 Research aims

The aims of this project were to examine the role of liposomes within antigen delivery and to investigate the factors influencing their overall efficiency and efficacy, within *in vitro* and *in vivo* situations. Efforts were focussed on the physiochemical properties, transfection efficiency and immunological characterisation of various liposome systems, for both plasmid-based vaccines and subunit protein antigens. This involved examining the effect of various lipid components, thus investigating the impact that bilayer composition, surface charge and liposome stability. In addition, the method of liposome preparation was assessed, in order to establish the affect that both liposome morphology and antigen spatial location, have on antigen protection and delivery.

2. Materials and methods

2.1 Materials

Chemicals	Supplier
Agarose	Sigma-Aldrich, Poole, Dorset, UK
Ampicillin	Sigma-Aldrich, Poole, Dorset, UK
2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)	Sigma-Aldrich, Poole, Dorset, UK
Boric acid	Biomedicals, Inc. Ohio, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, Poole, Dorset, UK
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	Promega, Madison, WI, USA
Chloroform	
Citric acid	Fisons, Loughborough, UK
Concanavalin A	Sigma-Aldrich, Poole, Dorset, UK
Deoxyribonuclease I	Sigma-Aldrich, Poole, Dorset, UK
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, Poole, Dorset, UK
Dulbecco's modified eagle medium (DMEM)	Gibco-Invitrogen, Carlsbad, CA
DuoSet® capture ELISA	DuoSet® capture ELISA
EcoR I ladder (1-10kb)	Promega, Madison, WI, USA
ELISA plates (flat bottomed, high binding)	Greiner Bio-One Ltd, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Poole, Dorset, UK
Ethidium bromide	Sigma-Aldrich, Poole, Dorset, UK
Foetal bovine serum (FBS)	Gibco-Invitrogen, Carlsbad, CA
Glycerol	Sigma-Aldrich, Poole, Dorset, UK
gWiz luciferase	Aldevron, Fargo, USA
Heparin	Sigma-Aldrich, Poole, Dorset, UK
Horseradish peroxidase-conjugated goat anti-mouse immunglobulin IgG, IgG_1 & IgG_{2a} subclasses	Oxford Biotechnology, UK
Hydrogen peroxide	Sigma-Aldrich, Poole, Dorset, UK
Incomplete Fruend's adjuvant	Sigma-Aldrich, Poole, Dorset, UK
Iodo-gen® pre-coated iodination tubes	Pierce Biotechnology, Rockford, IL
Kanamycin	Sigma-Aldrich, Poole, Dorset, UK
LB agar	Sigma-Aldrich, Poole, Dorset, UK
LB broth	Sigma-Aldrich, Poole, Dorset, UK

L-glutamine/Penicillin-Streptomycin	Gibco-Invitrogen, Carlsbad, CA
Library efficiency® DH5α™ competent cells	Gibco-Invitrogen, Carlsbad, CA
Lipofectin® Reagent	Gibco-Invitrogen, Carlsbad, CA
Loading buffer	Promega, Madison, WI, USA
Luciferase assay system	Promega, Madison, WI, USA
Magnesium chloride	Sigma-Aldrich, Poole, Dorset, UK
Marvel	Premier Int. Foods Ltd, Lincs, UK
Methanol	
Opti-MEM® I (Reduced serum medium)	Gibco-Invitrogen, Carlsbad, CA
Optiphase Hisafe	Perkin Elmer, USA
Phosphate buffer saline tablets (PBS)	Sigma-Aldrich, Poole, Dorset, UK
PicoGreen dsDNA quantitation reagent	Molecular Probes-Invitrogen, UK
Plasmid DNA (pRc/CMV HBS)	Aldevron, Fargo, USA
Potassium chloride	Sigma-Aldrich, Poole, Dorset, UK
Potassium phosphate	Sigma-Aldrich, Poole, Dorset, UK
Endotoxin-free plasmid purification kits	Qiagen Ltd, West Sussex, UK
Restriction enzyme buffer	Promega, Madison, WI, USA
Restriction enzyme (*Hind* III)	Promega, Madison, WI, USA
RPMI 1640 cell culture medium	Gibco-Invitrogen, Carlsbad, CA
Sephadex G-75	Sigma-Aldrich, Poole, Dorset, UK
Sodium chloride	Sigma-Aldrich, Poole, Dorset, UK
Sodium bicarbonate	Sigma-Aldrich, Poole, Dorset, UK
Sodium carbonate	Sigma-Aldrich, Poole, Dorset, UK
Sodium dodecyl sulphate	Sigma-Aldrich, Poole, Dorset, UK
Sodium ethylenediaminetetraacetic acid (Sodium EDTA)	Sigma-Aldrich, Poole, Dorset, UK
Sodium hydroxide tablets	Sigma-Aldrich, Poole, Dorset, UK
Sodium phosphate dibasic	Sigma-Aldrich, Poole, Dorset, UK
Sucrose	Sigma-Aldrich, Poole, Dorset, UK
Super coiled ladder DNA (2-10kb)	Promega, Madison, WI, USA
Synthetic hepatitis B surface antigen (HbsAg) (ayw subtype)	Aldevron, Fargo, USA
Tetramethylbenzidine	Sigma-Aldrich, Poole, Dorset, UK

[^{3}H]thymidine	Amersham plc, UK
Tris (ultra pure)	Biomedicals, Inc. Ohio, USA
Trizma base	Sigma-Aldrich, Poole, Dorset, UK
Triton X-100	Sigma-Aldrich, Poole, Dorset, UK
Trypan blue	Sigma-Aldrich, Poole, Dorset, UK
Trysin/EDTA	Gibco-Invitrogen, Carlsbad, CA
Tween 20	Sigma-Aldrich, Poole, Dorset, UK

Lipids	**Supplier**
Cholesterol (Chol)	Sigma-Aldrich, Poole, Dorset, UK
3β-[N-(N′,N′-Dimethylaminoethane)-carbamoyl] Cholesterol (DC-Chol)	Sigma-Aldrich, Poole, Dorset, UK
Dimethyldioctadecylammonium bromide (DDA)	Avanti Lipids,Alabaster, AL, USA
1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC)	Avanti lipids, Alabaster, AL, USA
1,2-Dimyristoyl-*sn*-Glycero-3-Phosphoethanolamine (DMPE)	Avanti lipids, Alabaster, AL, USA
1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC)	Avanti lipids, Alabaster, AL, USA
1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine (DOPE)	Avanti lipids, Alabaster, AL, USA
1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP)	Avanti lipids, Alabaster, AL, USA
1,2-Dipalmitoyl-3-Trimethylammonium-Propane (DPTAP)	Avanti lipids, Alabaster, AL, USA
1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine (DSPC)	Avanti lipids, Alabaster, AL, USA
Egg L-α-Phosphatidylcholine (PC)	Lipid products, Nutfield, Surrey, UK
Egg L-α-Phosphatidylethanolamine (PE)	Lipid products, Nutfield, Surrey, UK
α,α'-trehalose 6,6'-dibehenate (TDB)	Avanti Lipids, Alabaster, AL, USA

2.2 Plasmid DNA production

2.2.1 Amplification

Escherichia coli (*E.coli*) DH5α was the host strain used to amplify the pRc/CMV HBS antigen. Bacterial colonies of the *E.coli* DH5α, containing the required plasmid, were grown selectively on LB agar plates, containing 100 μg/ml ampicillin, overnight at 37°C. From this, a single colony was selected and added to 20 ml LB broth containing the appropriate selective antibiotic, ampicillin. This became the starter culture, which was grown for 8 hours at 37°C, with vigorous shaking (~300 rpm). The starter culture was then diluted 1:500 into LB broth and grown for 12-16 hours at 37°C, with vigorous shaking (~300 rpm).

2.2.2 Purification

The plasmid DNA was purified from *E.coli* DH5α by using QIAGEN® Plasmid Purification Mega Kit, supplied by QIAGEN Ltd, which has been approved by European countries and the USA. The bacterial cells were collected from the large cultures grown overnight, by centrifuging at 6250 rpm (~6000 g) in a Beckman JA-14 rotor, for 15 minutes at 4°C. After removal of the supernatant, the bacterial pellet was resuspended in Tris hydrochloride (50 mM, pH 8), containing EDTA (10 mM) and RNase A, which digests any RNA present within the solution. The bacterial cells were then lysed with sodium hydroxide (200 mM) containing 1% SDS, with the lysate subsequently being neutralised with the addition of potassium acetate (3 M, pH 5.5). The precipitated debris was removed from solution by centrifuging at 11,500 rpm (~15,000 g) in Beckman JA-14 for 30 minutes. This was followed by filtration, to ensure that the lysate is clear in order to establish good flow rates when loading onto the QIAGEN-tip, and to obtain a protein-free plasmid DNA preparation. The lysate, cleared of any insoluble complexes, was applied to a pre-equilibrated QIAGEN-tip and allowed to flow through by gravity flow. During the initial stages, the plasmid DNA remains within the columns resin, whereas the digested RNA, proteins and other contaminants flow through the column and are separated and removed from the plasmid DNA. Once the plasmid DNA has been purified, a high salt buffer, containing 1.25 M sodium chloride, 50 mM tris-chloride (pH 8.5) and 15 % isopropanol, was added to the column, eluting the purified DNA. Isopropanol was added to the eluted DNA to allow the process of precipitation to occur, producing a more concentrated sample. The volatile isopropanol was replaced by 70 % ethanol, which is easier to remove when the produced

pellet was air-dried. Finally, the dried pellet was redispersed and completely dissolved into 2 ml TE (pH 8).

2.2.3 Spectrophotometry

DNA yield was determined by measuring the concentration of nucleic acids in solution, where the nitrogenous bases in nucleotides have an absorption maximum at about 260 nm (A_{260}). Based on the extinction coefficient, the absorbance at 260 nm of a 50 μg/ml solution of double stranded DNA (dsDNA) is equal to 1, if measured in a quartz cuvette with a 1 cm light path. A 20 μl of DNA sample, produced by the Qiagen procedure, was made to 1ml with Tris-EDTA buffer (TE) (1:50 dilution) and measured at A_{260}. RNA has an absorbance of 280 nm (A_{280}), therefore, by measuring the DNA sample at A_{260} will provide an estimate of the protein contamination of the sample, and the 260 nm/280 nm ratio provides an estimate of the purity of a DNA sample. Further contamination was evaluated by measuring absorbance at 230 nm and 325 nm. With values of A_{230} higher than A_{260} indicate contamination by organic solvents and values at A_{325} greater than 0 indicate the presence of particulate matter.

2.2.4 Restriction enzyme analysis

Restriction enzymes are used to cleave the DNA at recognition sites along the double helix, generating linear DNA fragments. Therefore, when a gel electrophoresis analysis is performed, the cut plasmid is revealed as a single linear band. Restriction enzyme digest analysis was carried out to confirm the size, purity and structure of the plasmid pRc/CMV HBS produced as described in sections 2.2.1 and 2.2.2. 1 μg/ml of sample plasmid DNA was added to and mixed with 16.6 μl sterile deionised water, 0.2 μl acetylated bovine serum albumin, 2 μl restriction enzyme buffer (10x) and 0.5 μl restriction enzyme. In this instance, *Hind III* restriction enzyme was used to cleave pRc/CMV HBS at its specific recognition sites. The mixture was centrifuged in a micro centrifuge for a few seconds and then incubated at 37°C for 1-4 hours. Through the duration of the digest, an uncut experimental DNA (with no enzyme added to the mixture but with every other component consistent) was used as a control to identify and understand the mechanisms involved or any problems that may occur. After incubation, the samples were subjected to a 0.8% agarose gel, containing 1 mg/ml ethidium bromide, for 1.3 hours at 80 V. A supercoiled DNA ladder (2-10 kb) and Lambda DNA (*Ecor I*) (1 kb) were used in the gel analysis, to compare the bands with the

uncut control plasmid and digested plasmid, respectively, and confirm the size and purity of the amplified plasmid DNA.

2.3 Agarose gel electrophoresis

Samples of naked plasmid DNA and incorporated DNA were subjected to agarose gel electrophoresis in order to determine DNA retention of various liposome formulations. Agarose gel was made at a concentration of 0.8% (w/v) containing 10 µl ethidium bromide (1 mg/ml). Samples of free DNA and liposome incorporated DNA suspensions were mixed with gel loading buffer (bromophenol blue, 0.05% w/v; sodium dodecyl sulphate, 0.05% w/v; EDTA, 0.1 M at pH 8) and subjected to agarose gel electrophoresis for 1 hour at 80 V. DNA visualisation of the gels was carried out by using UV SynGene Bio Imaging (SynGene, Cambridge, UK).

2.4 Liposome production

2.4.1 Production of multilamellar vesicles (MLV)

MLV were prepared by the established film technique (i.e. the assembly of phospholipids into closed lipid bilayers within excess water) first observed by Bangham, in the 1960's (Bangham *et al.*, 1965; Bangham, 1983). The lipids used throughout these experiments were dissolved in a chloroform:methanol (9:1 v/v) solution at the desired concentrations. The required lipid solutions, at the desired concentrations, were placed in a 50 ml round-bottom spherical Quick-fit flask and the solvent was removed by rotary evaporation at about 37°C. This yields a thin lipid film on the walls of the flask, which was then flushed with oxygen-free nitrogen in order to ensure complete removal of all solvent traces. The dry lipid film was hydrated by addition of 2 ml of double distilled water (ddH_2O) and agitated vigorously until the thin lipid film was completely dissolved and transformed into a milky suspension. The hydration of the lipid film was maintained above the gel-liquid crystal transition temperature (Tc) of the phospholipid (>Tc). PC has a Tc of 0°C and DMPC has a Tc of 23°C, however, DSPC has a Tc above 55°C, therefore the water added to hydrate the lipid film should be pre-warmed to a temperature above 55°C and the liposome solution should be maintained at this temperature during liposome formation.

2.4.2 Production of small unilamellar vesicles (SUV)

To generate SUV, the MLV produced in section 2.4.1 were disrupted using sonic energy to fracture the large liposomes into smaller structures (<100 nm). In this instance, a probe sonicator (Soniprep 150) was used to produce SUV. The tip of the sonication probe (diameter ~ 4 mm) was placed onto the surface of the 2 ml MLV mixture and the amount of time required for sonication, varies depending on the lipid composition (approximately 2-6 minutes). The milky MLV suspension transforms into clear SUV suspension with a slight blue tint. The SUV suspension was then centrifuged at 3,500 g for 10 minutes to remove any titanium debris released from the probe during sonication.

2.4.3 Production of dehydration-rehydration vesicles (DRV)

The preparation of dehydration-rehydration vesicles (DRV) was first established by Kirby and Gregoriadis (1984). The empty SUV suspension was mixed with various quantities of antigen (and ^{125}I radio-labelled Ag85B-ESAT-6, the fusion protein antigen isolated from *M. tuberculosis* used as the subunit vaccine against tuberculosis, for protein antigen samples), which were subsequently frozen at -70°C for 1 hour and then placed in a freeze dryer over night, with a shelf temperature of -20°C. Additionally, empty SUV were freeze-dried overnight, to produce empty DRV. After the freeze-drying process, the lipid cake formed, under went controlled rehydration procedure, with a minimal amount of ddH_2O being added to the lipid cake (100 µl per 16 µmoles lipid), followed by vigorous agitation till all the dry lipid cake was redispersed and rehydrated. The suspension was left to stand for 30 minutes, $>T_c$, to allow vesicle formation, after which further ddH_2O was added and again left for a further 30 minutes, $>T_c$, in order to ensure complete rehydration. Thereafter, the milky suspension was diluted to the required lipid concentration and volume with phosphate buffer saline (PBS) and again left to stand for 30 minutes before any experimental analyses were carried out. As a consequence, DRV entrapping plasmid DNA form and stabilise.

2.4.4 Production of modified dehydration-rehydration vesicles (DRV+sucrose)

The technique for the preparation of small DRV by the addition of a cyroprotectant was developed by Zadi and Gregoriadis (2000). Four 2 ml samples of SUV, composed of 16 µmole PC, 8 µmole DOPE or Chol and 4 µmole DC-Chol were prepared as detailed in section 2.4. The four samples were pooled and subsequently, 1 ml was removed to produce four separate 1 ml samples of empty SUV containing a total lipid mass of 8.8 mg or 10.2 mg

for Chol-containing liposomes or DOPE-containing, respectively. To each sample, increasing amounts of sucrose (80 mg/ml) was added, as demonstrated in Table 2.1, to generate various sucrose to lipid mass ratios.

Lipid/Sucrose ratio (w/w)	Volume of SUV (ml)	Volume of Sucrose solution for DOPE liposomes (ml)	Volume of Sucrose solution for Chol liposomes (ml)
0	1	0	0
1	1	0.1273	0.1094
3	1	0.3820	-
5	1	0.6367	-

Table 2.1 Volume of sucrose solution added to SUV samples in accordance with the lipid/sucrose ratio (w/w).

Following the addition of sucrose, 50 μg plasmid DNA was added to each sample and subsequently freeze-dried for 72 hours, at a shelf temperature of -40°C. After freeze-drying, the samples were rehydrated with the addition of ddH_2O, mixed vigorously and left for 30 minutes > T_c. Additional ddH_2O was added and again mixed vigorously, followed by a further 30 minutes incubation > T_c. The solution was diluted to 0.5 ml with PBS and incubated > T_c for 30 minutes to allow stabilisation. The z-average diameter and zeta potential of each sample was determined (section 2.7 and 2.8). The percentage DNA entrapment and percentage recovery was measured and calculated as described in section 2.5.2.1. Each sample was subjected to a 0.8% agarose gel electrophoresis, for 1 hour at 80 V. The samples were loaded onto the gel in the presence of high concentrations of a competitive anionic component and sucrose (40% w/v).

2.5 Measuring antigen association (plasmid and protein)

2.5.1 Separation of non-associated antigen

Liposome formulations were washed in order to remove unincorporated antigen. Samples were either centrifuged at 19,000 rpm in a Beckman model J2-21 centrifuge (JA-20 rotor), at 4°C for 1 hour or at 50,000 rpm in a Beckman TL-100 ultracentrifuge (TLN 100 rotor). After centrifugation, the supernatants (containing free antigen) were removed carefully and

the pellets (containing entrapped or associated antigen) were resuspended to the required volume with PBS.

2.5.2 Quantification of antigen

2.5.2.1 Plasmid DNA

For each plasmid DNA antigen liposomal formulation, percentage plasmid DNA entrapped was determined. The samples were examined by removing, 25 µl sample for the unwashed and washed samples and diluting to 1 ml with PBS or in the case of measuring DNA content within the supernatant, 1 ml was directly removed from the supernatant, with no dilutions. From this, 100 µl was removed and again diluted to 1 ml with PBS. To this, 10 µl of 10% Triton X-100 (with final concentration of 0.1%) was added and mixed by vigorous shaking. Finally, 2 µl of PicoGreen reagent was added to the mixture, again mixed by vigorous shaking and incubated for 2-5 minutes at room temperature. After incubation, 200 µl of the mixture was removed and added to a 96 well black micro titre plate, with triplicate measurements for each sample measured. The samples were measured with an excitation maximum at 495 nm and an emission peak at 525 nm (Spectra Max Gemini XPS, Molecular Probes).

Entrapment values of DNA within liposomes was calculated using the following equation (including any dilutions):

$$\frac{\text{Fluorescence of washed sample}}{\text{Fluorescence of unwashed sample}} \times 100\ \%$$

To ensure reliability of entrapment values, the total recovery of DNA was also calculated using the following equation (including any dilutions):

$$\frac{\text{Fluorescence of washed sample} + \text{Fluorescence of supernatant}}{\text{Fluorescence of unwashed sample}} \times 100\ \%$$

2.5.2.2 Protein

For each protein antigen liposomal formulation, percentage protein entrapped was determined on the basis of ^{125}I radioactivity. The protein antigen Ag85B-ESAT-6, was radiolabelled using the Iodo-gen® pre-coated iodination tubes (Pierce Biotechnology, Rockford, IL). The protein antigen was diluted to 50 µl Tris-buffer (25 mM, pH 8) and added to the pre-coated iodination tube. A pre-determined activity of ^{125}I (3.7 MBq) was

diluted to 30 µl with Tris-buffer (25 mM, pH 8) and also added to the iodination tube, after which, the mixture was left for 15 minutes with intermittent shaking. The labelled antigen was separated from the unlabelled antigen by applying the mixture onto a 5 ml Sephadex G-75 gel column. The gel column prepared by, firstly soaking the Sephadex G-75 (1%, w/v) in double distilled water at 90°C for 1 hour. This swollen gel solution was then packed to a give a final gel volume of 5 ml and was then equilibrated with Tris-buffer (25 mM, pH 8). The antigen-^{125}I reaction mixture was added to and passed through the column, washing with Tris-buffer (25 mM, pH 8). 0.5 ml aliquots were collected and the gamma radioactivity was measured using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA). Additionally, each aliquot was spectrophotometrically analysed at A_{280} and the radioactivity and absorbance readings for each 0.5 ml aliquot were plotted to confirm the presence of radiolabelled protein antigen. The aliquots containing the radiolabelled protein antigen were collated and stored at –20°C.

Incorporated and unincorporated protein antigen within liposome suspensions were separated by diluting the suspension to 3.9 ml with PBS and centrifuging on an Ultra Combi centrifuge (Beckman TL-100). The amount of ^{125}I labelled protein antigen was measured using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA) prior to centrifugation and also within each fraction after centrifugation (i.e. pellet and supernatant). The amount of incorporated antigen was determined by the following equation: Entrapment values of DNA within liposomes was calculated using the following equation (including any dilutions):

$$\frac{\text{Radioactivity of washed sample}}{\text{Radioactivity of unwashed sample}} \times 100\ \%$$

To ensure reliability of entrapment values, the total recovery of protein antigen was also calculated using the following equation (including any dilutions):

$$\frac{\text{Radioactivity of washed sample} + \text{Radioactivity of supernatant}}{\text{Radioactivity of unwashed sample}} \times 100\ \%$$

2.6 Protection of DNA from degradation: Comparing naked and entrapped DNA

A standard solution of deoxyribonuclease I was prepared by reconstituting a vial of DNase I with 1 ml of cold sodium chloride (NaCl) (0.15 M). This produced a standard stock solution with a concentration of 2,000 Kunitz units/ml. From this, aliquots of 60 µl were pipetted into vials and frozen at -20°C for future use. 150 µl of magnesium chloride ($MgCl_2$) (5 mM) was added to 100 µl of naked DNA (10 µg). Samples were then incubated in the presence (i.e. positive test) or absence (i.e. negative test) of 100 units of DNase I, at 37°C for 10 minutes. 50 µl from the stock solution provides 100 units of DNase I. The same procedure was completed for the samples of DRV incorporating plasmid DNA. The reaction was stopped after 10 minutes with 100 µl of EDTA (0.1 M; pH 8.0). 8 µl from each sample was removed and 2.5% w/v SDS was added (1:1 v/v), in order to break open the liposomes to release their contents. The samples (i.e. positive and negative tests, free and entrapped DNA and with and without SDS) were then subjected to a 0.8% agarose gel for 1 hour at 80 V.

2.7 Measuring vesicle size

The z-average diameter of all liposome formulations and preparations were determined by dynamic light scattering using the photon correlation spectroscopy (PCS) technique. Measurements were performed on a ZetaPlus (Brookhaven Instruments Corporation, USA) at 25°C and for the viscosity and refractive index the value of pure water was used (0.89 Cp and 1.0, respectively). Liposome samples (50 µl) were diluted with 2 ml of ddH_2O, to maintain optimal vesicle count rate and concentration.

2.8 Measuring zeta potential

Zeta potential, which is an indirect measurement of vesicle surface charge as it can be termed as a remote effect of the surface charge, for all liposome formulations and preparations were measured on a ZetaPlus (Brookhaven Instruments Corporation, USA). The technique used was microelectrophoresis, where the measured velocity of a particle is said to proportional to the applied electric field. 50 µl of liposome suspension was diluted to 2ml with 0.001 M PBS and measurements carried out at 25°C. The zeta potential provides

information about the particles behaviour in solution, however, the zeta potential of a particle is highly dependent on the nature of both the particle itself and also the surrounding medium.

2.9 Morphological studies

2.9.1 Transmission electron microscopy (TEM)

Morphological analysis was carried out by TEM using a JEOL 1200EX TEM fitted with a LaB6 filament, with an operating voltage from 40 to 120 kV. A small drop of liposome suspension, (containing either empty liposomes, liposome-antigen complexes or liposomes entrapping antigen), was placed on a polymer filmed copper grid and allowed to stand for 2 minutes. The excess sample was removed using filter paper, followed by the addition of 10 µl of uranyl acetate. The grid was then allowed to stand for another 2 minutes, washed in distilled water and air dried, forming a thin film, which was viewed at 70 kV.

2.9.2 Environmental scanning electron microscopy (ESEM)

Liposome formulations were analysed using a Philips Electron Optics ESEM. This technique for imaging liposomes enables real time viewing of the liposomes in wet conditions, without exerting any mechanical stresses or damage, as other techniques, such as SEM (Mohammed *et al.*, 2004). A liposome suspension was placed within the ESEM sample holder, previously hydrated with PBS and examined under saturated water vapour conditions. Gradually the pressure within the samples chamber was reduced in order to examine the stability and rigidity of various liposome formulations. Through out viewing, temperature was maintained constant, at 5°C.

2.10 Protein release studies

Protein entrapment within liposome formulations was determined as detailed in section 2.5.2.2. Liposome preparations were then diluted to 15 ml with PBS and incubated in a 37°C water bath with shaking. At various time points (0, 2, 4, 24, 48, 96, 168, 336, 672 and 1008 hours), protein entrapment and release from liposome formulations was determined by removing 1 ml sample from liposome suspension, which was subsequently replaced with 1

ml PBS, in order to maintain sink conditions. From this 1 ml sample protein entrapment and amount of protein released was determined as detailed in section 2.5.2.2.

2.11 Liposome stability

Liposome formulations were prepared as detailed in section 2.4 and stored at either 4°C or room temperature. At various time points (0, 7, 14, 28, 42, 56, 77 and 98 days), 150 µl liposome sample was removed and protein entrapment was determined by ^{125}I radioactivity as detailed in section 2.5.2.2.

2.12 *In vitro* studies

2.12.1 Cell culture protocols

2.12.1.1 Resuscitation of frozen COS-7 cell line

An ampoule containing COS-7 cells (African green monkey kidney fibroblast cells) was removed carefully from liquid nitrogen storage and half submerged in a 37°C water bath for approximately 2 minutes, until cells have thawed. The outside of the ampoule was wiped with a tissue moistened with 70% isoproponal, before placing into a sterile microbiological safety cabinet in order to maintain sterile conditions. Under sterile conditions, the ampoule lid was removed and the cell solution was removed and slowly pipetted into a 75 cm^2 cell culture flask containing 20 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomyocin/L-Glutamine, in order to dilute out the toxic effects of Dimethyl Sulfoxide (DMSO). Incubate cells at 37°C under sterile conditions, in a humid 5% CO_2 conditions.

2.12.1.2 Subculture of COS-7 cells

DMEM was removed by careful suction, followed by the addition of 5 ml trypsin/EDTA, ensuring that all cells are covered, which were then incubated at 37°C for 5 minutes. Cells were examined under an inverted microscope to ensure all cells have detached from the flask. 5 ml of DMEM was added to the cell suspension to dilute the trypsin/EDTA solution and then the cell suspension was added to a 50 ml centrifuge tube and subsequently

centrifuged (Mistril 3000i) at 200 x g for 10 minutes at 15°C. After centrifugation, within sterile conditions, the supernatant was carefully removed and the pellet was resuspended in 10 ml DMEM. 1 ml of cell suspension is added to a 75 cm^2 containing 19 ml DMEM and incubated at 37°C under sterile conditions, under 5% CO_2.

2.12.1.3 Cell quantification

Adherent COS-7 cells were brought into solution as detailed in section 2.12.1.2. From this cell suspension, 200 µl was removed and to this 200 µl of Trypan Blue was added and mixed. Using a haemocytometer, cell viability and cell counts were determined. To each side of the cover slip of the haemocytometer, 10 µl of the Trypan Blue cell suspension was added and viewed microscopically. The bright cells (i.e. viable cells) were counted and from this the cells concentration was calculated (including dilutions), whereas the cells stained blue indicate non-viable cells.

2.12.1.4 Cryopreservation

Adherent cells were brought into suspension as previously described in section 2.12.1.2. From the cell suspension, a 0.2 ml sample was removed and a cell count was performed as detailed in section 2.12.1.2. The remaining cells were then centrifuged at 200 x g for 10 minutes at 15°C and the pellet was resuspended in FBS containing 10% DMSO containing a cell concentration of 4 x 10^6 cells/ml. 1ml aliquots of cell suspension was pipetted into cryopreservation ampoules, which were clearly labelled and frozen at -70°C overnight. The ampoules were then placed and stored in a liquid nitrogen storage container.

2.12.2 Transfection protocol

2.12.2.1 Cell preparation and plating for in vitro studies

Adherent COS-7 cells were brought into suspension and quantified as detailed in section 2.12.1.2 and 2.12.1.3. The cell suspension was then centrifuged at 200 x g for 10 minutes at 15°C and the pellet was resuspended to a cell concentration of 1 x 10^5 cells/ml with supplemented DMEM. For transfection experiments, 1 ml of cell suspension was pipetted per well on a 12-well cell culture plate. For cytotoxicity assay, 100 µl of cell suspension was pipetted per well on a 96-well plate. Culture plates were then incubated at 37°C for 24 hours.

2.12.2.2 Samples preparation and transient transfection of COS-7 cells

Cationic liposome-DNA complexes were prepared by incubating 17.5 µl liposome suspension in 0.35 ml serum-free and antibiotic free medium (opti-MEM) for 40 minutes at room temperature. After incubation, 0.35 ml of opti-MEM containing 3.5 µg plasmid DNA was added and gently mixed with the liposome suspension and incubated again for a further 15 minutes at room temperature. The resultant cationic liposome-DNA mixture was then diluted to a final volume of 3.5 ml with opti-MEM. COS-7 cells, plated 24 hours previously, were removed from the 37°C incubator and the media was removed, subsequently washed with 1 ml prewarmed opti-MEM. 1 ml of the cationic liposome-DNA complex suspension (100 µl for cytotoxicity assay on a 96-well plate) was added to the cells and incubated at 37°C for 5 hours in a humid CO_2 atmosphere. After the incubation period, the transfection suspension was removed and replaced with 1 ml prewarmed supplemented DMEM (100 µl for the cytotoxicity assay on a 96-well plate) and incubated at 37°C for a further 48 hours in a humid CO_2 atmosphere.

2.12.2.3 Luciferase assay

Working solution of 1x lysis buffer was prepared by adding 4 volumes of ddH_2O to 1 volume of 5x lysis buffer. Growth medium (i.e. DMEM) was removed from cells and washed with 1 ml PBS per well. 80 µl of 1x lysis buffer was pipetted to each well containing cells and incubated at room temperature for 1 minute. Attached cells were scraped from each well and 60 µl of detached cells in buffer were transferred into microfuge tubes and vortexed. Samples were centrifuged at 12,000 x g for 15 seconds at room temperature. 10 µl of each supernatant was removed and pipetted onto a 96-well plate. The 96-well plate was placed into a (Spectra Max Gemini XPS, Molecular Probes) luminometer and the plate was read 30 reads/well with auto mix prior to the addition of luciferase reagent. 100 µl of luciferase assay reagent was added to each well and the plate was read again with 30 reads/well with auto mix.

2.12.2.4 Cytotoxicity assay

To each well of a 96-well plate containing COS-7 cells, 20 µl of MTS reagent was added. The MTS reagent is bioreduced by the cells into a red formazan product, which indicates the presence of metabolically active cells. Plates were then gently mixed and incubated for 4

hours at 37°C, in a 5% humid CO_2 atmosphere. After 4 hours incubation, the quantity of formazan produced was measured on microplate reader (Bio-Rad, model 680) at A_{490}, with the absorbance reading being directly proportional to the number of living cells in the medium. Results are compared to and expressed as percentage of positive control (i.e. cells and medium). Negative control was also included which was cells incubated Triton X-100.

2.13 *In vivo* studies for DNA delivery

2.13.1 Immunisation protocol

Female Balb/c mice (6-8 weeks old), purchased from Charles River, UK, were placed in groups of 8, with a total of 9 groups. Each mouse, excluding the naïve group, were immunised subcutaneously with 200 µl containing 20 µg of pRc/CMV HBS plasmid DNA, either incorporated within liposomes (SUV-DNA complexes or DRV(DNA)) of various formulations or as free plasmid DNA, using disposable plastic syringes with 26G needles. As a positive control, one group of 8 mice were intramuscularly injected with Synthetic hepatitis B surface antigen (HbsAg) (ayw subtype) mixed with incomplete Freund's adjuvant. Blood samples were taken three weeks after last immunisation by creating a small cut in the tail vein and collecting 50 µl of blood sample within a capillary tube lightly coated with 1% heparin. The collected blood was then added to an eppendorf containing 0.45 ml PBS (1/10 dilution) and centrifuged (Micro Centaur) at 13,000 g for 5 minutes. The supernatant (i.e. sera) from each tube, representing each individual mouse was collected and placed into a clean eppendorf and stored at -20°C until the samples were required. Therefore, each eppendorf contains 20-fold dilution of sera (i.e. if it is assumed the haematocrit is 50%) for each individual mouse.

2.13.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA plates were coated with 60 µl of 2 µg ml^{-1} recombinant hepatitis B surface antigen in 0.05 M sodium carbonate (0.318 g Na_2CO_3 and 0.58 g Na_2HCO_3 in 250 ml ddH_2O, pH 9.6) and left overnight at 4°C. Plates were washed three times with PBST buffer (40 g NaCl, 1 g KCl, 1 g KH_2PO_4, 7.2 g $Na_2HPO_4(2H_2O)$ in 5 litres ddH_2O with approximately 0.4 ml Tween 20). To eliminate any non-specific antigen binding, the plates were coated (i.e.

blocked) with 100 µl of 1% (w/v) Marvel (dried skimmed milk powder) and incubated for 1 hour at 37°C, after which the plates were then washed again three times with PBST buffer. 120 µl of serum sample was added to the first well of each plate of round bottom dilution plates, which were then serially diluted and added to the ELISA plates. The plates were then incubated for 1 hour at 37°C. After incubation, the plates were then washed five times with PBST buffer. 50 µl of conjugate (horseradish peroxidase-conjugated anti-mouse isotype specific immunoglobulins; IgG, IgG_1 and IgG_{2a}) diluted 1:5,000 in PBS, was added per well, which is then incubated for 1 hour at 37°C. The plates were then washed five times with PBST. Colouring agent (three x 10 mg tablets of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) are added per 50 ml of citrate buffer (2.3 g citric acid, 4.89 g Na_2HPO_4 in 250 ml ddH_2O) incorporating 5 µl of hydrogen peroxide and then 50 µl of this substrate solution was added to each well. The plates were incubated for 20 minutes at 37°C and the absorbance was measured at 405 nm using a microplate reader (Bio-Rad, model 680). Positive sera and normal mouse sera were employed as positive and negative controls, respectively, on each ELISA plate.

2.12.3 Splenocyte and cell proliferation assays

Upon termination of the experiment, BALB/c mice were humanely culled and their spleens were aseptically removed and placed into ice-cold sterile PBS. A suspension of spleen cells was prepared by gently grinding the spleen on a fine wire mesh into 10 ml working media (RPMI 1640 supplemented with 10% Foetal bovine serum, 20 mM L-glutamine, 10^5 U of penicillin $litre^{-1}$ and 100 mg of streptomycin $litre^{-1}$). The cell suspension was left for approximately 5 minutes to allow the cells to settle, and then the liquid was transferred to sterile falcon tubes, being careful not to disturb the cellular debris at the bottom. The cell suspension was spun at 1200 rpm for 10 minutes, at 15°C. After centrifugation the supernatant was carefully removed and the pellet was resuspended to 10 ml with fresh supplemented RPMI, after which this cell suspension was again centrifuged at 1200 rpm for 10 minutes, at 15°C. The supernatant was carefully removed and the pellet was resuspended to 5 ml with fresh RPMI.

100 µl of sterile serially diluted antigen (in supplemented RPMI) was added per well of a sterile 96-well plate to give resultant HepB concentrations of 0 and 5 µg/ml and additionally

concanavalin A at a concentration of 1 μg ml^{-1} per well was added as a positive control. Subsequently, 100 μl of the splenocyte cell suspension was added per well and the plates were incubated for 72 hours at 37°C, in a humid 5% CO_2 environment. After 72 h ours incubation, 40 μl containing half a microcurie of [^{3}H]thymidine within sterile supplemented RMPI was added to each well of a 96-well plate and then incubated for a further 24 hours. The well contents were harvested onto quartz filter mats using a cell harvester (Titertek). The discs representing each well were punched from the filter mats into 5 ml volumes of scintillation fluid (Optiphase Hisafe) to measure the incorporation of [^{3}H]thymidine within the cultured cells using standard counting procedures.

2.13.4 Cytokine ELISA (sandwich ELISA)

Splenocytes were isolated and cells were plated onto 96-well plates as outlined in section 2.12.3. Cells were incubated for 40 hours at 37°C, in a humid (5% CO_2) environment, after which supernatants were removed and stored at minus 70°C for later analysis. Cytokine levels of IL-2, IL-5, IL-6, IL-10 and IFN-γ in the cell culture supernatants were quantified using the DuoSet® capture ELISA. The plates are coated with 100 μl capture antibody (1 μg/ml for IL-2 and IL-5, 2 μg/ml for IL-6 and IL-10, 4 μg/ml for IFN-γ, all in PBS) and incubated at room temperature over night. Plates were then washed three times with PBST buffer and blocked with 300 μl of 1% BSA (in PBS with 0.05% NaN_3) for IL-2 and IFN-γ, and 1% BSA (in PBS, pH 7.2-7.4) for IL-5, IL-6 and IL-10. Plates were then incubated for 1 hour at room temperature, followed by three washes with PBST buffer. 100 μl of reagent diluent for each cytokine was added to each well (for IL-2 and IFN-γ reagent diluent is, 0.1% BSA, 0.05% Tween 20 in Tris-buffer Saline (20 mM Trizma base, 150 mM NaCl) pH 7.2-7.4) (for IL-5, IL-6 and IL-10 reagent diluent is, 1% BSA in PBS, pH 7.2-7.4). Plates were then incubated at room temperature for 2 hours and again washed three times with PBST buffer. 100 μl detection antibody for the corresponding cytokine was added to each well and the plates were incubated for 2 hours at room temperature. Plates were once again washed three times with PBST buffer. 100 μl of working dilution of Streptavidin-HRP (horseradish peroxidase) was added per well, after which the plates were incubated at room temperature for 20 minutes, stored away from direct light. Plates were washed three times with PBST buffer and 100 μl of substrate solution was added per well (substrate solution is a 1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (tetramethylbenzidine (TMB) agent

(1 mg tablets), with 1 tablet dissolved in 1 ml DMSO and 9 ml of phosphate citrate buffer (0.05 M, pH 5)). Plates were then incubated at room temperature for 20 minutes, again avoiding direct light. Reaction was stopped with 50 µl solution of 2N H_2SO_4, and the optical density of each well was measured immediately using a microplate reader (Bio-Rad, model 680) set to 450 nm.

2.14 *In vivo* studies for subunit protein delivery

The immunisation protocol and immunological characterisation of the liposomal formulations, using the fusion protein Ag85B-ESAT-6, against tuberculosis were carried out by Statens Serum Institute (Copenhagen, Denmark), as detailed in Davidsen *et al.* (2005).

2.15 Statistical analysis

For all experiments, means and standard deviations were calculated. To determine statistical significance the one-way analysis of variance (ANOVA) was performed on all data, with the statistical significance determined to 0.05 confidence intervals ($P<0.05$). Tukey's post hoc test was conducted to determine which conditions differ significantly from each other.

3. Liposome-Mediated DNA Delivery: Initial Studies

3.1 Preparation and characterisation of plasmid DNA

When preparing any plasmid DNA for gene therapy *in vivo*, or even *in vitro*, it is crucial that the DNA is free of any contaminants, such as RNA, endotoxins and proteins. Therefore, throughout these studies the plasmids used (i.e. Figure 3.1; pRc/CMV HBS and gWiz expressing the luciferase gene) were amplified and purified based on the alkaline lysis procedure (Birnboin & Doly, 1979). The plasmid DNA consists of a gene encoding for the protein antigen that will elicit the specific protective immune response, a promoter sequence that plays a crucial role in initiating gene transcription of the antigen gene, which is usually derived from cytomegalovirus (CMV) (Patil *et al.*, 2005). In addition, each plasmid contains a gene for antibiotic resistance, to select the transformed bacteria during amplification of the plasmid within the bacteria (i.e. *Escherichia coli (E. coli)*. The bacterial cultures were selectively grown in the presence of a particular antibiotic, depending on the plasmid DNA being amplified. The plasmid pRc/CMV HBS contains a gene for antibiotic resistance to ampicillin, whereas, the plasmid gWiz contains a gene for antibiotic resistance to kanamycin (Figure 3.1).

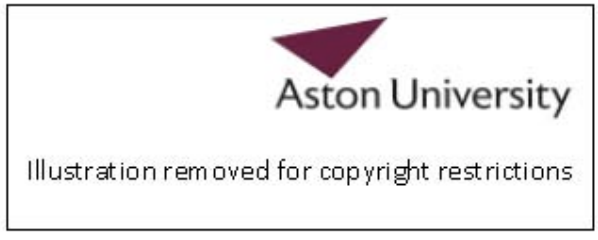


Figure 3.1 Plasmid DNA of pRc/CMV HBS and gWiz luciferase. The pRc/CMV HBS plasmid, under the control of the CMV promoter, expresses the hepatitis B surface antigen (i.e. S region). The gWiz luciferase plasmid, under the control of the CMV promoter, expresses the luciferase gene. Diagrams obtained from www.aldevron.com.

3.1.1 Purification of plasmid DNA

To validate the extraction and purification of plasmid DNA, Figure 3.2 monitors the process at various stages throughout the procedure where samples were collected and subjected to

agarose gel electrophoresis. The desired plasmid DNA is extracted from *E.coli* DH5α strain efficiently and separated from bacterial chromosomes as demonstrated by a clear lysate and by the presence of supercoiled and open circular plasmid DNA (Figure 3.2, lane 1). After application of the clear lysate to the ion-exchange resin column, traces of RNA flow through the column with the DNA binding to the resin (Figure 3.2, lane 2). The DNA, still bound to the column, is than washed to remove any remaining traces of RNA and contaminants (Figure 3.2, lane 3), followed by the addition of a high salt buffer to elute the pure plasmid DNA from the column (Figure 3.2; lane 4).

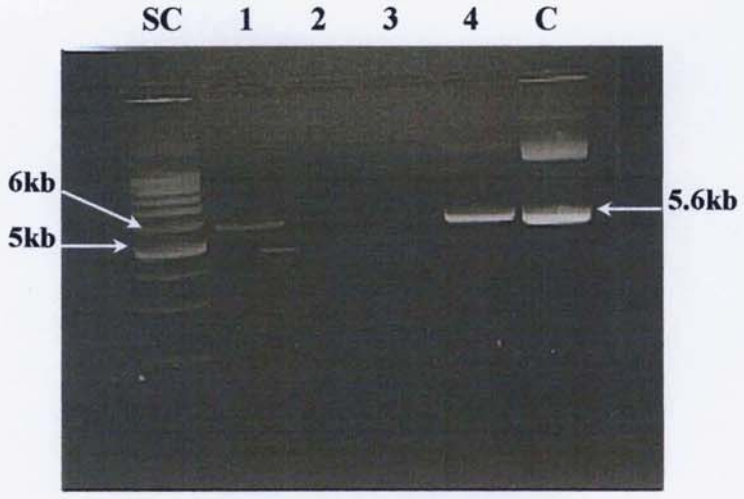


Figure 3.2 Agarose gel electrophoresis of samples taken at various stages throughout the purification process of the plasmid pRc/CMV HBS. Lane SC, supercoiled ladder DNA (2-10kb); Lane 1, clear lysate; Lane 2, flow through; Lane 3, wash; Lane 4, elute; Lane C, control DNA (0.1mg/ml).

3.1.2 Spectrophotometry of plasmid DNA: quantification and purity assay

The concentration and purity of the eluted DNA was measured by spectrophotometry. 20μl sample from the plasmid DNA solution was diluted 1:50 with TE buffer and the nucleic acid concentration of the purified DNA was determined by measuring the absorbance of the solution at A_{260}. The final concentration was calculated by multiplying the A_{260} by 50μg (as this concentration produces a reading of 1 at A_{260}) and then multiplying again by the dilution factor (i.e. 50).

The purity of all DNA samples produced using the Qiagen procedure was determined. Firstly the protein content within the solution was determined by measuring the absorbance at A_{280}. A ratio between A_{260} and A_{280} can be a good indicator of purity. Ahn *et al* (1996) stated that a reading between 1.7 and 2.0 is a good indicator of a 'clean' sample. All values determined at A_{280} when using the Qiagen procedure were approximately 1.85, indicating that all plasmid DNA samples produced and used during all experiments possessed good levels of purity. In addition, samples were also read at A_{230} in order to test for the presence of any organic solvent contamination, such as ethanol or isopropanol, however, as no values at A_{230} exceeded the A_{260} values, no contamination was found. At A_{325} any reading greater than 0 indicates the presence of any particular matter within the DNA solution, however, no form of particular matter was found within any plasmid DNA samples amplified. In addition, the QIAGEN procedure used removes endotoxins, (cell membrane components of *E. coli*) within the purified plasmid DNA ensuring the production of a very pure yield of DNA.

3.1.3 Restriction enzyme analysis to examine plasmid DNA purity

Restriction enzymes recognise and bind to specific base sequences of the dsDNA and cleave both strands of the DNA within that site. This generates a linear restriction fragment, which can be used to determine the purity of the DNA by comparing the single band generated by gel electrophoresis against a control DNA, which has known restriction fragment sizes.

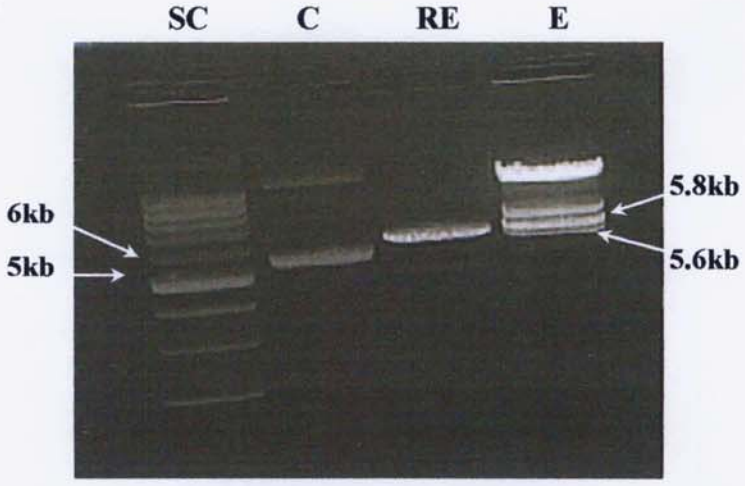


Figure 3.3 Agarose gel electrophoresis of plasmid DNA pRc/CMV HBS before and after restriction enzyme digestion. Lane C shows the undigested plasmid DNA (5.65kb) based on the supercoiled DNA ladder (2-10kb; lane SC). Lane RE shows the plasmid DNA after restriction enzyme digestion with *Hind* III, with linear plasmid monomer size between 5.6-5.8kb as based on the *EcoR* I ladder (1-10kb; lane E).

Figure 3.3 illustrates that the intact pRc/CMV HBS plasmid (5.65kb) has a size between 5 and 6kb, when measured against the supercoiled plasmid DNA ladder (1-10kb) (lanes C and SC, respectively). However, in the presence of the enzyme *Hind* III, the pRc/CMV HBS plasmid DNA is digested by the enzyme and cleaved at a single site for *Hind* III digestion, consequently generating a linear plasmid monomer, with a reformed size of between 5.6 to 5.8kb (lane RE), based on the *EcoR* I ladder (1-10kb) (lane E), illustrating the purity of the plasmid DNA.

3.1.4 Degradation of plasmid DNA by extracellular enzymes

Naked DNA, when injected directly, is rapidly degraded when exposed to a host of extra cellular degrading enzymes located within the biological milieu, rendering the plasmid DNA non functional and causing severe loss of its original structure. Deoxyribonuclease I is a degrading enzyme that promotes the hydrolysis of nucleic acids by recognizing the phosphodiester linkage within the backbone of the plasmid DNA (Patil *et al.*, 2005) acting as a catalyst to breakdown the plasmid DNA into smaller fragments i.e. DNA nucleotides. As a result, the application of synthetic DNA delivery systems, such as liposomes is required. Liposomes efficiently entrap plasmid DNA and act as a protective barrier whereby enzymes, such as deoxyribonuclease I, are unable to access and degrade the DNA entrapped within the bilayers consequently maintaining original DNA structure and function (Gregoriadis *et al.*, 1996; Gregoriadis *et al.*, 2000).

To confirm and emphasise the ability of liposomal vesicles to protect the plasmid DNA from degradation by digestive enzymes, plasmid DNA (pRc/CMV HBS) was exposed to DNase I as either naked or entrapped within cationic liposomes composed of PC:Chol:DC-Chol (16:8:4 μmole/ml), after which samples were subjected to agarose gel electrophoresis to examine DNA integrity from each study, naked or entrapped DNA. Figure 3.4 shows the undigested (lane 1) and digested (lane 2) naked DNA. The undigested anionic DNA migrates towards the cathode, at a rate dependent on its molecular weight and tertiary structure. After digestion by DNase I the naked DNA is completely degraded by the enzyme with no band visible on the agarose gel. However, in contrast, for liposome entrapped DNA, although there is no DNA band visible in lanes 3 and 4, the plasmid DNA is encapsulated within vesicle bilayers and therefore remains at the site of application along with the lipids, as indicated by the increased fluorescence within the wells of lane 3 and 4. It is not until the

lipid bilayer is lysed and broken down by sodium dodecyl sulphate (SDS; which as shown in lane 5 and 6 has no effect on naked DNA properties) that the DNA is released and migrates towards the cathode (lanes 7 and 8), confirming that the plasmid DNA remains intact during the digestion process with the liposome bilayer acting as a protective barrier between the DNA and enzyme (Wong et al, 2001; Fenske et al, 2002).

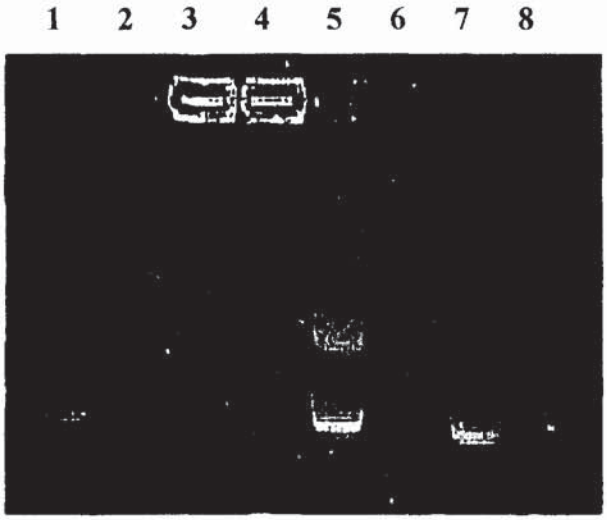


Figure 3.4 Gel electrophoresis of free and liposome entrapped plasmid DNA, pRc/CMV HBS, before and after exposure to digestive enzyme, DNase I. 100μg plasmid DNA (pRc/CMV HBS), either naked or entrapped within liposomal vesicles was exposed to the digestive enzyme DNase I, for a period of 10 minutes, after which the reaction was stopped with EDTA (0.1M). Lane 1, naked DNA without digestion; Lane 2, naked DNA with digestion; Lane 3, liposome entrapped DNA without digestion; Lane 4, liposome entrapped DNA with digestion; Lane 5, naked DNA in the presence of SDS without digestion; Lane 6, naked DNA in the presence of SDS with digestion; Lane 7, liposome entrapped DNA in the presence of SDS without digestion; Lane 8, liposome entrapped DNA in the presence of SDS with digestion. Liposome formulation composed of 16μmoles PC, 8μmoles Chol and 4μmoles DC-Chol.

This confirms and emphasises the need for a delivery system, which will offer protection for the plasmid DNA and efficient delivery to the target cell in order to initiate the appropriate immune response. When applied *in vivo*, a considerable amount the injected naked DNA will be readily degraded by interstitial enzymes, causing loss of plasmid DNA structure and function. Therefore, to provoke any substantial level of immunity, large doses of DNA would need to be administered and also relatively frequently (Perrie *et al.*, 2001). By entrapping the plasmid DNA within the liposomes lipid bilayers, the plasmid DNA is largely protected from degradation by enzymes (Gregoriadis *et al.*, 1996; Perrie *et al.*, 2001; Patil *et al.*, 2005), therefore, depending on the lipid composition and liposome characteristics, the

plasmid DNA can be efficiently delivered to the target cell or even act locally, whereby the DNA is slowly released from the liposomes that function as a depot system.

Despite the fact that liposomes are extremely effective at protecting the plasmid DNA from deleterious effects of the biological milieu, the efficiency of DNA delivery and gene expression is relatively low (Liu and Huang, 2002; Zhdanov *et al.*, 2002). To further understand the role of liposomes within DNA delivery the underlining mechanisms involved with successful delivery need to be determined. As liposomes can be formulated with an immense variety of different lipids, liposome physiochemical characteristics and activity can be transformed depending on the lipid composition employed and also influence the interactions with the plasmid DNA and cell membrane. Essentially every aspect of the liposomal system plays a crucial role in determining structure and the liposomes operation mechanism. Therefore, by analysing the contribution of each lipid component in terms of transfection efficiency, by means of formulating liposomes composed of a variety of different lipid mixtures and then subsequently applying these *in vitro*, the resultant level of transfection efficiency emphasises the role and efficacy of each lipid component within DNA delivery and in doing so, liposomes as a delivery system for DNA can be improved once the function of each lipid component is fully recognised.

3.2 Initial optimisation of transfection studies

Prior to any *in vivo* investigations, preliminary *in vitro* studies are required, which enable the development of a rapid screening process of various liposome formulations, when it is not feasible to test all formulations *in vivo*. Therefore, further to the *in vitro* studies (presented in chapter 4), a select few formulations will be taken forward and applied *in vivo*, in which the results are presented and discussed in chapter 6.

Transfection is a multifactorial process being influenced by both external and internal factors (Zuidam *et al.*, 1999). External factors, such as, cell type, medium used and experimental protocol were kept constant throughout these studies, therefore variation in transfection efficiency between formulations could be directly related to internal factors, such as lipid composition, lipid:DNA ratio, etc, all related to the physiochemical properties of the liposomal formulations. The objective of these *in vitro* experiments is to generate an

underpinning understanding of the mechanisms involved in liposome-mediated DNA transfection, in terms of liposome composition, lipid to DNA charge ratio, complex structure and function. Firstly, for successful transfection every aspect and condition of the transfection procedure was tested and optimised prior to the screening process of liposomal formulations.

3.2.1 Cell distribution studies

During the transfection procedure it is essential that the adherent cells are evenly distributed between wells, in order to minimise variations betweens formulations. In an initial investigation, COS-7 cells were seeded at a concentration of 1 x 10^5 cells/well on 12 well and 96 well plates and incubated at 37°C, until cells reached confluence. After 24 hours the cell medium was removed and the adherent COS-7 cells were stained with 0.4% (w/v) crystal violet. As shown in Figures 3.5 and 3.6, the cells were evenly stained within each well of a 12 well and 96 well plate respectively, revealing that there is an even cell distribution between wells, with a good level of confluence. With an even distribution of COS-7 cells, reproducible results can be obtained and transfection activity can be directly attributed to variations between liposome formulations.

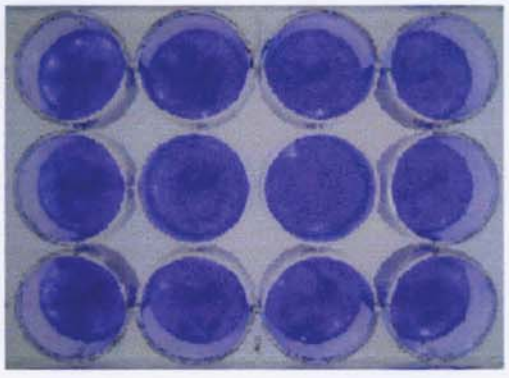


Figure 3.5 COS-7 cell distribution in a 12 well cell culture plate. Cells seeded at a concentration of 1 x 10^5 cells/well and stained with crystal violet in each well.

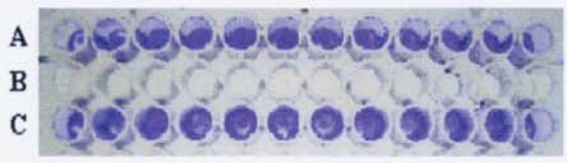


Figure 3.6 COS-7 cell distribution in a 96 well cell culture plate. Cells seeded at a concentration of 1 x 10^5 cells/well and stained with crystal violet in each well (lanes A and C). Empty wells (lane B).

3.2.2 Optimising cytotoxicity assay

One concern of liposomes as DNA vectors is the associated cellular toxicities that may occur after administration (Romoren *et al.*, 2005). The composition and lipid concentration within liposome systems is said to affect cell viability and cell integrity. Cationic liposomes formulated at higher lipid concentrations exhibiting increased levels of toxicity (Romoren *et al.*, 2005) and liposomes formulated with the cationic lipid stearylamine (SA) exhibited extremely high levels of cytotoxicity on a range of tumour cell lines (Mayhew *et al.*, 1987; Cambell, 1983). Although a trend of decreased transfection efficiency may be associated with an increase in cell toxicity and reduced cell viability (Romoren *et al.*, 2005), it is necessary to determine the level of cytotoxicity within each liposome system tested for *in vitro* transfection activity.

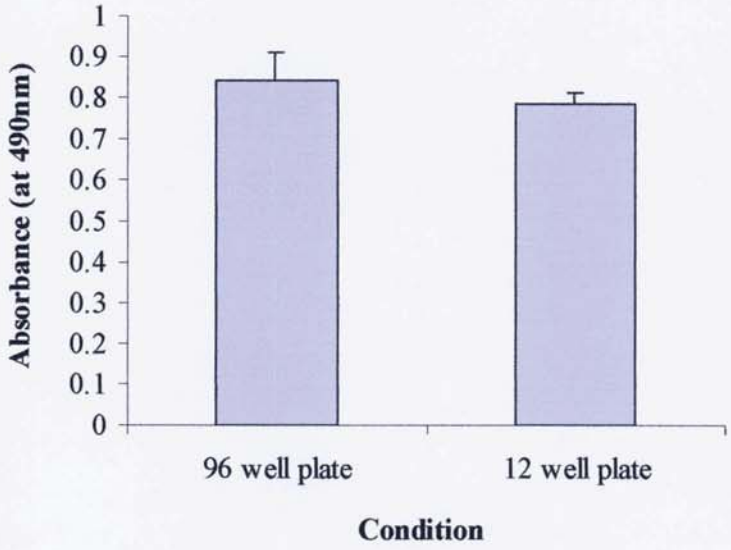


Figure 3.7 Effect of cell culture plate on absorbance at 490nm measured using the CellTiter 96® AQ_{ueous} One Solution Assay. COS-7 cells were plated on either a 96 well plate or 12 well plate at a cell concentration of $1x10^5$ cells/ml in supplemented DMEM. Cells were than incubated at 37°C in a humidified, 5%CO_2 atmosphere. After 72 hours, 20µl/well of a 96 well plate and 200µl/well of a 12 well plate of CellTiter 96® AQ_{ueous} One Solution Reagent were added. After 3 hours incubation, absorbance at 490nm was recorded. Results denote mean ± SD, $n = 8$.

The cytotoxicity assay used for determining the number of viable cells in proliferation is prepared and manufactured for use on 96 well plates. An initial study was carried out in which COS-7 cells were plated onto either 96-well or 12-well cell culture plates, at a cell

concentration of 1 x 10^5 cells/ml and the transfection procedure was carried out (section 2.12.2). 42 hours post transfection, the cytotoxicity assay was carried out with the addition of Cell Titer 96® AQ_{ueous} One Solution reagent (i.e. 20 µl/well on a 96-well plate and 200 µl/well on a 12-well plate). Figure 3.7 shows that the values from the cytotoxicity assay were not influenced by well size, as there were no significant differences in A_{490} between assays carried out on a 96 or 12-well plate.

In a further study, the cytotoxicity assay was carried out to ensure that the presence of liposomes, which may possibly be contained within the medium, does not interfere with absorbance readings at 490nm. Liposomes composed of DOPE:DC-Chol (1:1 molar ratio) were prepared and added to each well of a 96 well plate containing DMEM at the same volume of solution as used for the cytotoxicity assay. Absorbance reading of 0.173 ± 0.01 (n = 8) for liposome containing wells were compared to those wells only containing medium, producing a value of 0.170 ± 0.003 (n = 8) (results not shown). Therefore, with no significant differences between readings, the absorbance readings at 490nm are unaffected by the presence of liposomes which may still be present within solution, indicating that values are a true reflection of cell viability.

3.2.3 Optimising lipofectin concentration

In 1987, Felgner *et al.* first synthesised the cationic lipid DOTMA. This cationic lipid in combination with the helper lipid DOPE, at a 1:1 (w/w) ratio, is commercially available i.e. Lipofectin®. Lipofectin has been extensively used to transfect a wide variety of cells and has shown to be an effective transfection agent *in vitro*. The cationic liposome spontaneously interacts with the negatively charged plasmid DNA, causing the DNA strand to condense and compact within the lipid arrangement, forming liposome-DNA complexes (May *et al.*, 2000; Safinya, 2001). Throughout these transfection studies, lipofectin was utilised as the positive control, in which all liposome formulations tested within these studies were be evaluated against. Therefore, the optimum concentration of lipofectin reagent, which produces the highest levels of transfection efficiency, was determined using the manufacturers protocol (invitrogen). Transfection activity was measured by the luciferase assay system (Promega) as described in section 2.12.2.3.

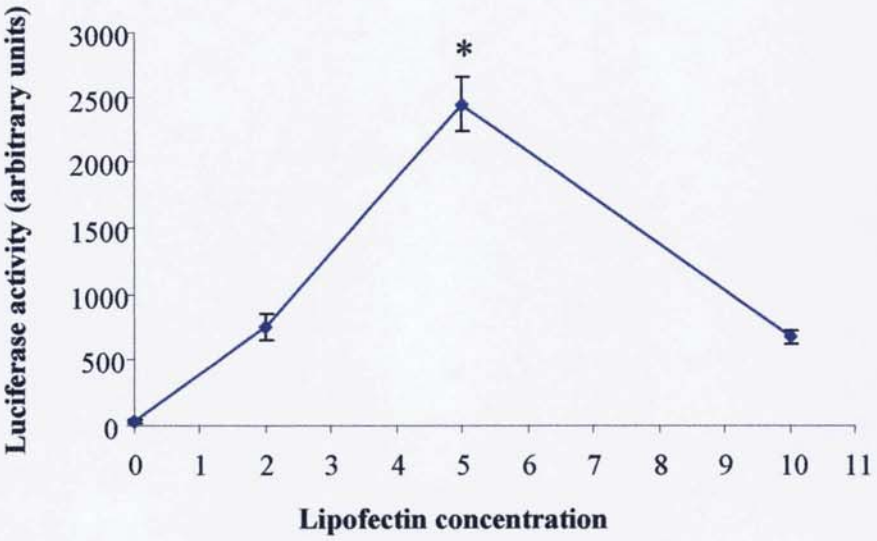


Figure 3.8 Effect of lipofectin concentration on luciferase activity. COS-7 cells were transfected with varying concentrations of lipofectin reagent (DOPE:DOTMA, 1:1 w/w ratio at a total lipid concentration of 1mg/ml). COS-7 cells were plated in a 12 well plate at a cell concentration of $1x10^5$cells/ml 24 hours prior to the transfection procedure. 1ml of lipofectin at different concentrations, 0, 2, 5 and 10 μg/ml, containing 1μg/ml plasmid DNA (gWiz luc) was added to the cells. After a 5 hour incubation, the medium was removed and replaced with fresh supplemented medium. Cell lysates were harvested 2 days post-transfection and luciferase activity was determined by luciferase assay. * signifies that Lipofectin at a lipid concentration of 5 μg/ml produces significantly greater ($P<0.05$, ANOVA) values of luciferase activity than lipid concentrations of 2 μg/ml and 10 μg/ml. Results denote mean ± SD, $n = 3$.

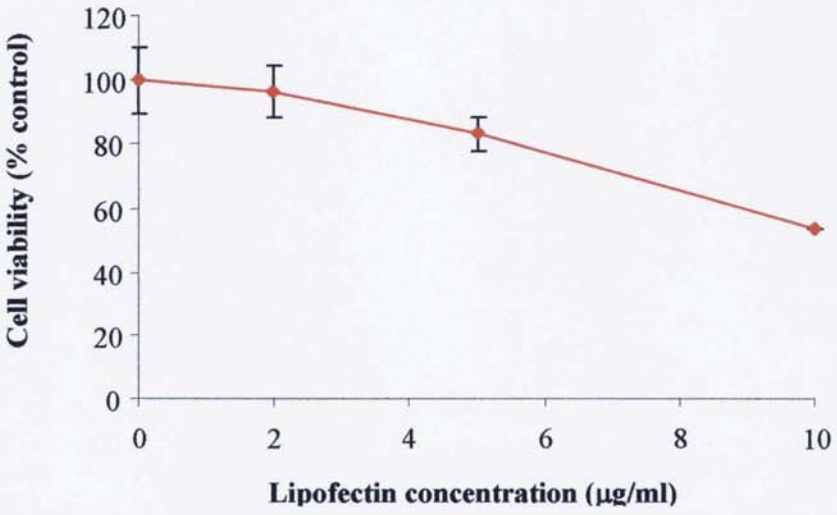


Figure 3.9 Effect of lipofectin concentration on COS-7 cell viability post transfection. The level of cytotoxicity was measured by the CellTitre 96® AQueous One solution cell proliferation assay (Promega). This is a colorimetric method for determining the number of viable cells in proliferation, which is measured by the amount of 490nm absorbance and this is directly proportional to the number of living cells in culture. Results are expressed as a percentage of positive control (cells and medium only). Results denote mean ± SD, $n = 8$.

Figure 3.8 shows that a lipofectin concentration of 5 μg/ml produced the highest values of luciferase activity, with values significantly ($P<0.05$, ANOVA) greater than both 2 μg/ml and 10 μg/ml. Furthermore, cytotoxicity assays were also carried out using the CellTitre 96® AQueous One solution cell proliferation assay (Promega), revealing that as the lipofectin concentration increases from 2 μg/ml to 10 μg/ml, the number of viable cells seems to decrease by almost half from 96% to 54% (Figure 3.9). Therefore, with an increase in lipid content the liposome-DNA complexes become toxic to the cultured cells (Romoren *et al.*, 2005). Overall, the low values of transfection activity of lipofectin at a lipid concentration of 10 μg/ml could result from lipid toxicity, where there are a reduced number of viable cells and a smaller amount of COS-7 cells to transfect.

For that reason, lipofectin reagent at a lipid concentration of 5 μg/ml will be used as the positive control within further transfection studies, in which transfection efficiency of liposome formulations will be expressed as a percentage of lipofectin control.

3.2.4 Sterile liposomes

In order to minimise any contamination of the cultured cells during the transfection procedure the ideal situation is to produce sterile carrier systems. However, liposome characteristics must remain unchanged throughout the sterilisation process. At present, there are a number of ways in which to produce sterile liposomes, including; exposure to chemical sterilising agents (Ratz *et al.*, 1989), autoclaving (Zuidam *et al.*, 1993) and sterile filtration (Freise, 1984). Incomplete removal of chemical agents and their residues may be toxic to cells and autoclaving by extreme heat treatment may alter the chemical and physical nature of the phospholipids and liposomal bilayer, therefore, the latter procedure of sterile filtration was adopted for these experiments.

During these *in vitro* transfection experiments, two morphologically different liposome systems will be tested. Firstly, SUV-DNA in which the plasmid DNA is complexed to liposomal vesicles as a consequence of electrostatic interactions between the anionic DNA and cationic liposomes. Secondly, DRV(DNA) in which the plasmid DNA is entrapped within the lipid bilayers of liposomal vesicles, due to a procedure of controlled dehydration-rehydration. Liposomes were sterilised by filtration, in which empty SUV (<100nm) were passed through a sterile filter of pore size 0.22μm, then to which sterile plasmid DNA was

added (i.e. SUV-DNA) and subsequently freeze-dried and rehydrated DRV(DNA), all carried out within sterile conditions. The physiochemical characteristics of non-sterilised and sterilised liposomes were measured and compared for all liposomal systems (Table 3.1).

The sterilisation process of liposomes by filtration is a non-disruptive method that does not alter liposome physiochemical characteristics. The size and zeta potential of empty SUV is similar for non-sterile (75 ± 16 nm and 35 ± 5mV; Table 3.1) and sterile (68 ± 8 nm and 33 ± 2 mV; Table 3.1). Even upon addition of plasmid DNA, the complexation process is unaffected producing non-sterile and sterile SUV-DNA complexes and DRV(DNA) liposomes which are relatively similar in size. Indicating that the orientation and ordering of lipids within the bilayer may remain uniform throughout sterilisation by this filtration process.

Preparation	Size (nm ± SD)		Zeta Potential (mV ± SD)	
	Non-sterile	Sterile	Non-sterile	Sterile
Empty SUV	75.2 ± 15.9	67.5 ± 7.7	34.8 ± 5.3	32.5 ± 1.9
SUV-DNA	287.9 ± 14.4	283.9 ± 19.3	37.4 ± 2.7	34.2 ± 2.0
DRV(DNA)	721.9 ± 32.3	655.7 ± 46.4	38.1 ± 1.9	34.9 ± 2.7

Table 3.1 Effect of liposome sterilisation by filtration on liposome physiochemical characteristics. SUV composed of 16μmoles PC, 8μmoles Chol and 4μmoles DC-Chol were prepared by sonication, followed by sterile filtration through a millipore with a mesh size of 0.22μm. There are no significant ($P<0.05$, ANOVA) differences in characteristics between non-sterile and sterile. Results denote mean ± SD, $n = 3$.

3.2.5 Effect of liposome preparation: MLV vs. SUV

Transfection efficiency of liposome-DNA complexes is said to effected by a variety of factors, including; type of cationic lipid used, lipid to DNA ratio and the type of neutral lipid used (Zabner, 1997). Ultimately, all these factors also influence the formation and resultant complex size of liposome-DNA complexes.

Liposomes composed of DOPE:DC-Chol at 1:1 molar ratio with a total lipid concentration of 1.56 μmole/ml, generate MLV with a z-average diameter of around 550-650nm. When plasmid DNA is added the liposomes tend to aggregate around the DNA producing much larger MLV-DNA complexes that have almost doubled in size 1024 ± 183 nm (Figure 3.10).

Due to the addition of plasmid DNA, electrostatic interactions occur between these positive MLV (44 ± 6 mV; Figure 3.11) and the anionic DNA, causing the MLV to complex or aggregate around the plasmid DNA. Zeta potential measurements reveal that MLV-DNA complexes are extremely negative (-34 ± 12 mV), implying that the cationic nature of the MLV is such that they did not sufficiently neutralise the negative charge of the plasmid DNA, thereby may not effectively protect and condense the plasmid.

Upon the addition of negatively charged DNA, the positive charges of the cationic lipids will be neutralised (Zuidam *et al.*, 1999) and even, as found here, the liposome-DNA complexes will possess a net negative charge where the DNA may be exposed or unincorporated within lipid bilayers. The zeta potential of liposome-DNA complexes is fully dependent on the total charge ratio between the cationic lipid and anionic DNA (Eastman *et al.*, 1997). However, irrespective of sonication time, vesicle size and morphology, zeta potential values of each liposome system (MLV vs. SUV) were similar for empty liposomes (39 to 44 mV; Figure 3.11) and also liposome-DNA complexes (-34 to –43 mV; Figure 3.11), demonstrating that the surface charge of these systems is not the determining factor for the differences found in transfection activity between MLV and SUV liposomes and it is more likely to be a result of their morphological nature (Zuidam *et al.*, 1999).

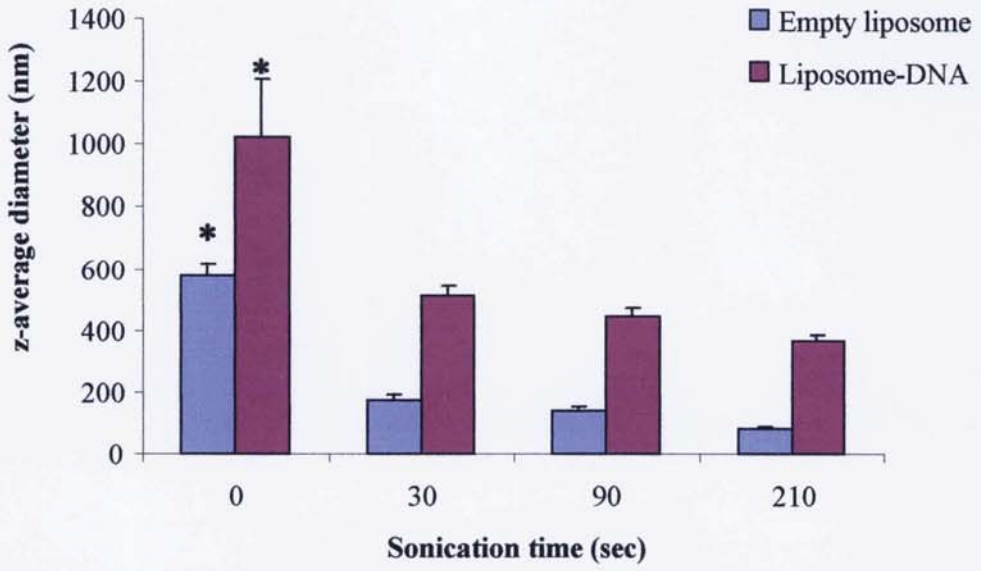


Figure 3.10 Effect of sonication time on z-average diameter of liposomes. Liposomes composed of DOPE:DC-Chol at a 1:1 molar ratio with a total lipid concentration of 1.56μmole/ml, were sonicated and samples were taken at various time points. * signifies that empty MLV and MLV-DNA liposomes were significantly ($P<0.05$, ANOVA) larger than liposomes at various sonication times. Results denote mean ± SD, $n = 3$.

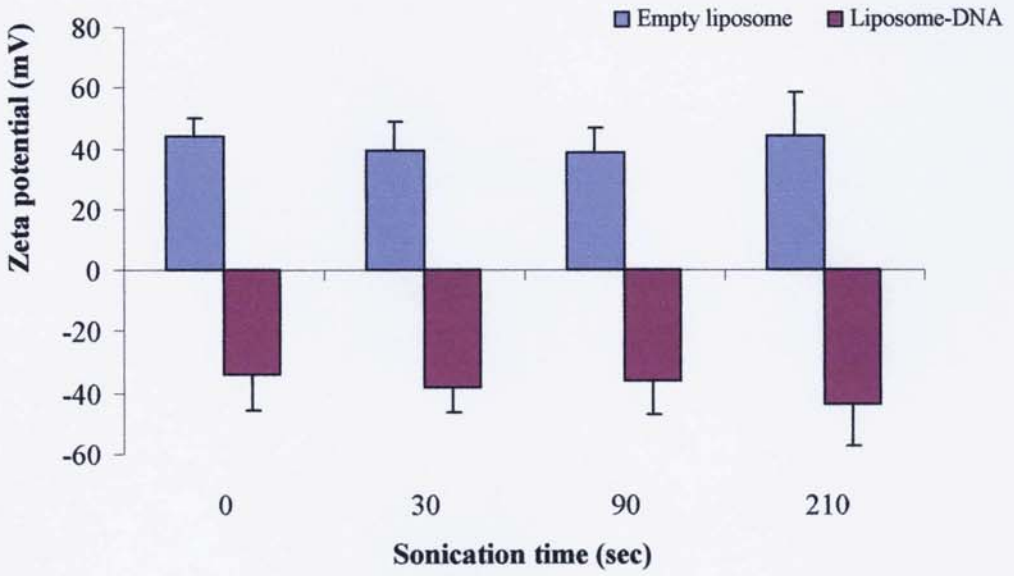


Figure 3.11 Effect of sonication time on zeta potential of liposomes. Liposomes composed of DOPE:DC-Chol at a 1:1 molar ratio with a total lipid concentration of 1.56μmole/ml, were sonicated and samples were taken at various time points. Results denote mean ± SD, $n = 3$.

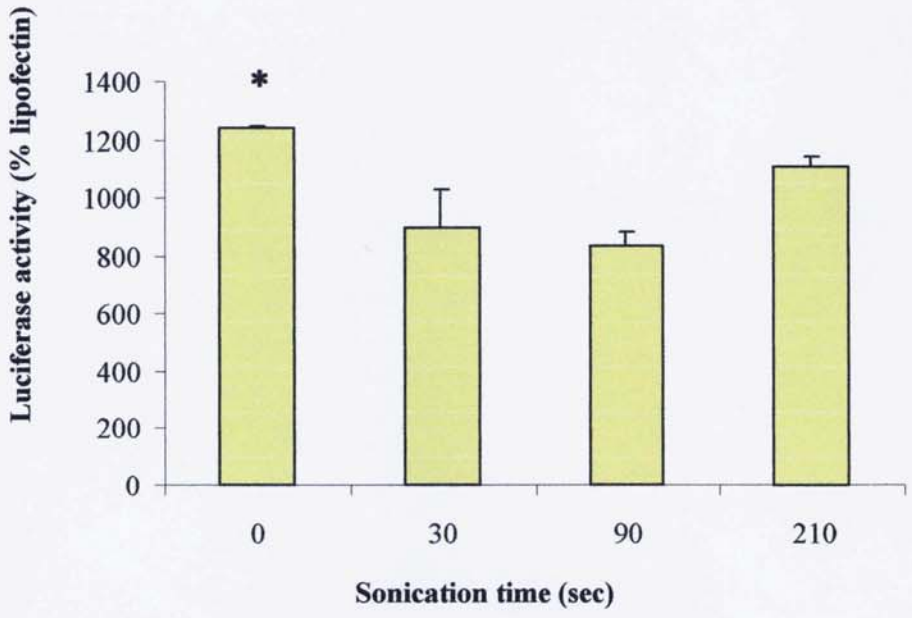


Figure 3.12 Effect of sonication of liposomes on transfection efficiency. Liposomes composed of DOPE:DC-Chol at a 1:1 molar ratio with a total lipid concentration of 1.56μmole/ml, were sonicated and samples were taken at various time points. 0.0078μmole of total lipid containing 1μg DNA was added to each well containing COS-7 cells. * signifies that liposomes prior to sonication (i.e. MLV) are better at transfecting COS-7 cells than liposomes that have been sonicated for any length of time (i.e. 30, 90 and 210 seconds). Results denote mean ± SD, $n = 3$.

Figure 3.12 shows that the method of liposome preparation influences transfection efficiency of the liposome-DNA complexes composed of DOPE:DC-Chol (1:1 molar ratio, with a total lipid concentration of 1.56 μmole/ml). MLV-DNA complexes where the liposomes were prepared without sonication prior to the addition of DNA, produced the highest values of transfection activity (1236 ± 8% of lipofectin control; Figure 3.12). Results presented in Figure 3.12 demonstrate that as the sonication time of the liposomes increases to 90 seconds there was a trend in decreased transfection efficiency (834 ± 45% of lipofectin control; Figure 3.12) although interestingly, as sonication time increases even further to 210 seconds producing SUV liposomes (79 nm; Figure 3.10) and following mixing with DNA (i.e. SUV-DNA, 363 nm), transfection efficiency is significantly enhanced once again to 1103 ± 36% of lipofectin control levels, although values of activity remain significantly lower ($P<0.05$) than the larger MLV-DNA complexes (Figure 3.12). These results are in line with previous studies when comparing MLV-DNA, SUV-DNA and LUV-DNA complexes, where complexes were either composed of the cationic lipid DOTAP alone or DOPE:DOTAP (1:1 molar ratio), although, MLV liposomes were more efficient irrespective of lipid composition (Felgner *et al.*, 1994; Liu *et al.*, 1997; Zuidam *et al.*, 1999). This increase in transfection efficiency could result from the increase in complex size enhancing liposome-cell interactions and DNA delivery. Chapter 4 examines such influential factors further, providing further detail and explanation of how these factors may contribute to DNA transport.

3.2.6 Transfection efficiency in the presence of serum

The inhibitory effect of serum on transfection efficiency has been previously been reported for several different cell lines (Ciccarone *et al.*, 1993; Escriou *et al.*, 1998). Although, it appears that the level of inhibition is dependent on the lipid composition and complex surface charge of the liposome-DNA complexes (Fanceca *et al.*, 2002).

To study the effect of serum on transfection efficiency within our model system, SUV-DNA complexes composed of DOPE:DC-Chol, Chol:DC-Chol and PC:DC-Chol (1:1 molar ratio, at a total lipid concentration of 1.56 μmole/ml) were prepared and 0.0078 μmole of total lipid, mixed with 1 μg plasmid DNA per ml were incubated with COS-7 cells in the presence of 10 % FBS.

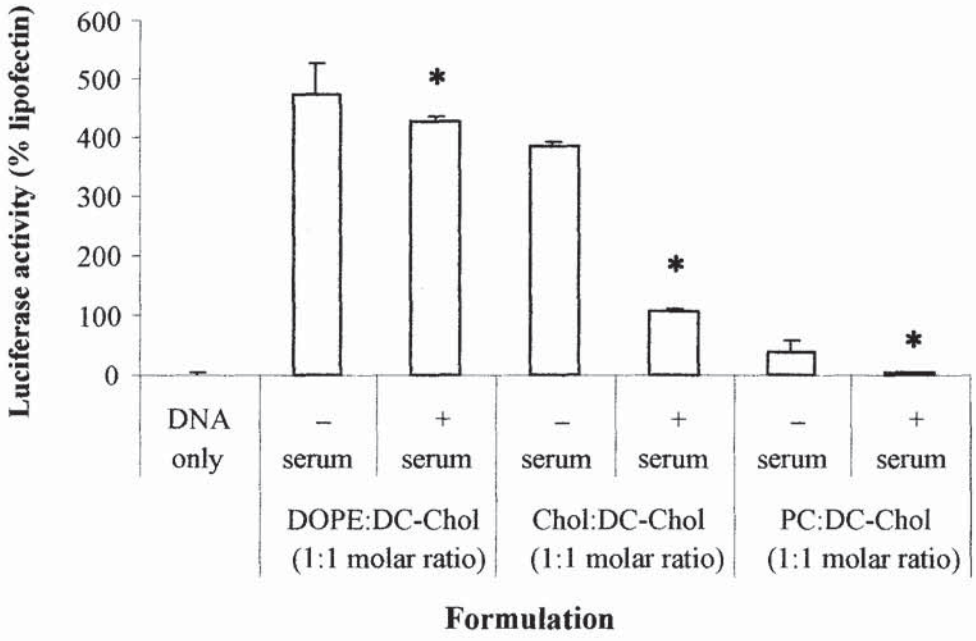


Figure 3.13 Transfection efficiency of liposome-DNA complexes of various compositions in the absence and presence of serum. SUV composed of Chol:DC-Chol and PC:DC-Chol, all at a 1:1 molar ratio with a total lipid concentration of 1.56 μmole/ml were formulated. 1ml suspension of liposome-DNA complexes consisting of 0.0078 μmole total lipid and 1 μg plasmid DNA was incubated with COS-7 cells in the absence or presence of 10% FBS, for a time period of 5 hours. Cell lysates were harvested 2 days post-transfection and luciferase activity was determined by luciferase assay. * signifies that transfection efficiency of complexes incubated in the presence of serum is significantly reduced ($P<0.05$, ANOVA) compared to those incubated in serum-free medium (i.e. OptiMEM). Results denote mean ± SD, $n = 3$.

Transfection efficiency of all liposome formulations tested; DOPE:DC-Chol, Chol:DC-Chol and PC:DC-Chol (1:1 molar ratio, at total lipid concentration of 1.56 μmole/ml) were reduced by the presence of 10% serum (Figure 3.13). Efficiency of Chol:DC-Chol complexes was significantly ($P<0.05$, ANOVA) decreased from 384% to 107%, and the efficiency for PC:DC-Chol complexes significantly ($P<0.05$, ANOVA) decreased from 72% to 2%, in the absence and presence of 10% serum, respectively. However, interestingly, transfection efficiency of DOPE complexes is less inhibited by 10% serum, than stabilized complexes containing Chol or PC. However, transfection efficiency of DOPE-containing complexes is still significantly reduced when incubated in 10% serum rather than serum free medium (i.e. opti-MEM). Usually it would be assumed that stable complexes are less affected and inhibited by serum proteins, due to their ability to generate membrane stability (Fancea *et al.*, 2002). Despite this, previous studies support this finding (Mok and Cullis, 1997; Kawaura *et al.*, 1998; Sternberg *et al.*, 1998). Sternberg *et al.*, (1998) found that Chol-containing complexes exhibited at least 20% lower *in vitro* activity than DOPE-containing

complexes that showed very high transfection activity (100%). The observed decrease in transfection efficiency in the presence of serum could be attributed to the presence of anionic serum proteins. The anionic molecules would electrostatically bind to the complexes and would either destabilise the liposome-DNA complex system, causing the complex to lose original structure and/or inhibit electrostatic binding to the cell membranes suggesting that the problems associated with *in vitro* transfection models will also occur within the *in vivo* studies.

3.3 Conclusion

These initial studies reveal that the plasmid DNA, amplified and purified by the Qiagen procedure, utilized throughout these studies was a pure form of the plasmid and additionally free from any contaminants. When the plasmid DNA is naked, it is readily degraded by digestive enzymes, whereas entrapment within liposomes fully protects the DNA, thus demonstrating the beneficial role of liposomes in contrast to naked DNA.

Optimisation studies of the transfection protocol show that the COS-7 cells were evenly distributed within and between wells of both a 12-well and 96-well plate. The cytotoxicity assays are unaffected by the presence of liposomes and generate similar absorbance values between a 12-well and 96-well plate. Therefore, these results reveal that any differences found between liposome formulations will result from the differences between formulations and not external variables.

4. Optimisation of liposome formulation and bilayer mechanics *in vitro*

4.1 Introduction

Although direct injection of naked antigen-encoded plasmid DNA by the intramuscular route has proved to be successful at transfecting cells and subsequently initiating protective humoral and cell-mediated immunity (Davis *et al.*, 1993; Chattergoon *et al*, 1998; Gregoriadis, 1998), the overall responses are minimal, with only a fraction of the DNA being taken up by cells. The small amount of plasmid DNA that is able to transfect cells efficiently are taken up by non-professional antigen presenting cells, such as myocytes, which lack essential co-stimulatory molecules, therefore are inefficient at presenting peptides in order to produce sufficient levels of antibodies against the antigen (Davis *et al.*, 1993; Perrie *et al.*, 2001). Inadequate cellular uptake of plasmid DNA is a result of a combination of several factors such as DNA charge, size and poor stability. To overcome such prevailing barriers the development of liposomes as a DNA delivery system, not only offers protection and stability for the DNA, but also enhances liposome-cell interactions (Gregoriadis, 1990).

One of the most important aspects when developing a successful liposome vesicle for antigen delivery is lipid composition. Cationic lipids are considered as the key component for gene delivery within liposomal formulations, as they interact with the anionic DNA through electrostatic interactions and act as condensing agents for the negatively charged DNA strands (May *et al.*, 2000). Without the cationic lipid present, liposome-DNA interactions are likely to be significantly reduced, however, this will be looked at in further detail in chapter 5. Various other lipids are also included within liposomal formulations, as these 'helper' lipids play an important role in determining liposome structure and physiochemical characteristics. Every phospholipid incorporated within the formulation plays an essential role in the physiochemical characteristics of the resultant liposome indicating that mechanical and surface properties of liposomes can be modulated through judicial selection of various bilayer components (Gregoriadis, 1990). As well as the lipid composition, transfection rates will also vary depending on the cationic liposome-DNA charge ratio (Ahearn and Malone, 1999).

The aim of this chapter is to investigate different chemical and physical aspects of the liposome composition (i.e. vesicle charge, lipid alkyl chain length, liposome transition temperature, etc) and identify the fundamental liposome characteristics that enhance transfection and expression of DNA within cells.

4.2 Importance of cationic lipid

The interaction and complex formation between the plasmid DNA and cationic liposomes is dependent on the type of cationic lipid present within the liposomal formulation. Since the pioneering development of Lipofectin® reagent, containing the cationic lipid DOTMA, there has been a considerable expansion in the synthesis of other cationic lipids, which may hopefully result in higher levels of activity. In an initial study, the transfection efficiency of SUV liposome formulations consisting of five different cationic lipids (i.e. DDA, DOTAP, DOTMA, DPTAP and DC-Chol) in combination with DOPE (at a 1:1 molar ratio) was preformed. All cationic lipids tested differ in their chemical structures with respect to their polar head group, hydrophobic tail and interlinking region of the lipid molecule. Therefore, their ability to transfect cells is likely to vary as a result of differences in their interactions with the helper lipid and DNA and potentially due to the structural features of the resultant cationic lipid-DNA complexes. As a screening process, five cationic lipids were chosen, including DOTAP, which has previously been shown within other studies to generate high levels of DNA transfection *in vitro* (Zuidam *et al.*, 1999; Regelin *et al.*, 2000). DPTAP, which is an analogue of DOTAP, was also tested to investigate whether substituting DOTAP with its derivative DPTAP could enhance transfection rates. The cationic lipid DDA has previously been reported as a good immunomodulator for eliciting both cell-mediated and humoural immunity *in vivo*, when used in combination with various protein antigens (Hilgers and Snippe, 1992; Brandt *et al.*, 2000; Holten-Andersen *et al.*, 2004; Davidsen *et al.*, 2005). Therefore the ability of DDA to promote effective DNA transfection *in vitro* was investigated.

As shown in Figure 4.1, the transfection efficiency of liposome-DNA complexes were in the order of DC-Chol > DPTAP > DDA ≈ DOTMA ≈ DOTAP, with efficiency of DC-Chol being around 6 times higher than those complexes composed of DOTAP, DOTMA or DDA. One of the reasons to which this may be attributed is the toxicity of these cationic lipids (Figure 4.2), such as DOTAP and especially DDA, which has been effectively used as an immuno-stimulatory agent (Anderson, 1994; Wedlock *et al.*, 2002; Cai *et al.*, 2005). Within their structures, DOTAP, DOTMA and DDA all contain quaternary amines that tend to inhibit protein kinase C (PKC) required for the endocytosis of the cell, which inversely correlates with efficacy (Farhood et al, 1992). Previous studies have shown there is an association between the inhibition of PKC activity by cationic lipids with respect to cellular

toxicity and impaired transfection efficiency (Li *et al.*, 1996). On the other hand, DC-Chol contains a tertiary amine group signifying that this cationic lipid has no PKC inhibitory activity suggesting an endosomal pathway for the entry of the complexes into the cells and consequently efficient transfection of the incorporated plasmid DNA.

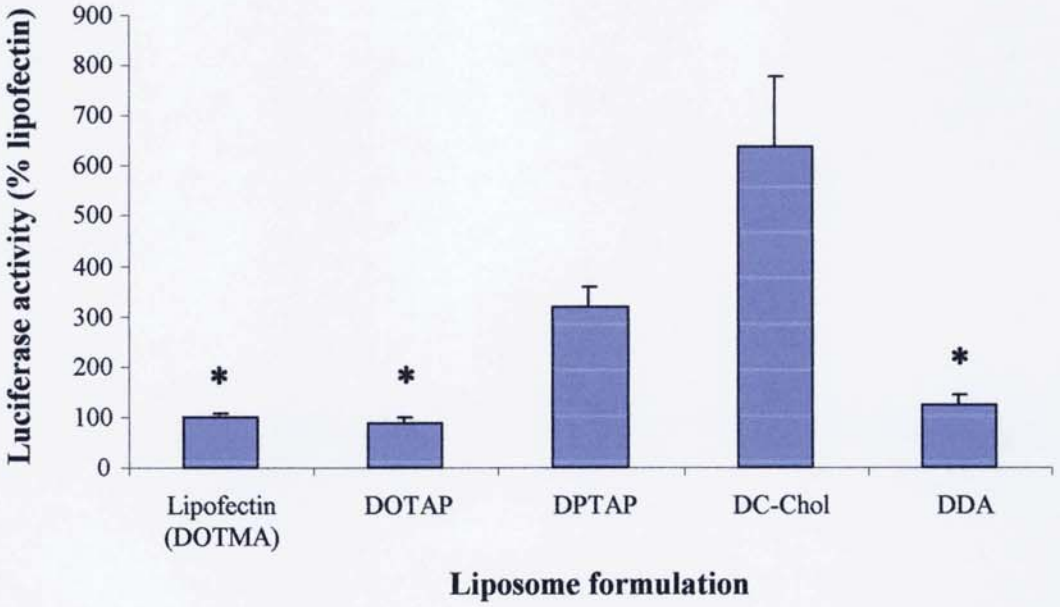


Figure 4.1 Transfection efficiency of SUV-DNA complexes composed of various cationic lipids in equimolar ratio with the helper lipid DOPE. Liposomes composed of 0.78:0.78μmole/ml DOPE:cationic lipid were complexed with gWiz plasmid DNA expressing firefly luciferase. 0.0078μmole of total lipid containing 1μg DNA was added to each well containing COS-7 cells. * signifies that the cationic lipid DC-Chol generates significantly ($P<0.05$, ANOVA) higher levels of activity than these cationic lipids (i.e. DOTAP, DDA and DOTMA). Results denote mean ± SD, $n = 3$.

Additionally, the variation in transfection efficiency between the different cationic lipids could be attributed to the manner in which the complexes form. By employing the dextran step gradient method, Eastman *et al.* (1997) found that even though the different cationic lipids all form complexes by charge neutralisation, the definitive characteristics of the resultant complex varies between the different cationic lipids, with variation in size and densities. The extent in which the cationic liposomes interact and bind with the plasmid DNA is also likely to have a large effect on they way in which the DNA dissociates from the complex. Studies have shown (Zuidam and Barenhloz, 1997) that DC-Chol vesicles are only moderately charged which as a consequence may make it easier for the DNA to dissociate from the complex without any great force. However, for quaternary amines such as DOTAP

or DOTMA, these vesicles are completely charged meaning DNA release is likely to require more energy and force which may provide an additional explanation of the lower transfection efficiency values of complexes containing cationic lipids such as DOTAP or DOTMA (Figure 4.1). This may also apply to the quaternary amine, DDA.

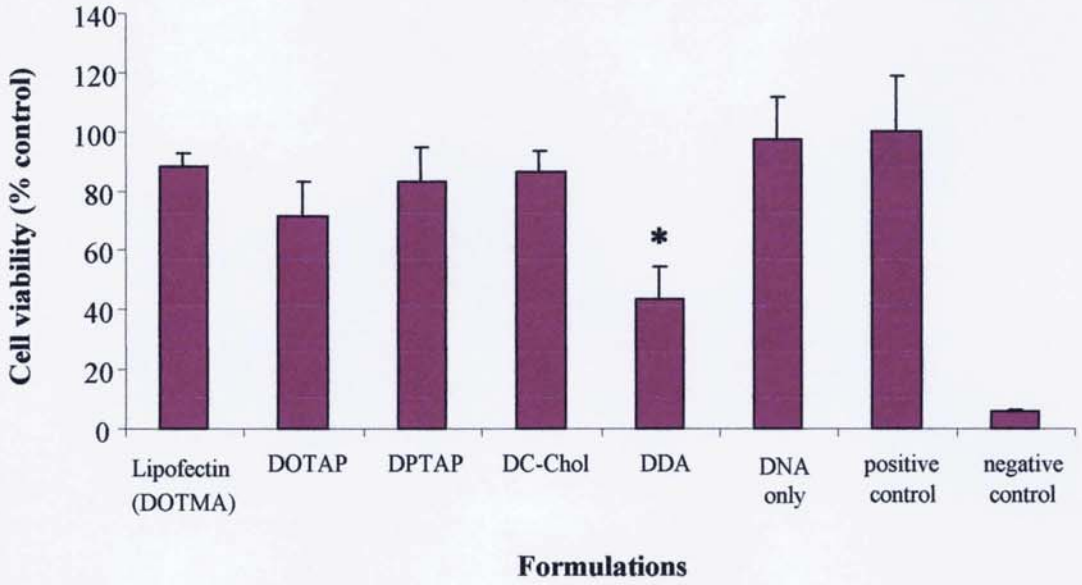


Figure 4.2 Cytotoxicity of various cationic lipids to COS-7 cells. The level of cytotoxicity was measured by the CellTitre 96® AQueous One solution cell proliferation assay (Promega). This is a colorimetric method for determining the number of viable cells in proliferation, which is measured by the amount of 490nm absorbance and this is directly proportional to the number of living cells in culture. Results are expressed as a percentage of positive control (cells and medium only). * signifies that the cationic lipid DDA is significantly ($P<0.05$, ANOVA) more toxic than all other formulations tested. Results denote mean ± SD, $n = 8$.

4.3 Effect of helper lipid

The importance of including a helper within cationic liposomes is to stabilise these cationic lipid suspensions as the cationic lipids alone will repel each other (Zuidam and Barenholz, 1998) and to additionally increase transfection rates, as studies have found depleted rates of transfection when omitting neutral helper lipids from the liposome formulation (Mui *et al.*, 2000). Previous reports have shown that the helper lipid, DOPE, assists in the release and escape of the complexed DNA from the endosome and thus entry into the cytosol by the 'flip-flop' mechanism. DOPE is reported to be responsible for destabilising the endosomal membrane whereupon, through lateral diffusion of anionic lipids from the cytoplasm-facing

endosomal monolayer, DNA is displaced from the complex and released into the cytosol for eventual episomal transfection (Szoka *et al.*, 1996; Zelphati and Szoka, 1998).

4.3.1 Transfection efficiency of DOPE and Chol containing liposome-DNA complexes and the effect of lipid to DNA charge ratio

At low lipid:DNA charge ratios (0.5:1), DOPE:DC-Chol liposomes exhibit minimal transfection levels (23% of Lipofectin control; Figure 4.3), whereas in comparison, Chol:DC-Chol liposome-DNA complexes formulated at the same lipid:DNA charge ratio gave significantly ($P<0.05$, ANOVA) higher transfection (121% of control; Figure 4.3). As the lipid:DNA ratio increased to 0.75:1, transfection activity increased to 170% and 370% (of control) for the DOPE and Chol liposomes respectively, again showing the enhanced ability of the cholesterol liposomes to promote transfection at these ratios. At these lower charge ratios, Chol as a helper lipid performs significantly ($P<0.05$, ANOVA) better than DOPE. Optimum transfection for both systems was achieved at a DNA charge ratio of 1.5:1, however there was a reversal in terms of efficiency with DOPE containing complexes producing greater levels of activity than Chol (738% vs 497% respectively; Figure 4.3). Increasing the lipid:DNA ratio further to 2:1 resulted in a major reduction of activity for DOPE complexes down to 453% of control while Chol containing complexes plateau at this level. These results demonstrate the importance of both; the type of neutral helper lipid incorporated within the formulation and also the cationic liposome-DNA charge ratio.

In terms of cell toxicity of these liposome formulations, the viability of the cells decreased slightly as the lipid content increased to give a charge ratio of 1, as would be expected (Figure 4.4), as cationic lipids can become toxic to the cells when in high concentrations, therefore by increasing lipid/DNA ratios, cell death increases (Guo and Lee, 2000; Hyvonen *et al.*, 2000). However, with a further increase in lipid content, giving a charge ratio of 1.5 and 2, the cells began to significantly reduce in viability, indicating that with more lipid present the formulation is becoming toxic to the cells, resulting in cell death.

4.3.2 Effect of zeta potential and complex size on transfection efficiency

Both the z-average diameter and zeta potential of DOPE:DC-Chol-DNA and Chol:DC-Chol-DNA complexes were dependent on the lipid:DNA charge ratio (Figures 4.5 and 4.6). As expected, initially at the lower ratios (i.e. 0.5 and 0.75) the zeta potential for both DOPE and

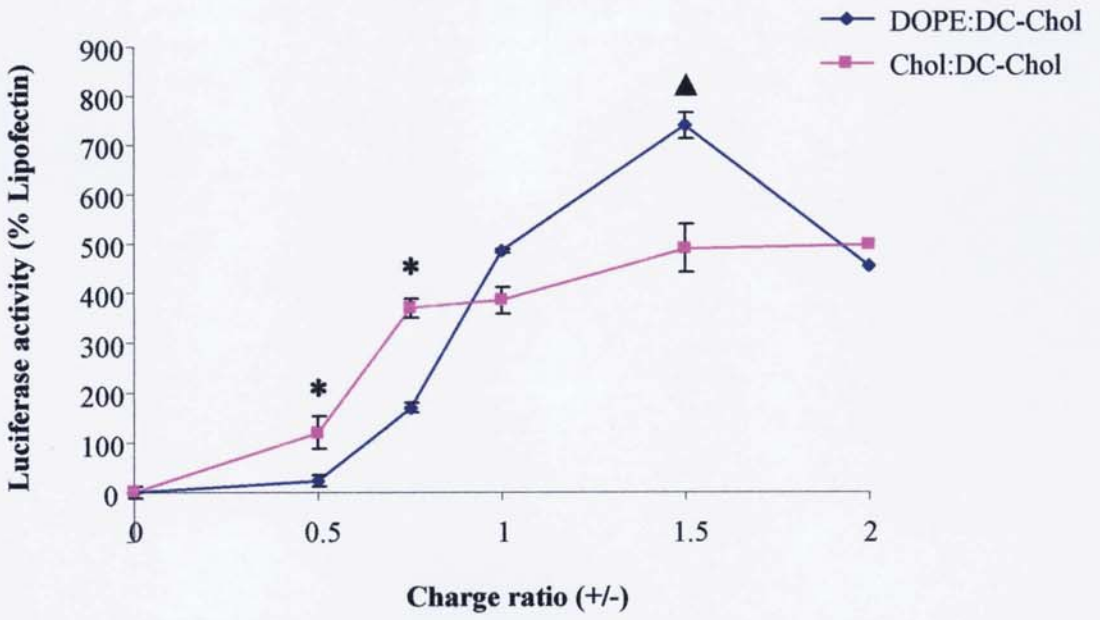


Figure 4.3 Effect of lipid-DNA ratio on transfection efficiency of liposome-DNA complexes. Liposomes composed of either DOPE:DC-Chol or Chol:DC-Chol (at equimolar ratios) formulated with increasing amounts of total lipid were mixed with a fixed amount of gWiz luciferase DNA (1μg/ml, of which was added to COS-7 cells) to give various lipid:DNA charge ratios (for full details see section 2.12.2). * signifies that Chol:DC-Chol complexes at lower charge ratios significantly ($P<0.05$, ANOVA) transfect cells better than DOPE:DC-Chol complexes. ▲ signifies that at a charge ratio of 1.5, DOPE:DC-Chol complexes significantly ($P<0.05$, ANOVA) transfect cells better than Chol:DC-Chol complexes. Results denote mean ± SD, n = 3.

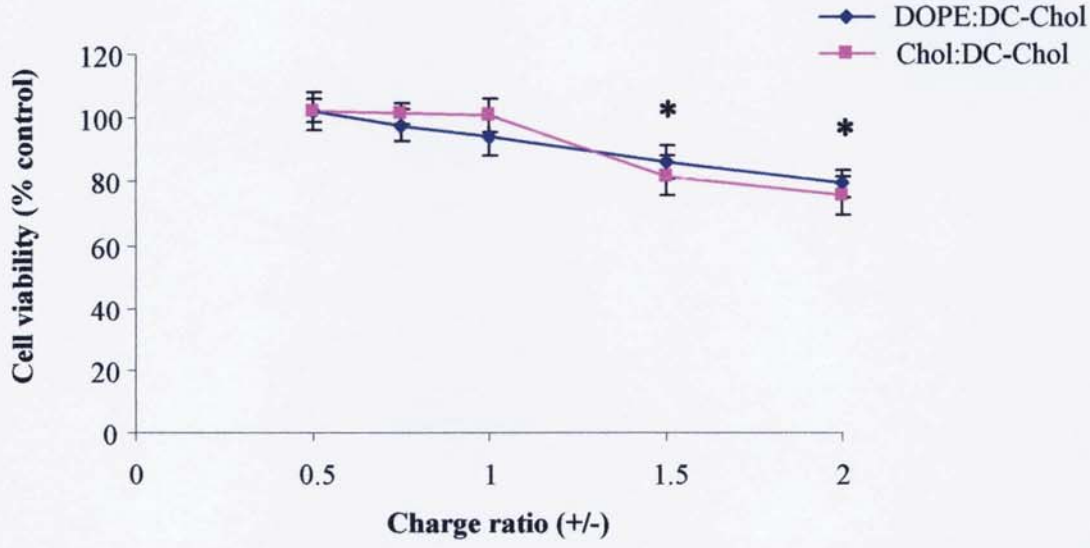


Figure 4.4 Cytotoxicity of liposome-DNA complexes composed of either DOPE:DC-Chol or Chol:DC-Chol (of equimolar ratio) of increasing lipid-DNA charge ratio. The viability of COS-7 cells after the transfection procedure was measured by the CellTitre 96® AQueous One solution cell proliferation assay (Promega). This is a colorimetric method for determining the number of viable cells in proliferation, which is measured by the amount of 490nm absorbance and this is directly proportional to the number of living cells in culture (for full details see section 2.12.2). * signifies that liposome-DNA complexes at a charge ratio of 1.5 and 2 significantly ($P<0.05$, ANOVA) reduce cell viability. Results denote mean ± SD, n = 8.

Chol formulations were highly negative due to the presence of more DNA then lipid, with no significant differences found between the two formulations at these ratios. However, when the lipid:DNA charge ratio increased further (i.e. 1, 1.5 and 2) the measured zeta potential of the two formulations significantly differ ($P<0.05$, ANOVA) revealing the contrast between these two helper lipids in the way in which their complexes interact with the DNA (Figures 4.5 and 4.6). Take DOPE for example, at charge ratios of 1.5 and 2, where there is more cationic lipid, the negative charges exhibit a gradual decrease towards neutrality and progressively become more positive (-10 mV and 13 mV, respectively; Figure 4.5), showing that these complexes progressively surround the DNA with the cationic lipid masking the negative charges. When more cationic lipid is added, at a charge ratio of 2, DOPE-containing complexes completely enclose and fuse around the anionic DNA, generating an overall positive complex (Figure 4.5). This can be attributed to the electrostatic interactions that occur during the complexation process, when cationic liposomes interact and bind onto the anionic surface of the plasmid DNA, structures previously described as being like beads on a string develop (Gershon *et al.*, 1993). At the low lipid:DNA charge ratios (0.5 and 0.75) where there is an excess of DNA to cationic lipid, high negative zeta potentials were measured (Figures 4.5), indicating that there is considerable amount of non-condensed and exposed DNA. However, as the lipid to DNA ratio increases, there are likely to be more liposomal vesicles present to bind to and surround more of the DNA strands and consequently, with the enhanced amount of cationic charges present, the anionic charges of the DNA will be neutralised resulting in a less negative or neutral zeta potential (Figures 4.5). In the case of Chol-containing complexes, the surface charge does not reach neutrality, even at a charge ratio of 2 (Figure 4.6), relating to the way in which these complexes form, which will be discussed in greater detail within section 4.3.3.

As confirmed by gel electrophoresis (Figures 4.7a and b), the amount of non-condensed and free DNA decreases as the lipid to DNA ratio increases, demonstrating that the complexation efficiency is dependent on the lipid to DNA ratio. Complexes exhibiting a negative zeta potential at the lower ratios contain a large amount of free DNA. However, as the zeta potential reaches neutral, most of the DNA is retained at the site of application along with lipid, indicating that the majority of the plasmid DNA is complexed and bound to the cationic charges of the liposomes (Perrie and Gregoriadis, 2000). To further confirm the presence of free DNA, gel electrophoresis was carried out within 40% sucrose, to ensure that

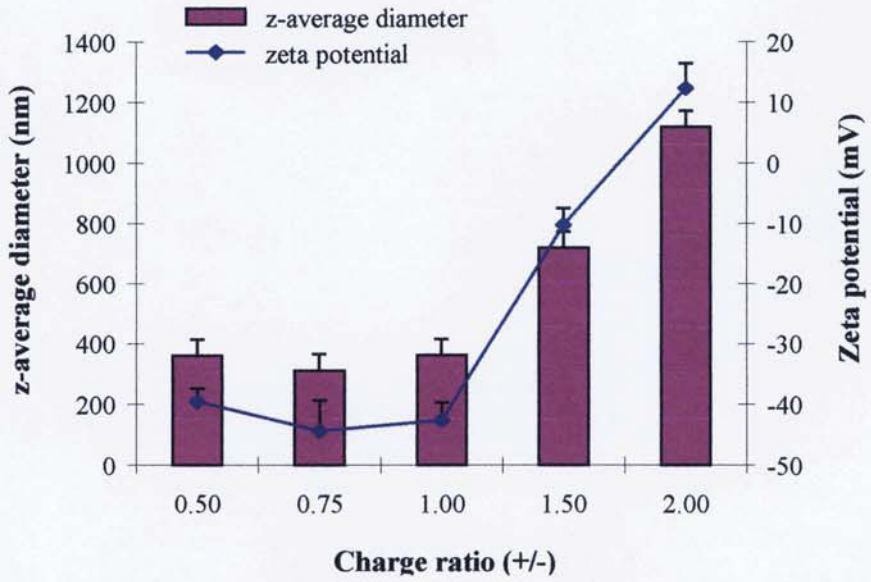


Figure 4.5 Effect of increasing the lipid-DNA charge ratio of DOPE:DC-Chol liposome-DNA complexes on z-average diameter and zeta potential. Size and zeta potential of each empty SUV and SUV-DNA complexes was measured on a ZetaPlus (Brookhaven Instruments), in ddH_2O and 0.001 M PBS, respectively, Results denote mean ± SD, n = 3.

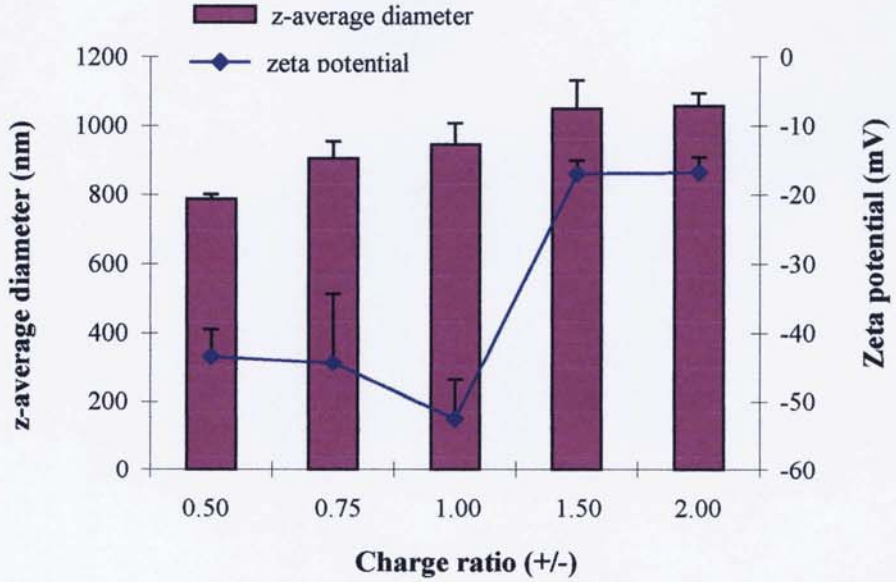


Figure 4.6 Effect of increasing the lipid-DNA charge ratio of Chol:DC-Chol liposome-DNA complexes on z-average diameter and zeta potential. Size and zeta potential of each empty SUV and SUV-DNA complexes was measured on a ZetaPlus (Brookhaven Instruments), in ddH_2O and 0.001 M PBS, respectively, Results denote mean ± SD, n = 3.

DNA displacement is not a consequence of the anionic components present within the loading buffer, electrostatically competing with the DNA. Once again upon gel electrophoresis, in the absence of anionic components, the level of DNA release decreases as lipid content increases (Figures 4.7c and d) with the level of DNA released being similar in the presence and absence of anionic components (Figures 4.7a vs c and 4.7b vs d for DOPE and Chol systems, respectively).

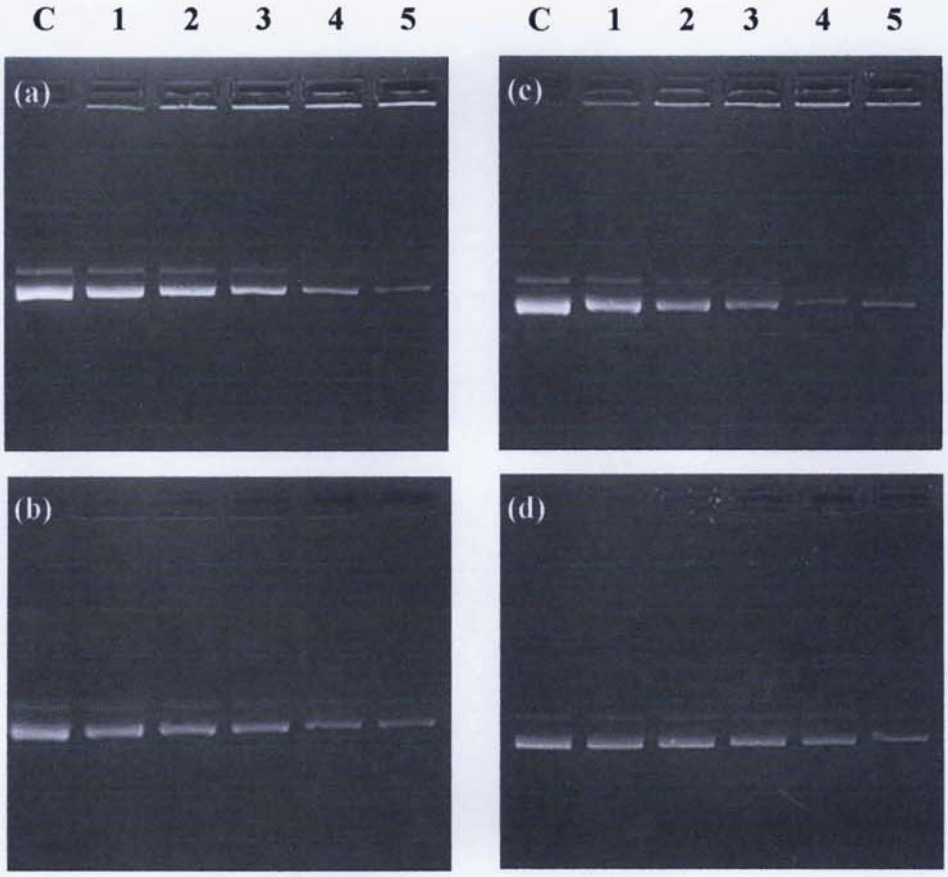


Figure 4.7 Agarose gel electrophoresis of liposome-DNA complexes composed of: DOPE:DC-Chol (a and c) and Chol:DC-Chol (b and d) at lipid-DNA charge ratios of 0.5 (lane 1); 0.75 (lane 2); 1.0 (lane 3); 1.5 (lane 4); 2.0 (lane 5). Lane C, naked DNA. (c) As in (a) but in the absence of anionic competition. (d) As in (b) but in the absence of anionic competition.

If we take the sizes of the liposome-DNA complexes into consideration, there is a much more interesting relationship with transfection efficiency. As previously reported (Birchall *et al.*, 1999) upon addition of DNA, small cationic liposomes undergo dramatic changes in configuration whereby the oppositely charged DNA causes the liposomes to aggregate and fuse, eventually forming larger structures (e.g. DOPE-containing liposomes at a charge ratio of 1.5 produce empty SUV with sizes of 76 nm vs complexes with sizes of 756 nm). As

shown in Figure 4.5, the z-average diameter of DOPE containing complexes plateaus until a charge ratio of 1 is reached and subsequently increases gradually as the lipid:DNA ratio increases further, with these results corresponding to the surface charge of the complexes. Size changes of the cationic liposomes are a result of fusion between individual small liposomes induced by plasmid DNA (Almofti *et al.*, 2003). As more cationic lipid is added, fusion increases, with the liposomes eventually fusing around and surrounding the DNA, causing neutralisation of the DNA's negative charges, at the higher lipid:DNA charge ratios. For DOPE containing complexes the increase in size, at a charge ratio of 1.5, correlates with the decrease in the negative surface charge of the vesicles (Figure 4.5). As zeta potential becomes less negative and nearer to neutral, the liposome-DNA complexes are gradually reaching what is termed the isoelectric point, where there is a balance between the total lipid and total DNA charge. As the complexes begin to reach this point, the van der Waals' attractions are able to overcome existing weak electrostatic repulsions between vesicles (Safinya *et al.*, 1998; Pedroso de Lima *et al.*, 2001) causing them to interact and aggregate into larger structures surrounding the plasmid DNA.

In the case of Chol complexes, the trend in surface charge and size is less pronounced, as even at the lower ratios, the liposome-DNA complexes are significantly larger in size when compared to DOPE complexes and exhibit a slower rate of increase in size as lipid:DNA ratio is increased. For Chol containing complexes, an interesting observation is the correlation between the size (Figure 4.6) and transfection efficiency (Figure 4.3) as these results run in parallel. Additionally, DOPE:DC-Chol-DNA complexes exhibited enhanced activity as complex size increased. Indeed, at the low charge ratios (i.e. 0.5 and 0.75), where Chol:DC-Chol-DNA complexes were larger in size (between 750 nm and 900 nm; Figure 4.6) revealed greater levels of activity than the smaller DOPE:DC-Chol-DNA complexes (between 300 nm and 350 nm; Figure 4.5). Optimum transfection of DOPE containing complexes was achieved when complex size reached sizes around 700-800 nm. In this instance vesicle size appears to play a more predominant role in transfection efficiency and is an important parameter to consider when formulating a successful liposomal gene delivery system. In agreement with previous reports (Felgner et al, 1995; Kawaura *et al.*, 1998), liposome-DNA complexes with diameters ranging from 700-1000 nm produced elevated levels of activity when compared to smaller complexes of 300-350 nm. Larger complexes are more likely to be denser than smaller complexes, which may favour their sedimentation

over the cell surface *in vitro*, as a consequence of gravity, promoting liposome-DNA complex cell interactions, increasing endosomal entry into the cytoplasm and delivery of plasmid DNA. Faneca *et al.* (2002) also concluded that large complexes contain an abundance of DNA copies, thus delivering an ample amount of DNA to the cell and as previously reported, in order for complexes to transfect cells efficiently they need to incorporate and carry a sufficient amount of DNA to the cell surface.

Nevertheless, in addition to this, upon reaching the cell the complex should possess certain properties in order to mediate entry of the DNA into the cytoplasm. It is at this stage in which the helper lipid may play an influential role in mediating successful transfection. By incorporation of DOPE into the bilayer of the cationic lipid delivery system, transfection activity is enhanced, producing the highest level of activity overall at a 1.5:1 ratio. This is in agreement with previous studies, although with a different cationic lipid (Almofti *et al.*, 2003). DOPE is the common helper lipid used for *in vitro* transfection of complexes due to its high non-bilayer forming activity and ability to enter the inverted H_{II} hexagonal phase after endocytosis within acidic conditions, as a result of its inverted cone-shaped structure. Liposome-DNA complexes enriched with DOPE promote membrane fusion thus facilitating the destabilisation of the endosomal membrane and escape of plasmid DNA into the cytosol (Farhood *et al.*, 1995). Studies have shown that this fusion process is facilitated by the phospholipid PE present within DOPE, as replacement with DOPC, which shows to have no destabilising effect on endosomes (Farhood *et al.*, 1995) dramatically reduces transfection efficiency *in vitro* (Legendre and Szoka, 1993). The mechanism for intracellular entry of liposome-DNA complexes is still not fully understood, as initial studies reported that complexes fused with the plasma membrane enabling the delivery of DNA into the cytoplasm (Almofti *et al.*, 2003), whereas other studies suggest that another possible mechanism of entry is by endocytosis (Farhood *et al.*, 1995). These fusogenic properties are absent in Chol:DC-Chol-DNA complexes and although activity is still relatively high, overall its optimum level of activity is significantly lower than DOPE:DC-Chol-DNA complexes at the same optimum charge ratio of 1.5 (Figure 4.3). This highlights the benefit of the destabilising effect of DOPE containing complexes and emphasizes its ability to facilitate entry of plasmid DNA into the cytosol.

At the higher charge ratio of 2, transfection activity either reduces or plateaus for DOPE and Chol respectively (Figure 4.3). At this ratio, the zeta potential for both helper lipids reaches near neutral or becomes positive (13 mV for DOPE and –17 mV for Chol; Figures 4.5 and 4.6), demonstrating that the plasmid DNA, within DOPE complexes, has been completely condensed and surrounded by the cationic liposomes. Therefore, the reduction in activity for DOPE may be due to the high intensity of DNA condensation, where the DNA is very tightly packed and bound to the cationic charges of the liposomes, rendering it difficult for dissociation. Consequently this prevents unloading of the DNA from the complex, reducing efficient DNA delivery to the cytosol (Faneca *et al.*, 2002). Additionally, this reduction in activity could also be a consequence of high instability as a result of complex size (Zuidam and Barenholz, 1998).

4.3.3 Structural analysis of DOPE vs. Chol liposome-DNA complexes by TEM.

DOPE containing complexes that produced the highest transfection activity are enriched with heterogeneous particles, as shown by TEM (Figures 4.8a), demonstrating the presence of both aggregated liposomes (Figure 4.8b) and fused liposomes (Figure 4.8c). However, although this may seem problematic, Zabner (1997) found that transfection levels were highest when the liposome-DNA complex preparations contained heterogeneous structures compared to small homogenous particles. Although, this does make it difficult to identify between the 'active' and 'non-active' structures, however this heterogeneity may indicate that a variety of complex structures is required for successful transfection, therefore this needs further investigation.

Empty liposomes composed of DOPE:DC-Chol are small homologous population of vesicles (Figure 4.8a). As shown by TEM images of DOPE:DC-Chol-DNA complexes (1.5:1 ratio) (Figures 4.8b-c), the two morphological structures that were found could be described as the 'meat ball' and 'spaghetti-like' structures reported previously using freeze-fracture electron microscopy (Sternberg *et al.*, 1994). At a high cationic lipid:DNA charge ratio (1.5), cationic liposome aggregation occurs possibly surrounding the anionic DNA, forming a 'meat ball' structure (Figure 4.8b), which is further substantiated by zeta potential results showing relatively higher values (Figure 4.5). Another morphology of DOPE:DC-Chol-DNA complexes revealed 'spaghetti-like' structures (Figure 4.8c) formed by the adherence of cationic liposomes along the DNA strand. As the cationic lipid:DNA ratio increases, as

previously demonstrated by Gershon *et al.* (1993) with DOPE:DOTMA complexes, the cationic liposomes completely fuse around the DNA, causing the DNA to become entrapped within a hexagonal lipid bilayer. Gershon *et al.* (1993) concluded that this observed liposome fusion is induced by the DNA, causing the DNA to collapse, condense and become entrapped forming rod-like structures. This morphology could facilitate the transfection of DNA more efficiently into the cell membrane promoting an intimate contact with electrostatic attractive forces acting between the complex and the negatively charged cell membrane and in addition to expanded morphology of the liposome-DNA complex possessing a greater surface area allowing it to fuse with the cell membrane much more efficiently than the 'meat ball' structure.

As shown in Figures 4.8d-f, at a low lipid:DNA ratio (0.5:1), the cationic liposomes begin to aggregate around and along the DNA strand, showing initial signs of fusion between individual liposomes (Figure 4.8d), with the DNA behaving as a fusogenic agent and neutralising the repulsive forces acting between the liposomes (Li *et al.*, 1996). However, at this ratio (0.5:1) the preparation showed relatively small homogeneous particles (Figure 4.8e), due to the low amount of lipid coated with DNA, thus preventing aggregation (Eastman *et al.*, 1997), with a large amount of excess, free DNA within solution. This presumably explains the lower levels of transfection efficiency determined at this charge ratio (Figure 4.3).

On the other hand, one of the reasons for low transfection of Chol:DC-Chol-DNA complexes at a similar charge ratio as that of its DOPE counter part, could possibly be explained by the non-fusogenic properties of the helper lipid Chol as seen in the Figures 4.9a-c. Chol complexes do not exhibit the fusogenic properties as DOPE, instead the inclusion of Chol rigidifies the lipid bilayer and increases the stability of liposomal vesicles (Gregoriadis & Davis, 1979; Gregoriadis, 1993). Empty liposomes are visualised as solid spherical structures (Figure 4.9a). Upon addition of DNA to these complexes, it appears that these solid liposomes aggregate around the DNA, but do not show any signs of liposome-liposome fusion and therefore these complexes are unlikely to structurally enclose the DNA (Figure 4.9b and c) (Birchall et al, 1999). Considering this, complexes containing Chol as the helper lipid may bind along and surround the DNA strand eventually forming large aggregates, which coincides with the size reported earlier (Figure 4.6). Chol:DC-Chol-DNA complexes also revealed several morphologies (Figures 4.9b and c) resembling the structures for

DOPE:DC-Chol-DNA complexes (i.e. 'meatball' and 'spaghetti-like' structures) with sizes matching those measured by photon correlation spectroscopy (Figure 4.6).

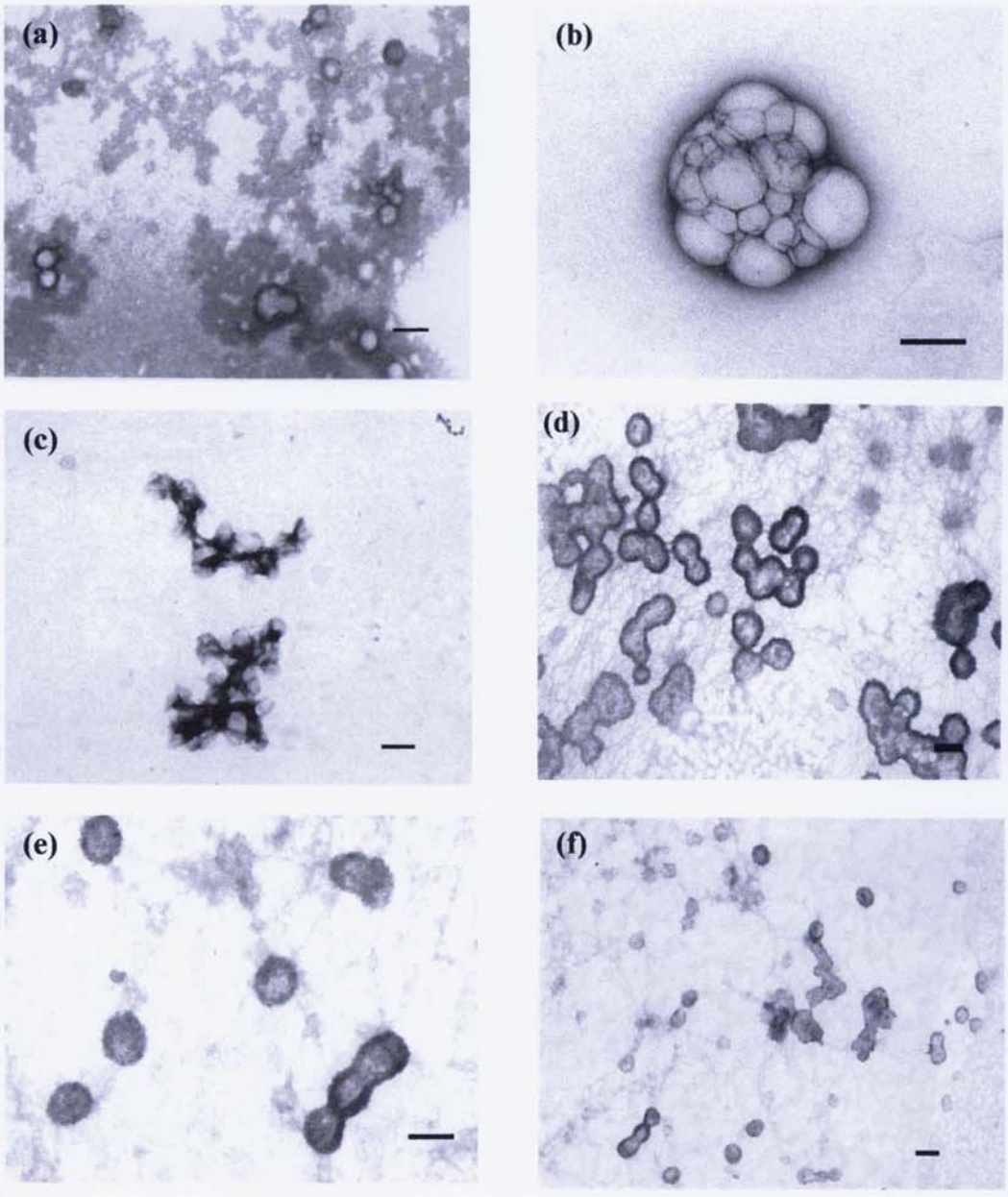


Figure 4.8 Transmission electron microscopy (TEM) of DOPE:DC-Chol liposomes. Empty liposomes (a, bar = 200 nm) and liposome-DNA complexes composed of DOPE:DC-Chol at a charge ratio of 1.5 (b, bar = 100 nm and c, bar = 500 nm) and 0.5 (d and e and f, bar = 100 nm). 1μg gWiz plasmid DNA was added to each liposome formulation (0.0078μmole). Samples were stained with 2% uranyl acetate and viewed with a JEOL 1200EX TEM.

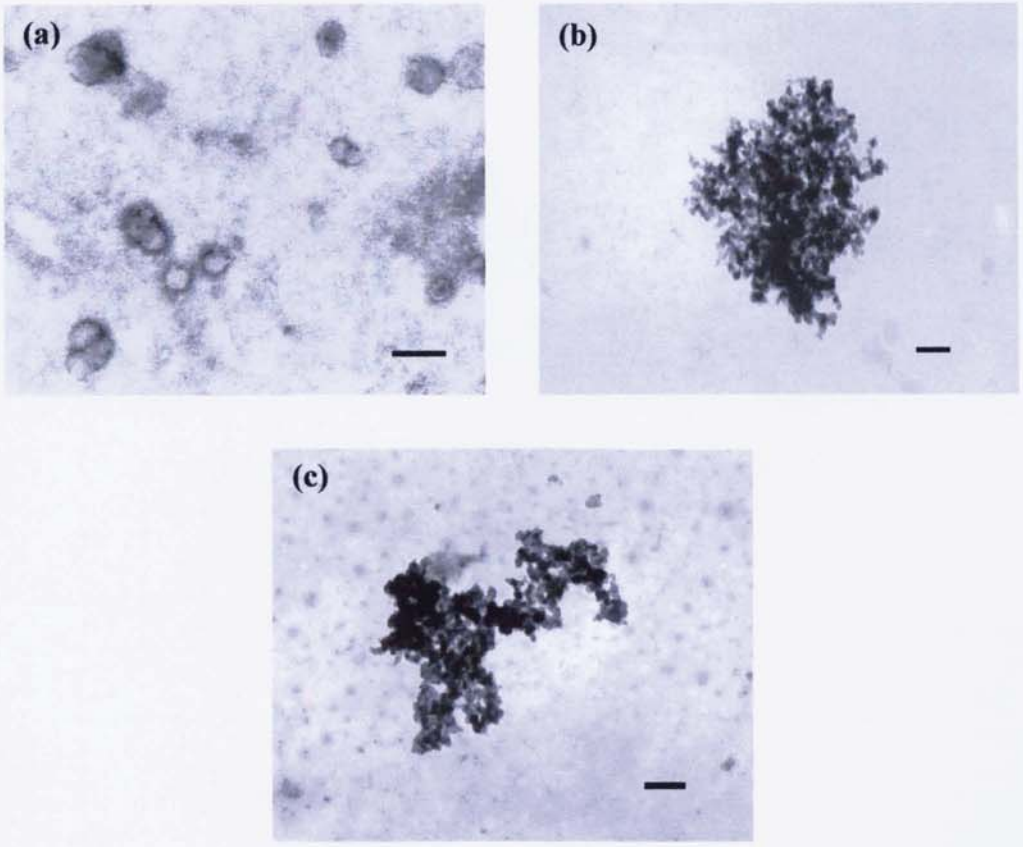


Figure 4.9 Transmission electron microscopy (TEM) of Chol:DC-Chol liposomes. Empty liposomes (a, bar = 150 nm) and liposome-DNA complexes composed of Chol:DC-Chol at a charge ratio of 1.5 (b and c, bar = 200 nm). 1μg gWiz plasmid DNA was added to each liposome formulation (0.0078μmole). Samples were stained with 2% uranyl acetate and viewed with a JEOL 1200EX TEM.

In addition to the TEM micrographs, environmental scanning electroscopy was used to observe the morphology of DOPE:DC-Chol liposome-DNA complexes. The advantage of using ESEM is the ability to observe liposome structures in real time within wet conditions and without prior sample preparation. Other microscopy techniques require prior sample preparation, such as SEM where the sample needs to be dried beforehand, therefore reducing the sample quality and damaging the vesicles characteristics. ESEM has previously been utilised for various other nanoparticle systems, including; polymeric surfactant micelles (Cao and Li, 2002) and dendrimers (Sui *et al.*, 2000).

Figure 4.10a and b show ESEM micrographs of liposome-DNA complexes composed of DOPE:DC-Chol (1:1 molar ratio, at a charge ratio of 1.5:1). The ESEM micrographs reveal extremely large liposome structures, which may result from the instability of this liposome formulation. As the aqueous environment within the chamber evaporates, these liposomes

may fuse together to generate larger liposome structures. Once again, 'meatball' structures were visible within the sample, however, at present, the nature of the small, dark spherical structures within the vesicles (Figure 4.10a) and the discrepancies in size between vesicles measured in the two techniques is uncertain and therefore further investigation is required. Within these ESEM micrographs there is an indication of fusion of liposomes leading to formation of a 'lipid coat' surrounding the plasmid DNA strand, possibly with inverted hexagonal lipid orientation around the DNA (Figure 4.10b). The liposomes fuse together and form a rod-like structure (Gershon *et al.*, 1993) that consists of a smooth lipid surface. The shape of these complexes shown in Figures 4.10a and b, are similar to those shown in the TEM micrograph (Figure 4.8b and c), although ESEM provides a 3D overview of the liposome-DNA complex structure.

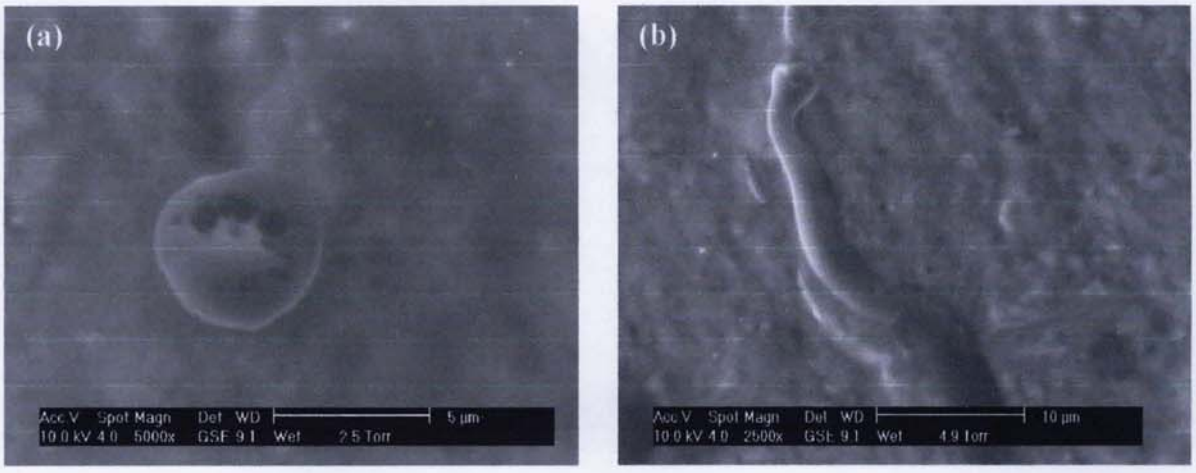


Figure 4.10 Environmental Scanning Electron Micrograph (ESEM) of liposome-DNA complexes composed of DOPE:DC-Chol (1:1 molar ratio, at a charge ratio of 1.5:1).

4.4 Helper activity

It is regarded that levels of transfection efficiency of liposome-DNA complexes are greatest when the complex is composed of a neutral helper lipid and a cationic lipid (Spack and Sorgi, 2001; Hirsh-Lerner *et al.*, 2005). As shown in section 4.3 of this chapter, at certain cationic lipid:DNA charge ratios DOPE, in combination with the cationic DC-Chol (at a 1:1 molar ratio) was extremely efficient at transfecting COS-7 cells *in vitro*, compared to other systems tested. As discussed previously, this is attributed to the fusogenic properties of this non-bilayer forming lipid (Farhood *et al.*, 1995), therefore, by increasing the amount of

DOPE within the formulation could this enhance the destabilising effect of this fusogenic lipid and accordingly increase transfection efficiency further?

Liposome-DNA complexes composed of DOPE:DC-Chol were formulated at either 1:1 or 2:1 helper lipid to cationic lipid molar ratios and were then tested for their ability to transfect COS-7 cells. Additionally, a further formulation also composed of DOPE:DC-Chol at a 2:1 molar ratio was made, however, total lipid concentration was increased considerably to 60 nmole/ml, as further studies detailed in chapter 5 utilize liposomes at this high lipid concentration.

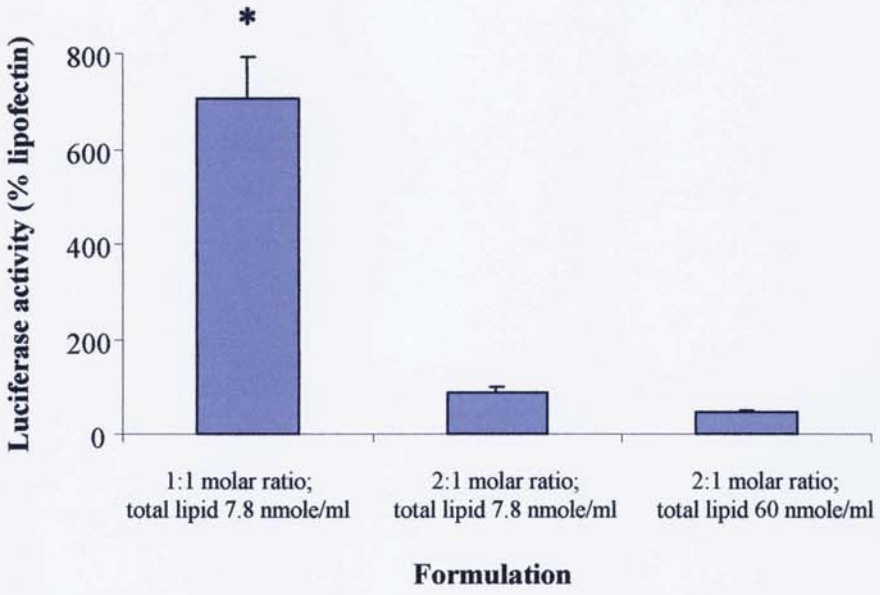


Figure 4.11 Effect total lipid concentration and helper lipid to cationic lipid molar ratio on transfection efficiency of COS-7 cells. SUV composed of DOPE:DC-Chol were formulated at different molar ratios and lipid concentrations. 1μg/ml of plasmid DNA (gWiz luc) was mixed with each formulation and than subsequently incubated with to COS-7 cells for 5 hours (for full details see section 2.12.2). Transfection activity was determined by luciferase assay, with results expressed as percentage of control (i.e. lipofectin transfection activity). * signifies DOPE:DC-Chol complexes at a 1:1 molar ratio and a total lipid concentration of 7.8 nmole/ml produced significantly higher ($P<0.05$, ANOVA) values of luciferase activity than DOPE:DC-Chol formulations at a 2:1 molar ratio (at 7.8 nmole/ml and 60 nmole/ml). Results denote mean ± SD, $n = 3$.

Figure 4.11 shows that an increase in the molar ratio of DOPE to DC-Chol significantly reduces transfection efficiency (from 708% for 1:1 molar ratio to 86% for 2:1 molar; Figure 4.11), demonstrating that an equal combination of neutral lipid and cationic lipid is required to determine optimum levels of efficiency. Therefore, the helper activity once seen with DOPE containing complexes is reduced, which could be related to the high instability of

these liposomes due to their high DOPE content. Although DOPE is known to enhance transfection efficiency by exerting a strong tendency to revert to an inverted hexagonal phase thereby facilitating endosomal disruption and release of DNA (Wimley & Thompson, 1991; Bailey & Cullis, 1997), it seems efficiency is dependent on the cationic lipid:DOPE mole ratio in which a mole ratio of 1 seems to be the optimum (Zuidam & Barenholz, 1998). Transfection efficiency was not affected by lipid concentration, as increasing the total lipid concentration of the formulation from 7.8 nmole/ml to 60 nmole/ml did not alter activity (Figure 4.11). However, despite this, the number of viable cells significantly ($P<0.05$) reduced to 71% when the total lipid concentration was increased to 60 nmole/ml (Figure 4.12), once again demonstrating that the higher lipid content becomes toxic to the cultured cells.

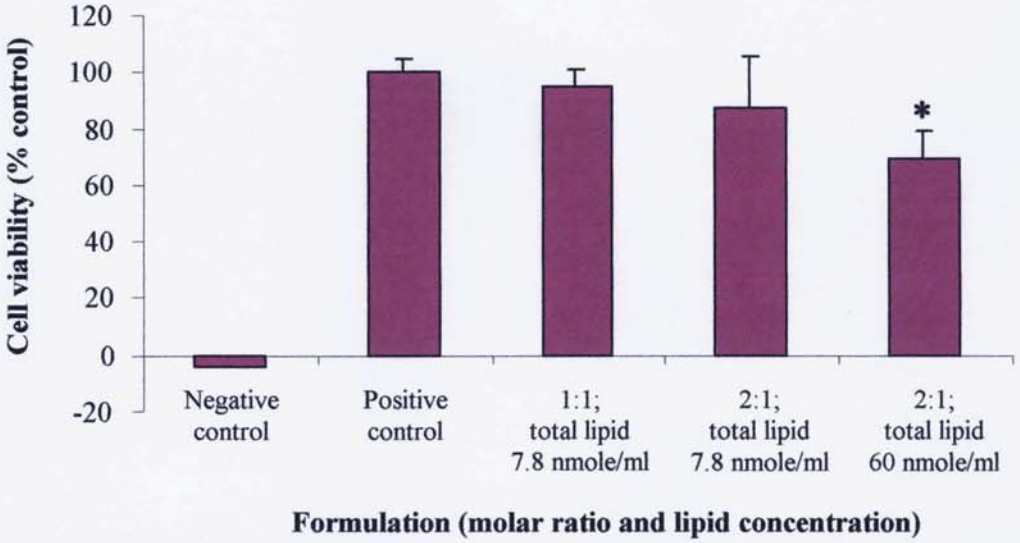


Figure 4.12 Effect of neutral lipid to cationic lipid molar ratio and total lipid concentration on cell viability. The viability of COS-7 cells after the transfection procedure was measured by the CellTitre 96® AQueous One solution cell proliferation assay (Promega). This is a colorimetric method for determining the number of viable cells in proliferation, which is measured by the amount of 490nm absorbance and this is directly proportional to the number of living cells in culture (for full details see section 2.12.2). Results are expressed as a percentage of positive control (cells and medium only). * signifies DOPE:DC-Chol complexes at a 1:1 molar ratio and a total lipid concentration of 60 nmole/ml significantly ($P<0.05$, ANOVA) reduce cell viability compared to positive control. Results denote mean ± SD, $n = 8$.

4.5 Inclusion of phosphotidylcholine

In some instances cationic liposomal formulations consist of a parental phospholipid, such as phosphotidylcholine (PC) in order to stabilise and rigidify the lipid bilayer (Gregoriadis, 1990). PC is a naturally occurring phospholipid that is a major constituent of cell membranes and has surfaced as the main phospholipid component, in respect to the pharmaceutical

application of liposomes, due to its non-toxic biodegradable profile (Storm and Crommelin, 1998).

Cationic liposome-DNA complexes were formulated where PC was either excluded or included within DOPE-containing liposomes or Chol-containing liposomes and in a further formulation with liposomes composed of both DOPE and Chol, at varying molar ratios. The transfection efficiency of these various formulations was measured (Figure 4.13), as well as liposome-DNA complex physiochemical characteristics, including size and zeta potential (Table 4.1) and morphology by TEM (Figures 4.17 and 4.18).

Previously, in section 4.3, it was demonstrated that liposomes composed of DOPE:DC-Chol, at an equal molar ratio, exhibited high levels of transfection efficiency. Conversely, when PC is incorporated within the vesicle bilayer to improve liposome stability, interestingly there is a significant reduction in DNA transfection to 123% (of lipofectin control; Figure 4.13). Previous reports, by X-ray studies, have revealed the existence of two distinct lipid arrangements within liposome-DNA complexes; lamellar ('sandwich'; L_α) and hexagonal ('honeycomb'; H_{II}) (Lasic *et al.*, 1997; Radler *et al.*, 1997; Salditt *et al.*, 1997; Dias *et al.*, 2002). The type of lipid phase and structure that forms is dependent on the shape of the lipid molecules, which in turn determines the natural curvature of the lipid membrane (Safinya, 2001). Therefore, liposomal structure is subject to the packing order of lipids, indicating that lipid orientation is highly dependent on the lipid composition of liposomes and their subsequent interactions with the plasmid DNA strand. When the helper lipid DOPE is included within the liposome formulation, due to its relatively small head group and two large bulky alkyl chains, liposome bilayers enriched with DOPE prefer the packing geometry of the inverted hexagonal phase (H_{II}) (May & Ben-Shaul, 1997; May *et al.*, 2000). In this instance, transfection activity increases due to the instability of this lipid and vesicle structure enabling complexes enriched with DOPE to fuse with cell membranes (Litzinger and Huang, 1992; Koltover *et al.*, 1998) and to destabilise endosomal membranes (Zhou and Huang, 1994; Farhood *et al.*, 1995). However, conversely with the inclusion of PC it seems that the formation of L_α is favoured, where the lipid layers remain in a planer and lamellar configuration with the formation of stable lipid bilayers, where the plasmid DNA is densely packed between these lipid bilayers (Templeton *et al.*, 1997; Battersby *et al.*, 2002). Due to its stable bilayers, these liposome-DNA complexes with an L_α configuration will not

effectively fuse with cell membranes or if cell entry is gained, these stable complexes will remain stable once inside cells (Safinya, 2001) and therefore with ineffective entry into the cytosol of the cell or inefficient DNA release, gene delivery and expression is relatively low (Tros de Ilarduya and Düzgünes, 2000), as confirmed by results shown in Figure 4.13. Indeed, previous studies (Pires *et al.*, 1999) have shown that while liposomes composed of PC in combination with the cationic lipid, DOTAP, do not inhibit cell binding, the ability of these cationic liposomes to fuse with cells is totally abolished, therefore preventing DNA entry and subsequent gene expression.

However, it should be stressed that lipid composition of liposome-DNA complexes is likely to be very different from free liposomes due to the structural reorganisation of lipids upon the addition of plasmid DNA (May *et al.*, 2000). Transfection efficiency of PC:DC-Chol complexes is enhanced with the addition of DOPE, which could be attributed to numerous factors; firstly, addition of DOPE into the lipid bilayer could cause a slight destabilisation of the lipid bilayer, causing the lipid membranes to 'soften' (May *et al.* 2000) and cause the once stable PC liposomes to possess fusogenic properties within the H_{II} configuration. Depending on the total lipid composition, it seems that within mixed planer bilayers there is an induced structural transition from L_α into H_{II} packing geometry upon the addition of plasmid DNA, due to its strong electrostatic interaction with the cationic lipid (May and Ben-Shaul, 1997; Safinya, 2001). Therefore, even within the presence of PC the H_{II} phase may still dominate within this formulation, with the presence of DOPE controlling the spontaneous curvature of the lipid membranes and inducing the L_α to H_{II} transition (Safinya, 2001). This may provide an explanation of the increase in transfection efficiency when DOPE is added to PC liposomes (1:1:1 molar ratio). However, an efficiency of 123 ± 1% (of lipofectin control) is still approximately 3-4 times lower than for DOPE:DC-Chol (1:1 molar ratio) alone (485%; Figure 4.3), indicating that the inclusion of PC is causing an inhibition of transfection efficiency of these liposome-DNA complexes. Therefore, secondly, the liposome-DNA complex suspension composed of PC:DOPE:DC-Chol (1:1:1 molar ratio) could contain a very heterogeneous population of vesicles with a coexistence of complexes differing in structure and composition, where there is a coexistence of both L_α and H_{II} structures (May *et al.*, 2000). By reducing the DOPE content within liposomes composed of PC:DOPE:DC-Chol (0.5:0.5:1 molar ratio), transfection efficiency is significantly reduced to 85 ± 5% (of lipofectin control), which is intermediate between PC:DC-Chol omitting DOPE

and PC:DOPE:DC-Chol formulated at a 1:1:1 molar ratio ($P<0.05$, ANOVA). This confirms that the inclusion of DOPE within PC-based formulations enhances transfection, as with an increase in DOPE content transfection efficiency increases accordingly (Figure 4.13).

Table 4.1 shows that the negative zeta potential for PC:DC-Chol (1:1 molar ratio) complexes is significantly lower than all other PC-containing complexes. The plasmid DNA within the lamellar structures (i.e. liposome-DNA complexes composed of PC:DC-Chol) is reported to be intercalated within the aqueous spaces situated between the stacked lipid bilayers (Gregoriadis, 1998; May *et al.*, 2000), therefore the anionic charges of the plasmid DNA will be concealed within lipid bilayers.

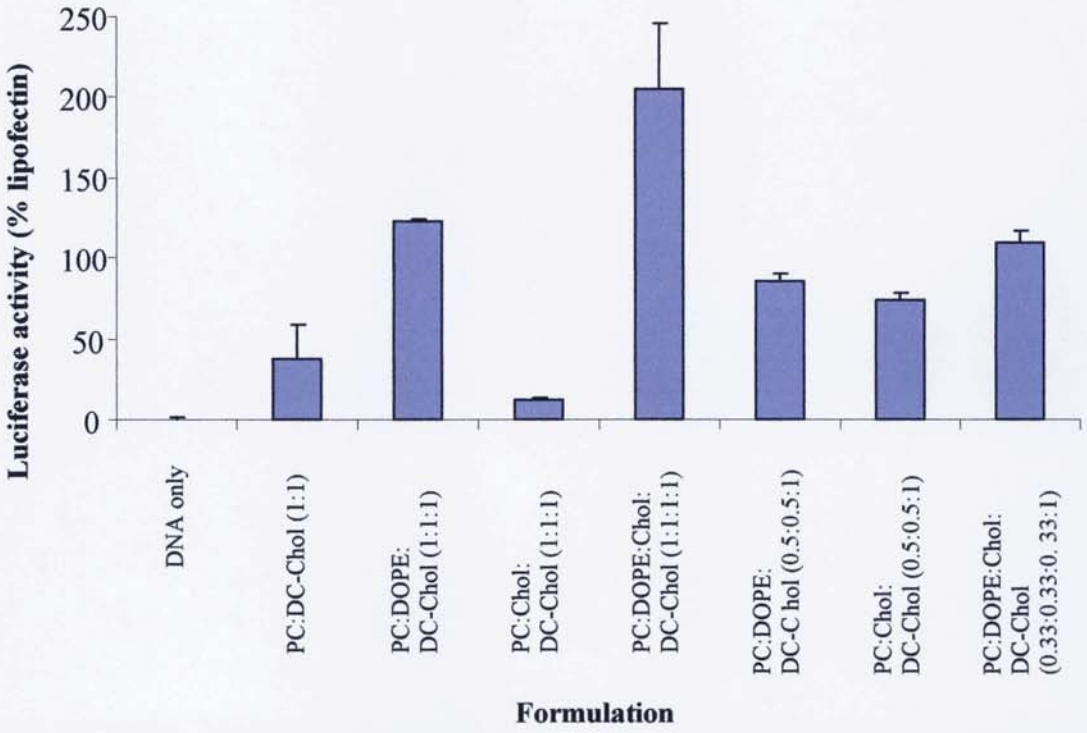


Figure 4.13 Effect of incorporating PC within the liposomal bilayer on transfection efficiency of various formulations. Liposomes composed of DOPE:DC-Chol and Chol:DC-Chol were formulated with or without PC, with varying molar ratios. 1μg/ml of plasmid DNA (gWiz luc) was mixed with each formulation and than subsequently incubated with to COS-7 cells for 5 hours (for full details see section 2.12.2). Transfection activity was determined by luciferase assay, with results expressed as percentage of control (i.e. lipofectin transfection activity). Results denote mean ± SD, $n = 3$.

With regard to Chol:DC-Chol liposomes formulated at a 1:1 molar ratio, the addition of PC significantly reduces transfection efficiency from 385% to 12% (Figures 4.4 vs. 4.13), with

efficiency being even lower than PC:DC-Chol, without the presence of a helper lipid (Figure 4.13). Although, in this instance for liposomes composed of PC:Chol:DC-Chol (0.5:0.5:1), when the molar ratio of Chol and PC were reduced, the values of transfection efficiency were significantly higher than PC:Chol:DC-Chol at a 1:1:1 molar ratio (74% vs. 12%). Results presented in section 4.3 of this chapter, reveal that when DNA is added to liposomes composed of Chol:DC-Chol (1:1 molar ratio), large aggregated structures form with the plasmid DNA thought to be entrapped between vesicles within these aggregates, producing relatively high levels of transfection activity (Figure 4.3 and 4.6). When PC is added to the formulation, there is a considerable reduction in complex size from 945 ± 63 nm for Chol:DC-Chol (1:1 molar ratio) to 391 ± 36 nm for PC:Chol:DC-Chol (1:1:1 molar ratio), which is also associated with an immense decrease in transfection activity (from 385 ± 10% to 12 ± 2%, respectively), again emphasising that smaller complexes are poor transfecting agents. However, for PC-containing complexes, transfection efficiency is greatest when both DOPE and Chol are included within the formulation, showing that a combination of the two helper lipids enhances activity.

Formulation	Size (nm ± SD)		Zeta Potential (mV ± SD)	
	SUV	SUV-DNA	SUV	SUV-DNA
PC:DC-Chol (1:1)	62.3 ± 1.3	384 ± 43.0	44.3 ± 2.2	-23.3 ± 1.1*
PC:DOPE:DC-Chol (0.5:0.5:1)	68.5 ± 1.4	347 ± 31.6	41.7 ± 14.0	-38.8 ± 2.1
PC:Chol:DC-Chol (0.5:0.5:1)	75.0 ± 3.5	346 ± 44.2	41.1 ± 9.3	-34.0 ± 0.3
PC:DOPE:DC-Chol (1:1:1)	55.2 ± 6.3	365 ± 15.8	41.2 ± 6.3	-45.3 ± 4.9**
PC:Chol:DC-Chol (1:1:1)	64.6 ± 4.4	391 ± 36.4	40.7 ± 3.8	-39.7 ± 7.8
PC:DOPE:Chol:DC-Chol (1:1:1:1)	71.2 ± 2.7	345 ± 15.0	45.1 ± 2.2	-36.3 ± 1.2

Table 4.1 Physiochemical characteristics of empty SUV and SUV-DNA complexes of various PC formulations. SUV composed of DOPE:DC-Chol, Chol:DC-Chol and Chol:DOPE:DC-Chol were formulated with or without PC, of varying molar ratios. Size and zeta potential of each empty SUV and SUV-DNA complexes was measured on a ZetaPlus (Brookhaven Instruments), in ddH_2O and 0.001 M PBS, respectively, at 25°C. * signifies that liposomes composed of PC:DC-Chol (1:1 molar ratio) produce significantly ($P<0.05$, ANOVA) lower values of negative zeta potential than all other PC formulations. ** signifies that PC:DOPE:DC-Chol (1:1:1 molar ratio) produces significantly higher values of negative zeta potential than liposome-DNA complexes composed of PC:DOPE:Chol:DC-Chol (1:1:1:1 molar ratio). Results denote mean ± SD, $n = 3$.

For empty SUV, there were no significant ($P>0.05$, ANOVA) differences in zeta potential values between formulations, therefore any significant differences found between formulations for SUV-DNA complexes can be attributed to the addition and presence of plasmid DNA (Table 4.1).

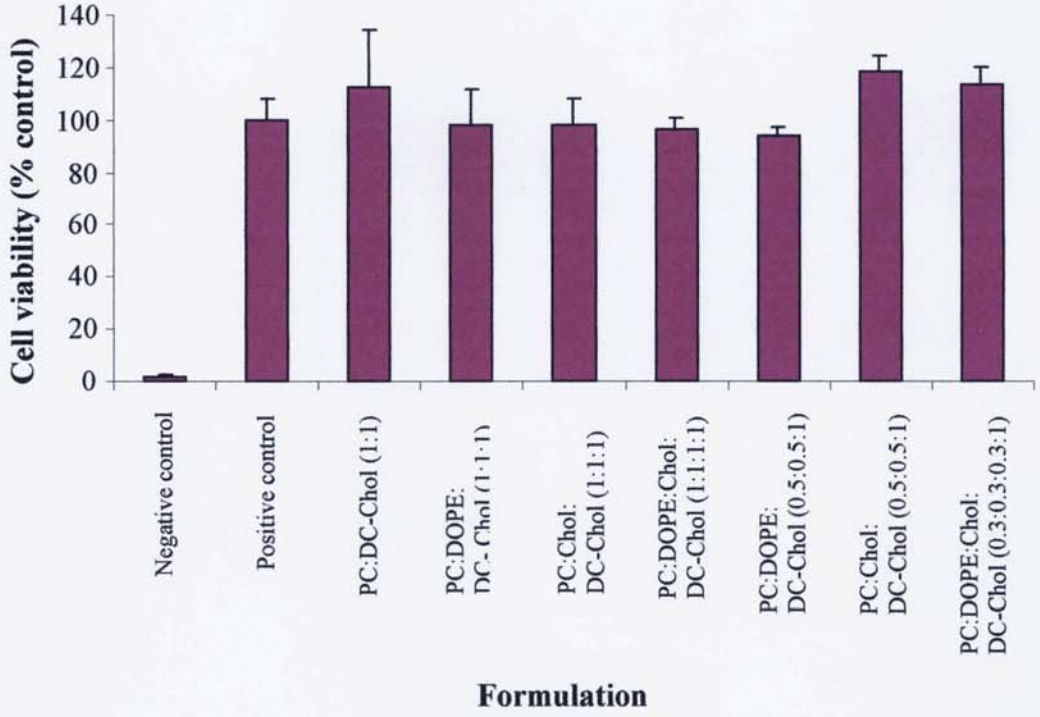


Figure 4.14 Cytotoxicity of various PC-based liposome-DNA complexes. Viability of COS-7 cells after the transfection procedure was measured by the CellTitre 96® AQueous One solution cell proliferation assay (Promega). This is a colorimetric method for determining the number of viable cells in proliferation, which is measured by the amount of 490nm absorbance and this is directly proportional to the number of living cells in culture (for full details see section 2.12.2). Results are expressed as a percentage of positive control (cells and medium only). Results denote mean ± SD, $n = 8$.

For all PC-containing liposome-DNA complexes, cell viability of COS-7 cells remained above 95% (of control; Figure 4.14), indicating that all formulations tested were not toxic to the cells at the lipid concentrations used.

The level of DNA condensation within each formulation was analysed and confirmed by gel electrophoresis (Figure 4.15) on the basis of intensity in the absence (40% sucrose) or exposure to anionic competition. The anionic components will knock off any surface bound, uncondensed DNA, which will then migrate towards the cathode (Perrie *et al.*, 2000). In the

presence of anionic competition (Figure 4.15a), plasmid DNA was dissociated from all formulations (PC:DC-Chol, DOPE:DC-Chol, PC:DOPE:DC-Chol, Chol:DC-Chol and PC:Chol:DC-Chol). On the other hand, in the absence of anionic competition a reduced amount of DNA can be seen migrating towards the cathode (Figure 4.15b) especially when compared to the control free DNA, demonstrating that a sufficient amount of DNA is condensed and complexed onto the liposomal surface. To some extent DOPE:DC-Chol (1:1 molar ratio) in lane 2 shows slightly less DNA migration, indicating that more plasmid DNA is attached and complexed to this formulation in comparison to the other formulations where there seems to be very slightly more free DNA within solution. More DNA complexed to the surface of DOPE:DC-Chol (1:1 molar ratio) liposomes, allows for more DNA to be delivered to the cells, therefore enhancing transfection efficiency (as shown in Figure 4.3). However, even though these gels reveal the level of DNA condensation the intensities between each DNA band are slightly difficult to distinguish.

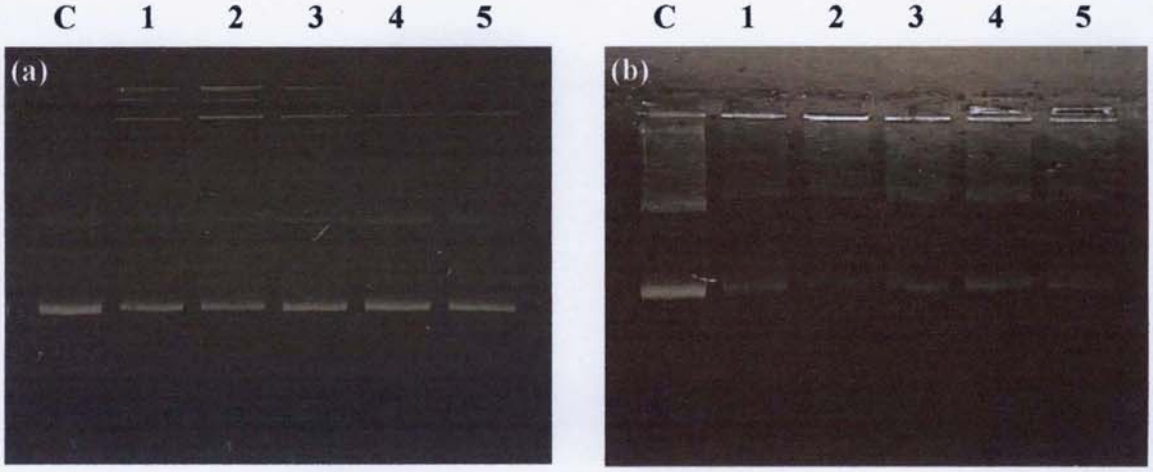


Figure 4.15 Agarose gel electrophoresis of PC based stabilised liposomes. Liposomes composed of DOPE:DC-Chol or Chol:DC-Chol were formulated with or without PC, were subjected to gel electrophoresis in the presence (a) and absence (b) of competitive anionic components. (Lane C, control naked plasmid DNA (1 μg/ml); lane 1, PC:DC-Chol (1:1 molar ratio); lane 2, DOPE:DC-Chol (1:1 molar ratio); lane 3, PC:DOPE:DC-Chol (1:1:1 molar ratio); lane 4, Chol:DC-Chol (1:1 molar ratio); lane 5, PC:Chol:DC-Chol (1:1:1 molar ratio)).

The considerable decrease in transfection efficiency due to the inclusion of PC is extremely intriguing and was examined further. To study the full extent of this effect preparations were made in which increasing amounts of PC was added to DOPE:DC-Chol (1:1 molar ratio) SUV liposomes and transfection efficiency was measured.

Interestingly, transfection efficiency of liposome-DNA complexes appears to decrease at a linear rate as PC content increases (Figure 4.16). This reduction in efficiency can be attributed to the stabilising effect of PC on membrane bilayers (Campbell *et al.*, 2001), and presumably, depending on the amount of PC present, the ability of the fusogenic helper lipid DOPE to form an inverted hexagonal phase and promote membrane-membrane fusion (Litzinger and Huang, 1992) appears to be diminished. Results presented here provide further support for the statement detailed above (for Figure 4.13). In the absence of DOPE, PC:DC-Chol (1:1 molar ratio) liposome-DNA complexes, as shown previously, produce negligible levels of activity (71% of lipofectin control; Figure 4.13) demonstrating further the beneficial role of the fusogenic properties of DOPE and the inability of stable PC liposomes-DNA complexes to disrupt endosomal membranes and to facilitate plasmid DNA entry into the cell.

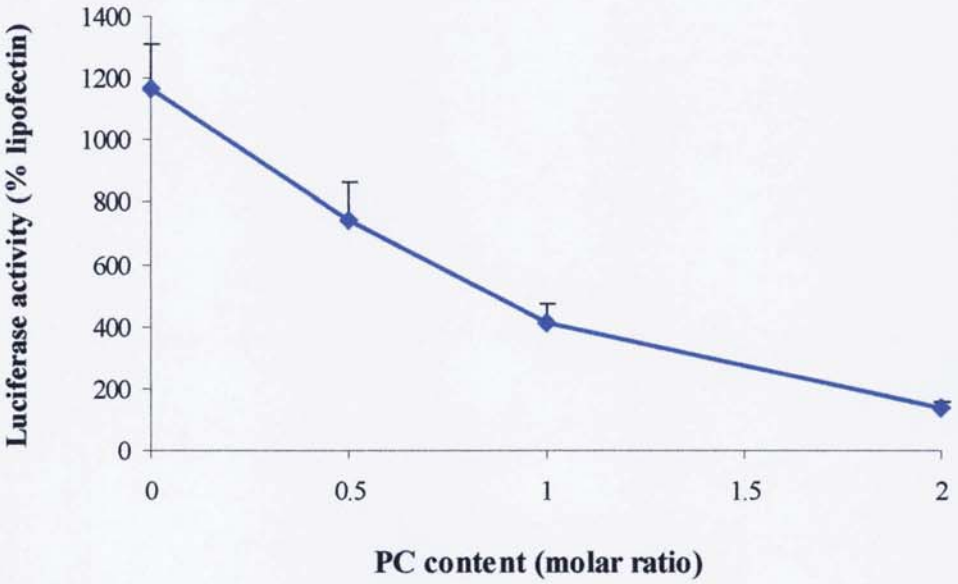


Figure 4.16 Inclusion of phosphotidylcholine within membrane bilayers of DOPE:DC-Chol liposomes and the effect on transfection efficiency. Increasing μmoles of PC was added to liposomes composed of DOPE:DC-Chol (1:1 molar ratio; total lipid of 1.56 μmoles/ml), to give varying molar ratios of PC. 1μg of plasmid DNA (gWiz luc) was mixed with each formulation (0.0078μmoles) and than subsequently incubated with to COS-7 cells for 5 hours (for full details see section 2.12.2). Transfection activity was determined by luciferase assay, with results expressed as percentage of control (i.e. lipofectin transfection activity). In the absence of DOPE, PC:DC-Chol (1:1 molar ratio) liposome-DNA complexes produce negligible levels of activity (71% of lipofectin control). Results denote mean ± SD, $n = 3$.

However, when formulating liposomes it is desirable for the vesicle to remain stable within its surrounding buffer and serum when applied *in vivo*, which can be achieved by

substituting the helper lipid DOPE with Chol (Sternberg *et al.*, 1998) and additionally the inclusion of PC. It is well recognised that complexes must be stable enough to endure the passage towards and across the plasma membrane in order to gain entry into the cytosol of the cell. Once inside the cell, it is essential that these liposome-DNA complexes destabilise and decompose to enable its DNA content to dissociate from the complex into the cytoplasm (Langner, 2000). By gradually increasing PC content, the complex is deemed too stable and rigid, and although this is beneficial for transporting the DNA, the successful release of plasmid DNA is prevented thus impeding efficient gene expression.

From a different perspective, when applied *in vivo* it is essential that liposome vesicles are rigid and stable in order to withstand the biological milieu of the host. The liposome has to protect the plasmid DNA from degradation by nucleases and needs to deliver their contents directly to antigen presenting cells (APC) infiltrating the site of injection (Gregoriadis *et al.*, 1997; Gregoriadis, 1998; Perrie *et al.*, 2001). Liposomes composed of PC stabilise the lipid bilayer and in spite of these liposome vesicles being poor at transfecting cells *in vitro*, previous studies have demonstrated that when applied *in vivo* these liposomes have the ability to withstand the inhibiting factors within the biological milieu and deliver its contents more efficiently (Perrie *et al.*, 2004). This highlights the multitude of properties that a successful liposomal formulation should possess in order to effectively deliver its therapeutic content.

Size values, in Table 4.2, show that PC:DOPE:DC-Chol with a molar ratio of 2:1:1 produce significantly ($P<0.05$, ANOVA) larger complexes than any other formulation (440 nm vs. 313-365 nm; Table 4.2). This may be a result of increased total lipid content within the formulation, as there are no significant differences between any other formulations containing PC and DOPE. Therefore, regardless of liposome composition, small distribution in size ranges, from approximately 310nm to 440nm, were measured for all liposome-DNA complexes tested (Table 4.2). This indicates that the presence or absence of PC or DOPE at any lipid concentration had very little effect on vesicle size, which in turn signifies that transfection efficiency in this instance is not affected by complex size.

Although complex size of PC:DC-Chol (1:1 molar ratio) and DOPE:DC-Chol (1:1 molar ratio) complexes were similar, the way in which these liposome formulations interact with

plasmid DNA may be extremely distinct. Therefore, transmission electron microscopy was used to observe any morphological differences between PC-containing and DOPE-containing complexes (Figures 4.17 and 4.18).

Formulation	Size (nm ± SD)	Zeta Potential (mV ± SD)
DOPE:DC-Chol (1:1 molar ratio)	364.9 ± 23.2	-42.6 ± 2.9
PC:DOPE:DC-Chol (0.5:1:1 molar ratio)	314.7 ± 30.0	-31.1 ± 7.6
PC:DOPE:DC-Chol (1:1:1 molar ratio)	312.8 ± 19.9	-44.0 ± 4.0
PC:DOPE:DC-Chol (2:1:1 molar ratio)	440.3 ± 44.5*	-32.8 ± 10.6
PC:DC-Chol (1:1 molar ratio)	361.2 ± 54.3	-23.3 ± 1.1

Table 4.2 Effect of PC concentration on vesicle size and zeta potential of SUV-DNA formulations. Plasmid DNA (gWiz; 1 μg/ml) was complexed with SUV of various formulations. Size and zeta potential of each vesicle was measured on a ZetaPlus (Brookhaven Instruments), in ddH_2O and 0.001 M PBS, respectively, at 25°C. * signifies that liposome-DNA complexes composed of PC:DOPE:DC-Chol formulated at a molar ratio of 2:1:1, produce significantly ($P<0.05$, ANOVA) larger complexes than all other liposome formulations. Results denote mean ± SD, $n = 3$.

TEM images of DOPE:DC-Chol (1:1 molar ratio; Figures 4.17 a and b) or PC:DC-Chol (1:1 molar ratio; Figures 4,18 a-d), reveal complexes with similar structures, indicating in this instance size and overall complex structure is not a determining factor in transfection efficiency, and is most likely due to bilayer composition and activity. However, although at first glance these complexes composed of DOPE:DC-Chol and PC:DC-Chol appear similar in shape (Figures 4.17 b & c and Figures 4.18 a-d) on closer inspection, further TEM images (Figures 4.18e and f) reveal distinct lipid bilayer membranes present within PC-containing complexes, possessing an L_α configuration, as detailed previously. Even as empty SUV (Figure 4.18a), lipid bilayers are visible on the TEM images with PC:DC-Chol exhibiting stable lamellar structures. As Figure 4.18 shows, the heterogeneous population of complexes (Figure 4.18b) essentially remain as individual liposomal vesicles, although with each individual vesicle situated in very close proximity, bonded together due to the presence of plasmid DNA causing high electrostatic interactions and resulting in liposome-liposome aggregation (Figures 4.18c and d). Even upon addition of DNA these lamellar structures still remain visible while surrounding the plasmid DNA, with the DNA intercalated between the stacked lipid bilayers (Figures 4.18e and f). Although, the lipid bilayers do show some

bilayer folding, due to the strong electrostatic interactions occurring between the cationic lipid and anionic DNA causing slight structural reorganisation. This is in line with previous reports using cyroelectron microscopy (Huebner *et al.*, 1999), where liposomes composed of DMPC and DC-Chol (3:2 molar ratio) mixed with DNA formed clusters of vesicles and at the point of liposome-liposome contact the lipid bilayers were slightly deformed or flattened.

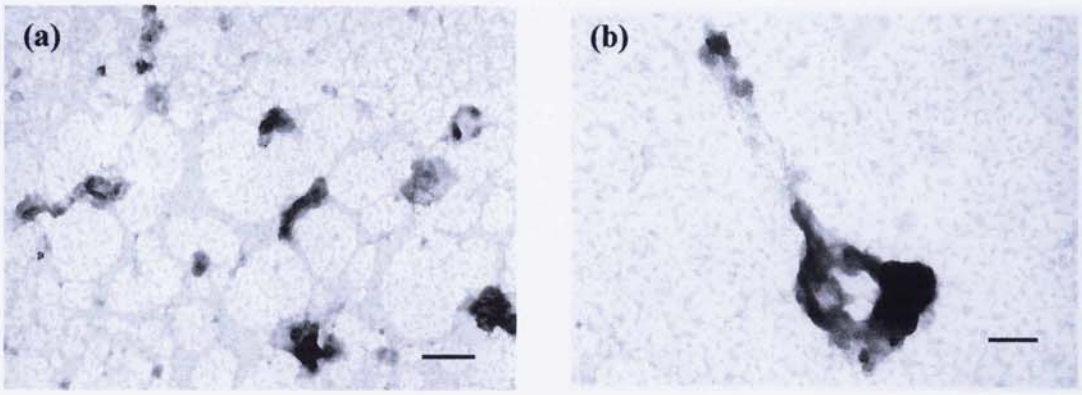


Figure 4.17 Transmission electron micrographs (TEM) assessing the morphology of DOPE:DC-Chol liposomes. SUV liposomes composed of DOPE:DC-Chol (1:1 molar ratio) mixed with DNA (0.0078μmole lipid to 1μg DNA), were stained with 2% uranyl acetate and imaged using a JEOL 1200EX TEM. (a) SUV-DNA complexes, bar = 300nm; (b) SUV-DNA complexes, bar = 100nm.

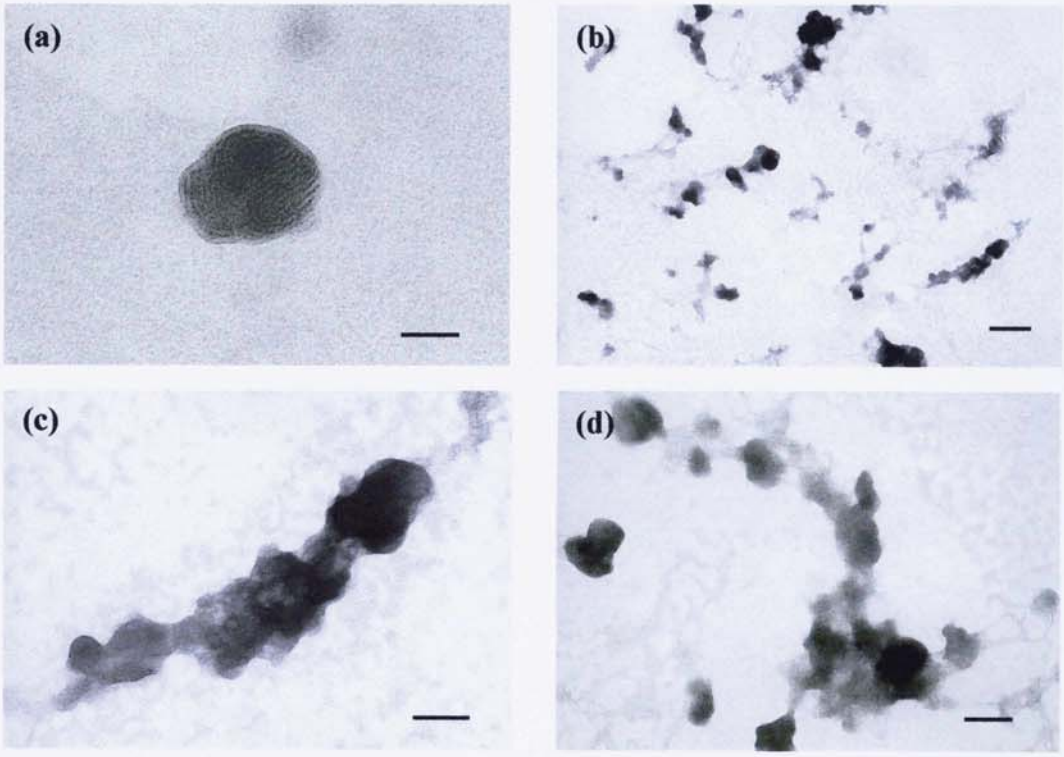


Figure 4.18 Transmission electron micrographs (TEM) assessing the morphology of PC:DC-Chol liposomes, continued on next page. SUV liposomes composed of PC:DC-Chol (1:1 molar ratio) mixed with DNA (0.0078μmole lipid to 1μg DNA), were stained with 2% uranyl acetate and imaged using a JEOL 1200EX TEM. (a) empty SUV, bar = 25nm; (b) SUV-DNA complexes, bar = 300nm; (c) and (d) SUV-DNA complexes, bar = 100nm.

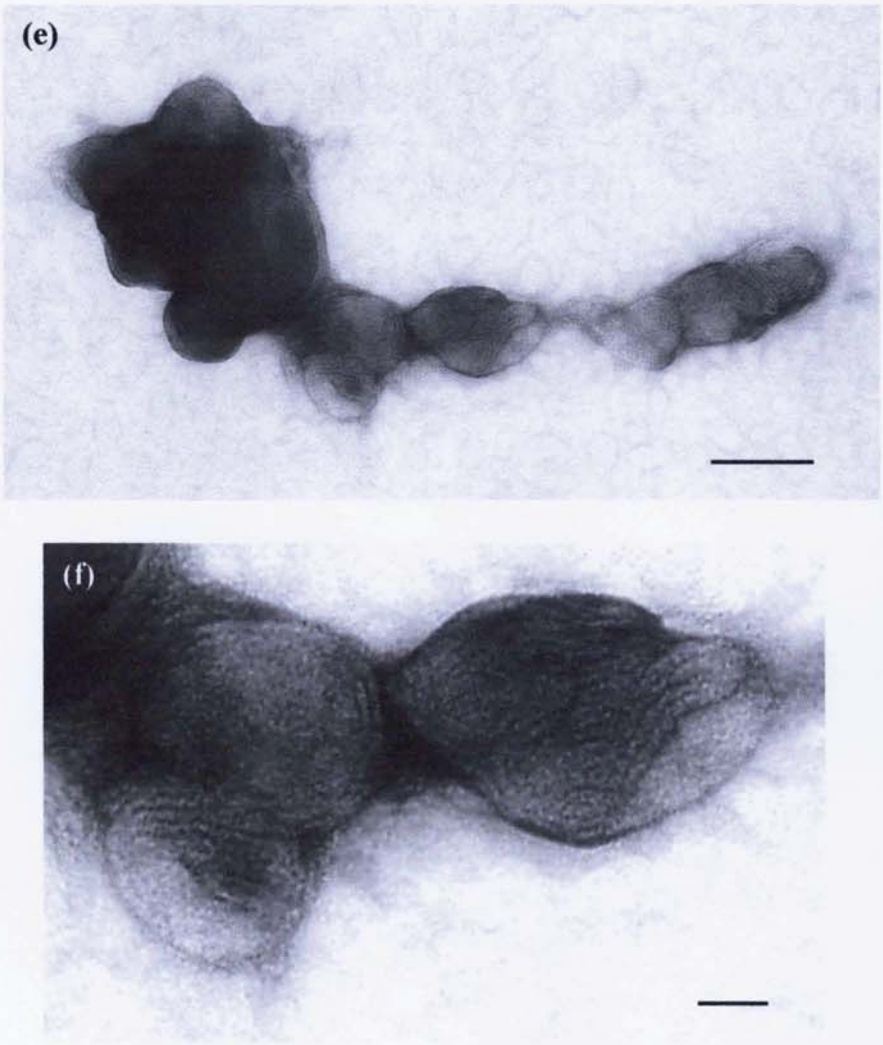


Figure 4.18 continued from previous page. Transmission electron micrographs (TEM) assessing the morphology of PC:DC-Chol liposomes. SUV liposomes composed of PC:DC-Chol (1:1 molar ratio) mixed with DNA (0.0078μmole lipid to 1μg DNA), were stained with 2% uranyl acetate and imaged using a JEOL 1200EX TEM. (e) SUV-DNA complexes, bar = 100nm; (f) SUV-DNA complexes, bar = 25nm. Images reveal complexes showing stable lipid bilayer membranes and structures.

4.5.1 Effect of alkyl chain length

The ability of liposome-DNA complexes to efficiently transfect cells *in vitro* seems to distinctly correlate with the alkyl chain length of the lipid component incorporated within the lipid bilayer. It is well recognised that as the hydrocarbon chain length of the phospholipid increases, the phase transition temperature is elevated. This consequently increases the rigidity of the lipid bilayer (Felgner *et al.*, 1994; Mohammed *et al.*, 2004), which is then associated with a decrease in transfection efficiency (Akao *et al.*, 1991). This is in agreement with results presented in Figure 4.19, where liposome-DNA complexes containing PC, a

phospholipid with a relatively short chain length, exhibits significantly higher levels of transfection in contrast to those liposome-DNA complexes where PC is replaced by lipids comprising of longer hydrocarbon chains, such as DMPC and DSPC. An apparent trend in the order of PC > DMPC > DSPC shows decreasing transfection efficiency with increasing alkyl chain length and also increasing phospholipid transition temperature. With an increase in lipid hydrocarbon chain length, the phase transition of the lipids also increases due to a greater ordered state of the phospholipids, where the van der Waals interactions between lipid chains become stronger, thus require more energy to disrupt the stable lipid bilayer (Mohammed *et al.*, 2004). Therefore, as the transfection procedure is carried out at physiological temperature of 37°C, lipids that have a high transition temperature, such as DSPC (T_c = 55°C), will be in the ordered gel phase and retain stable bilayers. As a consequence these stable and 'strong' bilayers impede the ability of these liposomes to fuse with the cell and endosomal membranes and thus inhibit successful delivery and release of plasmid DNA *in vitro*. Several studies assessing various antigens have also shown that by replacement of PC with a phospholipid possessing a high transition temperature generates lower levels of immune responses *in vivo* (Gregoriadis *et al.*, 1987; Perrie *et al.*, 2001), as these liposomes composed of phospholipids with saturated long alkyl chains are known to be extremely stable (Kirby & Gregoriadis, 1981; Senior & Gregoriadis, 1983). In contrast, other studies have also found that transfection efficiency *in vitro* is better with lipids consisting of shorter hydrocarbon chains, whereas the longer hydrocarbon chains allow for better transfection and DNA delivery *in vivo* (Floch *et al.*, 2000). When applying liposomes for delivering plasmid DNA *in vivo* other factors come into consideration, such as; route of administration, biological milieu, rate of transfection, etc. Therefore, transfection efficiency *in vivo* for various lipids can vary depending on the sole purpose and required effect of the liposomal delivery system.

On the whole, transfection efficiency *in vitro* of liposome-DNA complexes composed of the phospholipid DSPC, transfect COS-7 cells at the same efficiency as free DNA, as there is no significant difference between the two ($P>0.05$, ANOVA). Therefore, clearly shows the inability of these complexes to enhance DNA delivery *in vitro*.

In addition, previous reports have shown that the level of DNA condensation and compaction increases with an increase in lipid alkyl chain length (Matulis *et al.*, 2001;

Miguel *et al.*, 2003). Therefore more energy and force is required for DNA release from the complex. Transfection efficiency in the order of PC > DMPC > DSPC correlates with alkyl chain length and thus may be a reflection on the level of DNA compaction within complexes, with DNA very tightly compact within complexes formulated with DSPC.

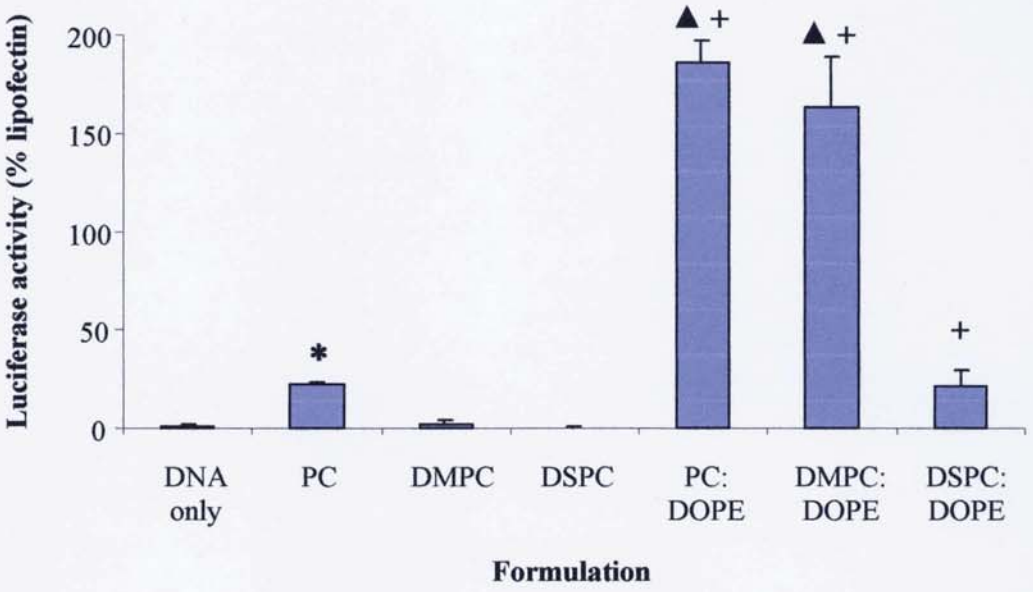


Figure 4.19 Effect of alkyl chain length on transfection efficiency of PC-containing liposomes, with and without the helper lipid DOPE. Liposomes were prepared as SUV, containing either PC, DMPC or DSPC in equimolar ratio with the cationic lipid DC-Chol, which were mixed with gWiz plasmid DNA so that there is 0.0078μmole/ml total lipid to 1μg DNA/ml. Alternatively, SUV containing either PC, DMPC or DSPC in equimolar ratio with both DOPE and DC-Chol were formulated at a 1:1:1 molar ratio, with 0.0117μmole/ml of total lipid and 1μg/ml plasmid DNA. 1ml of each formulation was added per well of COS-7 cells and incubated for 5 hours (for full details see section 2.12.2). Transfection activity was determined by luciferase assay, with results expressed as percentage of control (i.e. lipofectin transfection activity). * signifies that PC:DC-Chol complexes significantly ($P<0.05$, ANOVA) transfect COS-7 cells more efficiently than liposomes containing DMPC:DC-Chol or DSPC:DC-Chol. ▲ signifies that DOPE:DC-Chol complexes containing PC or DMPC produce significantly higher levels of luciferase activity than DSPC. + signifies that liposomes composed of PC, DMPC or DSPC in combination with DOPE significantly ($P<0.05$, ANOVA) transfect cells better than in the absence of DOPE. Results denote mean ± SD, $n = 3$.

In another experiment, liposomes were again formulated with DC-Chol and either PC, DMPC or DSPC, however, in this instance the helper lipid DOPE was incorporated within the formulations. With the addition of DOPE, there was a 10-fold increase in transfection efficiency from the previous formulations omitting this lipid (Figure 4.19), with activity increasing considerably from 23% to 186% for PC, 2% to 163% for DMPC and 0.5% to 22% for DSPC. Once more this demonstrates the ability of DOPE and its fusogenic

properties at promoting effective DNA transfection. Previously, as shown in Figure 4.19, activity of DSPC:DC-Chol SUV-DNA complexes was equivalent to DNA only, however it can be seen that by the inclusion of DOPE within the formulation, transfection efficiency increases, resulting in luciferase activity values that are significantly higher ($P<0.05$) than DNA only. The addition of DOPE to these formulations may decrease bilayer stability and increase bilayer flexibility. This could be attributed to the possibility that addition of DOPE decreases the phospholipids transition temperature, thereby reducing the rigidity of liposome bilayer composed of lipids with high transition temperatures such as, DMPC or DSPC. As detailed above, strong van der Waals interactions occur between phospholipids with long hydrocarbon chains, which then require more energy in order to disrupt the ordered packing of these lipids thus the phase transition temperature increases. However, with the inclusion of DOPE into the lipid bilayer, the DOPE molecule will intercept between these strong van der Waals interactions between the lipid chains, thereby reducing the ordered packing of these lipids, while simultaneously decreasing the phase transition temperature enabling more flexible lipid bilayers. This could be tested further by DSC analysis.

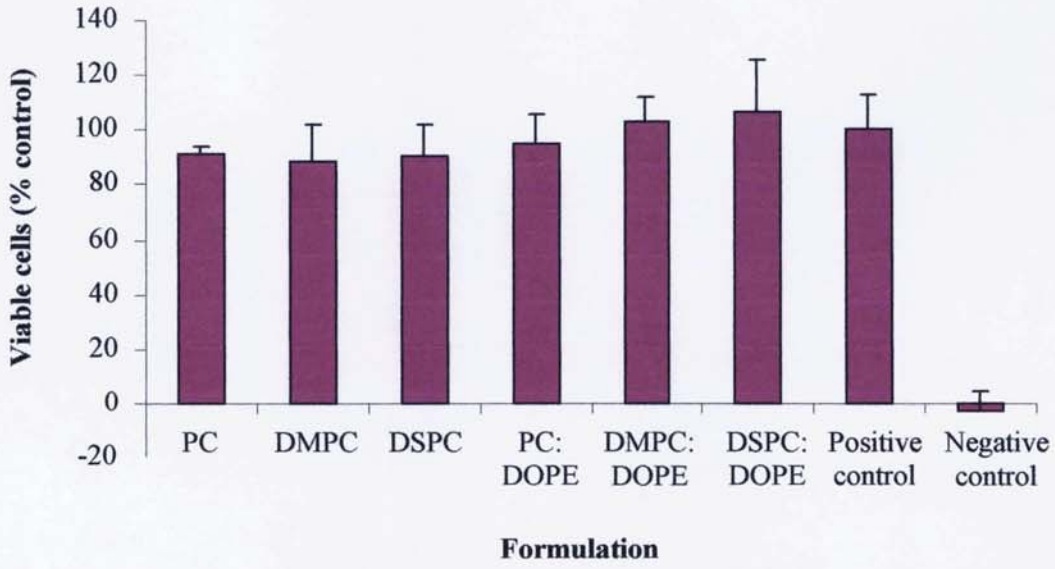


Figure 4.20 Effect of alkyl chain length of various PC-containing liposomes on cell viability of COS-7 cells. The viability of COS-7 cells post-transfection was determined and measured by the CellTitre 96® AQueous One Solution cell proliferation assay (Promega). This is a colorimetric method for determining the number of viable cells in proliferation, which is measured by the amount of 490nm absorbance and this is directly proportional to the number of living cells in culture (for full details see section 2.12.2). Results are expressed as a percentage of positive control (cells and medium only). Results denote mean ± SD, $n = 8$.

However, the prominent transfection levels previously determined with DOPE:DC-Chol SUV-DNA complexes (Figure 4.3), were diminished when phosphatidylcholines are added to the formulation. So although DOPE increases the levels of efficiency for these PC-containing complexes by decreasing the phase transition temperature and bilayer rigidity, conversely, once again the PC-based systems inhibit the transfection efficiency of DOPE-containing complexes, presumably due to these stable lipids impeding the transition to the inverse hexagonal phase. For liposome-DNA complexes composed of phosphatidylcholines, such as, PC, DMPC or DSPC, the number of viable cells remained above 85% (Figure 4.20), therefore showing no significant toxicity effects to COS-7 cells.

4.5.2 Effect of phospholipid head group

As an effective gene delivery system, liposomes must possess a multitude of properties in order to facilitate a magnitude of interactions, such as; cationic/phospholipid interactions, cationic liposome/DNA interactions and cationic liposome-DNA/cell interactions (Wong *et al.*, 2003). It has previously been shown that liposomes enriched with phospholipids containing an ethanolamine head group enhance both liposome/DNA interactions and mediate liposome-DNA complex/cell interactions and have been shown to be more efficient at transfection *in vitro* than those composed of choline head group (Chu *et al.*, 1990; Legendre & Szoka, 1993; Campbell *et al.*, 2001; Wong *et al.*, 2003). Transfection experiments were carried out in which COS-7 cells were transfected with a variety of phospholipids that differ in head group (i.e. consisting of either an ethanolamine or choline head group), and also lipid alkyl chain.

Figure 4.21, in agreement with previous reports, shows that the type of phospholipid head group plays an essential role in mediating effective transfection *in vitro*. Phospholipids containing the ethanolamine head group exhibit significantly higher levels of transfection activity than their phospholipid counterparts containing choline head groups, with the same alkyl chain composition (Figure 4.21). The transfection efficiency of liposome-DNA complexes composed of PE and DMPE decrease from 85% and 45% (of lipofectin control) to 23% and 2% for PC and DMPC, respectively. With regard to DOPE, high transfection efficiency of 581% is reduced considerably to 11% (of lipofectin control) when substituted with the phospholipid DOPC. Once again, this highlights the inability of lamellar forming PC-containing complexes to transfect cells effectively and furthermore confirms the ability

of membrane destabilising PE phospholipids in enhancing transfection efficiency *in vitro* (Farhood *et al.*, 1995; Campbell *et al.*, 2001; Wong *et al.*, 2003). However, for DOPE-containing complexes efficiency is significantly higher than all formulations studied, suggesting that there are other factors which may contribute to enhancing transfection efficiency *in vitro*, in which the architectures of the dioleoyl alkyl chain may play a role. Indeed, Figure 4.22 shows that the presence of additional PE does not have any influence on transfection efficiency, with the formulations PE:DC-Chol (1:1 molar ratio) and PE:DOPE:DC-Chol (0.5:0.5:1 molar ratio) producing significantly lower values of transfection efficiency (104% and 215%, respectively; Figure 4.22) than those formulations containing more DOPE (450-474%; Figure 4.22). The trend in increase of activity is a result of the gradual addition of more DOPE within the formulation, with PE:DOPE:DC-Chol at a 1:1:1 molar ratio producing equivalent values of transfection efficiency to DOPE:DC-Chol at a 1:1 molar (450% vs. 474%), showing that the presence of PE had no influence on transfection efficiency, neither reducing or enhancing activity.

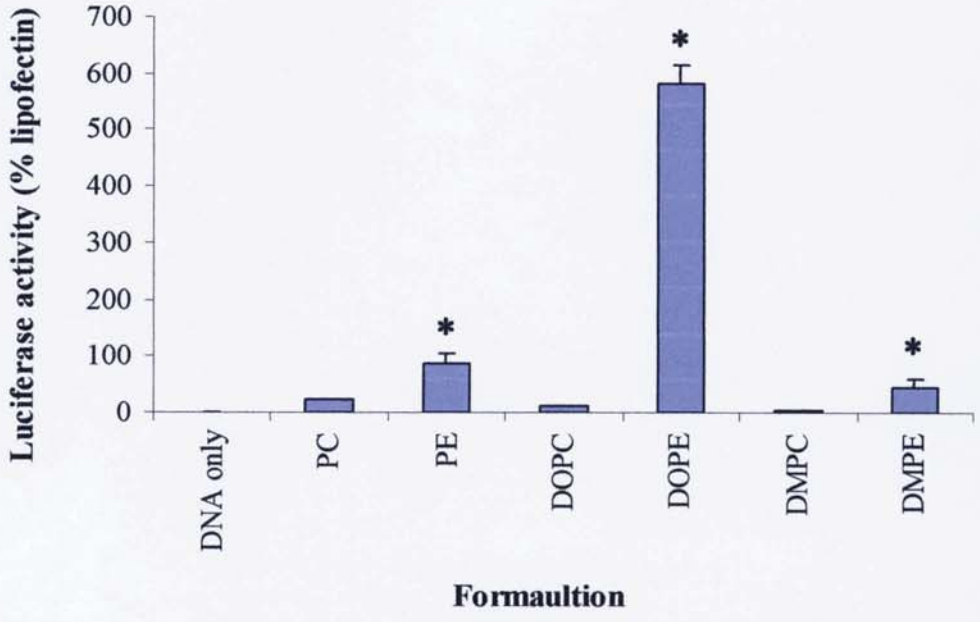


Figure 4.21 Effect of phospholipid head group type on transfection efficiency. SUV composed of DC-Chol in combination with different phospholipids of varying lipid head groups at a 1:1 molar ratio (total lipid concentration of 0.0078μmole/ml), were mixed with plasmid DNA (1μg/ml) and 1ml of SUV-DNA solution was added to each well (for full details see section 2.12.2). Transfection efficiency was determined by luciferase assay and results are expressed as percentage of positive control (i.e. lipofectin). * signifies that transfection activity values are significantly ($P<0.05$, ANOVA) higher for lipids containing a phophatidylethanolamine head group than those containing a phosphatidylcholine head group. Results denote mean ± SD, $n = 3$.

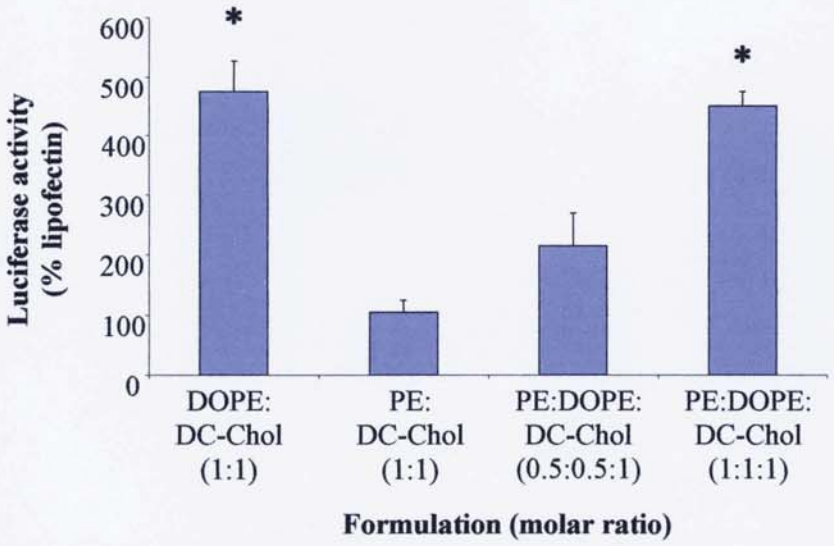


Figure 4.22 The effect of the neutral lipid PE within cationic liposome-DNA complexes on transfection efficiency *in vitro*. Cationic liposomes composed of DC-Chol in combination either DOPE or PE or both, were formulated with different lipid ratios. Transfection efficiency was determined by luciferase assay and results are expressed as percentage of positive control (i.e. lipofectin) (for full details see section 2.12.2). * signifies that liposome-DNA complexes composed of DOPE:DC-Chol at a 1:1 molar ratio and PE:DOPE:DC-Chol at a 1:1:1 molar ratio produced significantly ($P<0.05$, ANOVA) higher values of luciferase activity than liposome-DNA complexes composed of PE:DOPE:DC-Chol at a 0.5:0.5:1 molar ratio and PE:DC-Chol at a 1:1 molar ratio. Results denote mean ± SD, $n = 3$.

The effectiveness of DOPE established in these experiments is in line with previous investigations, suggesting that the architectures of the lipid DOPE facilitates fusion and disruption of the lipoplexes with cellular and endosomal membranes (Farhood *et al*, 1995) through their ability to invert into hexagonal lipid arrangements. However it is apparent this can only occur in combination with the PE head group, which may facilitate the dissociation of the lipoplexes after internalisation. This has been reported (Pedroso *et al*, 2001) to be due to a weakening of the otherwise strong cationic lipid-DNA electrostatic binding as a result of the PE amine group interacting with the DNA phosphate groups. Indeed, other studies have reported that lipids with a small PE head group destabilise the cationic lipid-DNA complex, as the amine group of the lipid interacts with both the phosphate groups within and between lipid bilayers and additionally with the phosphates within the DNA (Wong *et al.*, 2003). In contrast, PC lipids do not disrupt the electrostatic interaction and complex formation (Wong *et al.*, 2003). This combination of alkyl chain and head group within the lipid may promote successful release of the DNA into the cytosol.

4.6 Conclusion

The toxicity of the cationic lipid reduces the efficiency of DNA transfection, due to the inhibition of protein kinase C activity (PKC), subsequently blocking vital transfection pathways, such as endocytosis. High levels of transfection efficiency were achieved with cationic liposomes containing DC-Chol, proving to be at least 6 times more efficient than DOTMA, DOTAP and DDA, which can be attributed to the combination of the level of DNA condensation and level of lipid associated cytotoxicity. The inclusion of a helper lipid induces high levels of transfection, but the efficiency of transfection varies depending on the type of helper lipid incorporated within the liposome bilayer. When comparing the transfection efficiency of the helper lipids DOPE and Chol in combination with DC-Chol (1:1 molar ratio), overall the optimum level of transfection efficiency was achieved with DOPE at a charge ratio of 1.5:1, confirming the efficacy of DOPE as a transfecting agent, which greatly facilitates transfection efficiency *in vitro* (Felgner *et al.*, 1987; Farhood *et al.*, 1995; Almofti *et al.*, 2003). However, at the lower charge ratios Chol produced significantly higher levels of activity compared to DOPE, demonstrating that even in the absence of fusogenic properties, DNA can be delivered efficiently to cells by other methods. This highlights that transfection efficiency is effected by a combination of both the type of helper lipid utilised and the lipid:DNA charge ratio of those formulations. As expected, as lipid:DNA charge ratio increased, with cationic lipid content increasing, the zeta potential of the complexes became less negative and towards neutrality causing vesicle size to increase due to the reduction in electrostatic repulsions. Visualising the complexes by TEM confirm vesicle size measurements and reveal the lipid to DNA and also the lipid-to-lipid interactions. However, the ESEM micrographs reveal extremely large liposome structures, which may result from the instability of this liposome formulation. As the aqueous environment within the chamber evaporates, these liposomes may fuse together to generate larger liposome structures. Moreover, the increased stability of the liposome-DNA complexes, by inclusion of stabilising phospholipids, such as PC, DMPC or DSPC, inhibit transfection efficiency of plasmid DNA *in vitro* by impeding membrane fusion. A trend of decreasing transfection efficiency is associated with an increase in lipid hydrocarbon chain length and lipid transition temperature, in which DSPC is less efficient than PC or DMPC. Although, lipid stability can be reduced and the fusogenic properties can be enhanced by the inclusion of DOPE within these PC-containing liposomes systems.

5. Assessment of the dehydration-rehydration (DRV) procedure

5.1 Introduction

There are various ways in which to associate and transport plasmid DNA by liposomes, though encapsulating the DNA within DRV, by the dehydration-rehydration procedure (DRV) (Kirby and Gregoriadis, 1984), is a method which has shown to promote greater humoural and cell-mediated immune responses against the encoded antigen in immunized mice in contrast to naked DNA or DNA complexed to preformed liposomes (Gregoriadis *et al.*, 1997; Perrie *et al.*, 2001). With regard to SUV-DNA complexes, previous reports and also results presented within chapter 4, demonstrate that the transfection efficiency of liposome-DNA complexes *in vitro* are much higher than naked DNA, therefore there is a lot of work supporting the use of SUV-DNA complexes *in vitro* (Felgner *et al.*, 1987; Farhood *et al.*, 1995; Zuidam *et al.*, 1999; Ahearn and Malone, 1999; Regelin *et al.*, 2000). However, various reports have shown that when applied *in vivo*, gene expression is relatively low with these liposome-DNA complexes, resulting in immune responses that were only moderately higher than naked DNA (Sedegah *et al.*, 1994; Gregoriadis *et al.*, 1996). Therefore, entrapment of DNA within liposomes offers an attractive alternative liposome system for the delivery of DNA. The DRV procedure is considered as a mild procedure (Gregoriadis, 1994; Perrie and Gregoriadis, 2000; Gregoriadis *et al.*, 2002), as the entrapment of the solute occurs in the absence of any destructive components, such as sonication, organic solvents or detergents. The procedure is able to quantitatively entrap a wide range of solutes, including; small drugs (Kirby and Gregoriadis, 1984), interleukins (Gürsel and Gregoriadis, 1997), antigens (Gregoriadis *et al.*, 1987) and plasmid DNA (Perrie and Gregoriadis, 2000; Perrie *et al.*, 2001).

5.2 Incorporation of plasmid DNA within liposomes

The dehydration-rehydration procedure (DRV), originally reported by Kirby and Gregoriadis in 1984, involves mixing the solute to be entrapped, together with small unilamellar vesicles (SUV) prior to freeze-drying, producing SUV-DNA complexes. Accordingly, this allows intimate contact between the entire lipid content and all of the solute, subsequently facilitating a high degree of entrapped solute. By employing the DRV procedure, entrapment values previously reported for numerous antigens have shown to be consistently high, depending on liposome composition, in which incorporation values are expected to be higher when a cationic lipid in present within the lipid bilayers (Gregoriadis *et al.*, 1996;

Gregoriadis *et al.*, 2000). Additionally, it was shown that the antigen required for entrapment can have an influence, with *Mycobacterium* leprosy protein (pCMV4.65) exhibiting lower entrapment values (29-68%) than the hepatitis B surface antigen (pRc/CMV HBS) (56-89%), presumably due to the larger size of the pCMV 4.65 plasmid (~9kb) (Gregoriadis, 1998; Gregoriadis *et al.*, 2000). Further to chapters 3 and 4, which employed the production of SUV-DNA complexes, this chapter explores the influence of lipid composition and surface charge on the DRV procedure for entrapping plasmid DNA.

5.3 The effect of liposome preparation method

5.3.1 Physiochemical characteristics

The stage at which plasmid DNA is added to the liposomal formulation clearly affects the physiochemical characteristic (i.e. vesicle size and surface charge) of these vesicles (Table 5.1). Plasmid DNA (pRc/CMV-HBS) was added to various stages throughout the DRV procedure as shown in Figure 5.1.

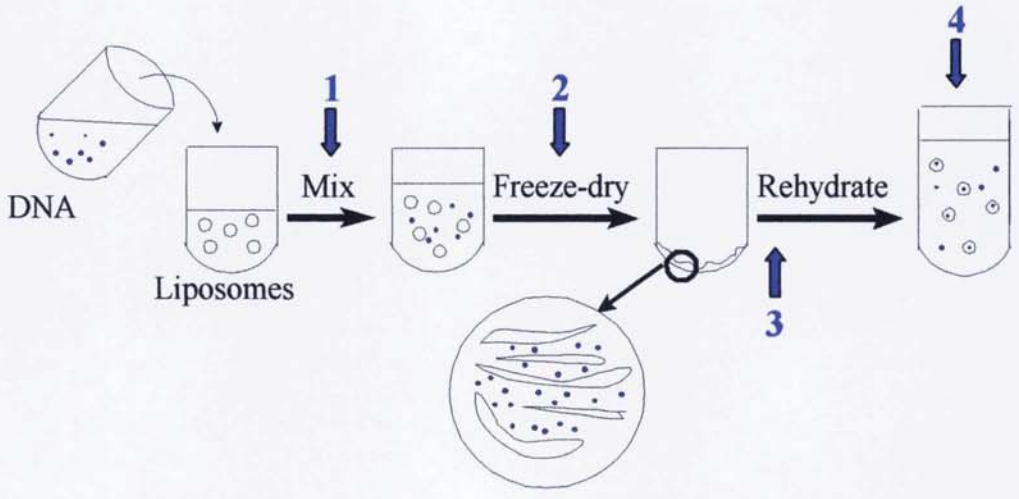


Figure 5.1 Six liposomal preparations each consisting of PC:DOPE:DC-Chol (16:8:4μmoles). 1, Empty small unilamellar vesicles complexed with DNA (SUV-DNA) after 30 minutes incubation at room temperature; 2, Dehydration-rehydration vesicles prepared with DNA present during controlled dehydration and rehydration (DRV(DNA)); 3, DRV prepared by rehydration with DNA solution (RV(DNA)); 4, Preformed empty DRV complexed with plasmid DNA (DRV-DNA) after 30 minutes incubation at room temperature. (Insert illustrates the close contact between flattened liposomal membrane structures and the DNA in a dry environment).

The addition of DNA to cationic preformed SUV composed of PC:DOPE:DC-Chol (16:8:4 μmole/ml), leads to the formation of complexes, where the plasmid DNA is complexed to the surface of the liposomes. The measured vesicle size of the SUV after addition of DNA increases from 98 nm to 259 nm (Table 5.1), indicating that the addition of DNA significantly alters the structural characteristics of the vesicles, due to the formation of SUV-DNA complexes, where the anionic DNA binds electrostatically to the cationic charges (Felgner *et al.*, 1987; Zhu *et al.*, 1993). In contrast, empty DRV and DRV dehydrated and rehydrated in the presence of DNA (DRV(DNA)) have similar vesicle sizes (884 nm and 889 nm, respectively; Table 5.1) and show no significant differences, suggesting that a high proportion of the DNA is entrapped within the vesicles, causing negligible alterations in size. However, when preformed cationic DRV is mixed with 100 μg DNA, to produce DRV-DNA complexes, large aggregates form since, as with SUV-DNA, the DNA binds to the cationic charges of the vesicle, causing structural configuration, where the bilayer strives to surround the plasmid DNA. This is further supported by zeta potential studies, which assist to evaluate the extent of cationic liposome and anionic DNA interactions, as the zeta potential provides an indirect reflection of the net surface charge of the liposomes. In the instance of surface complexed DNA, such as SUV-DNA and DRV-DNA, both preparations show a significant reduction in cationic charge, in contrast to their empty liposomal counterparts. This again supports the proposal that there is more DNA complexed on the vesicle surface thus causing neutralisation of the vesicles cationic charges. Furthermore, the presence of DNA during the dehydration and rehydration stage appears to be beneficial as there is less surface neutralisation by the anionic DNA of the cationic DC-Chol head groups and that with the DNA entrapped (DRV(DNA)) a considerable amount of DNA is located within the liposomes, presumably bound to the cationic charges of the inner bilayers.

Furthermore, when DRV were dehydrated in the absence of DNA but rehydrated with a solution of 100 μg DNA, (RV(DNA)), results show (Table 5.1) that the vesicles exhibit aggregation causing the formation of larger structures and a reduction in zeta potential in contrast to empty DRV. Interestingly, the DRV rehydrated with a solution of DNA (RV(DNA)) show characteristics intermediate between DRV(DNA) and DRV-DNA, which may suggest that these structures promote some DNA entrapment however less efficiently than when DNA is present during both the dehydration and rehydration process.

Liposome preparation	DNA incorporation (% of used ± SD)	Size (nm ± SD)	Zeta potential (mV ± SD)
SUV	–	105 ± 25	43.0 ± 1.5
SUV–DNA	92.5 ± 4.9	259 ± 81*	38.4 ± 2.1*
DRV	–	884 ± 77	42.0 ± 1.9
DRV(DNA)	93.3 ± 3.3	889 ± 89	41.4 ± 2.8
RV(DNA)	91.0 ± 4.0	1590 ± 104**	37.1 ± 2.0**
DRV–DNA	94.1 ± 2.9	2700 ± 876**	34.5 ± 3.0**

Table 5.1 Effect of liposome preparation procedure on DNA incorporation, size and zeta potential of liposomes composed of 16μmoles of PC, 8μmoles of DOPE, and 4μmoles of the cationic lipid DC-Chol. Dehydration and rehydration of SUV in the absence of DNA resulted in the formation of "empty" water containing DRV(DNA). Incubation of these preformed DRV with a solution of plasmid DNA (100μg) for 30 min resulted in the formation of DRV with surface complexed DNA (DRV–DNA). Dehydration and rehydration of SUV (freeze-dried in the absence of DNA) with a solution of 100μg DNA produced RV(DNA). Incorporation values were based on PicoGreen analysis. The vesicle z-average diameter and zeta potential of each liposomal preparation was measured in double-distilled water or 0.001 M PBS, respectively, at 25°C using a Brookhaven ZetaPlus. * signifies that the characteristics (i.e. size and surface charge) of SUV-DNA complexes are significantly ($P<0.05$, ANOVA) different from empty SUV. ** signifies that the characteristics (i.e. size and surface charge) of DRV-DNA and RV(DNA) are significantly ($P<0.05$, ANOVA) different from empty DRV. Results denote mean ± SD, $n = 3$.

Entrapment values were determined by PicoGreen analysis. PicoGreen is an intercalating cyanine dye that selectively binds to double stranded plasmid DNA with a linear detection range extending over more than four orders of magnitude of DNA concentration. There are very low variations between signals (Singer *et al.*, 1997) and is considered as a sensitive and practical alternative to other methods (Ahn *et al.*, 1996). Entrapment values measured for DRV(DNA) using PicoGreen analysis strongly correlate with entrapment values previously reported by ^{35}S-radioassay (Gregoriadis *et al.*, 1996; Perrie & Gregoriadis, 2000; Perrie *et al.*, 2001). When plasmid DNA was mixed with preformed empty SUV (SUV-DNA) or empty DRV (DRV-DNA) the DNA incorporation values were equally as high as DRV(DNA) (Table 5.1). Nevertheless, the spatial location of the DNA can significantly influence DNA retention and protection *in vivo*. Even though the incorporation values are similar, once administrated *in vivo*, the biological milieu is likely to displace the surface bound DNA and therefore possibly reduce the DNA content within these complexes (Perrie and Gregoriadis, 2000). Previous studies (Felgner *et al.*, 1987) have shown that *in vivo*, surface loading of DNA to preformed cationic liposomes can result in the formation of DNA

vectors, which display insufficient stability and transfection efficiency, due to the presence of sera. This suggests that the spatial location of DNA within these preparations may play an important role in their ability to protect and deliver their DNA payload.

5.3.2 Spatial location of plasmid DNA

The spatial location of the DNA within each of these systems was further investigated using gel electrophoresis in the absence and presence of an anionic component (Figures 5.2a and b). Anionic components, like SDS for example, have been previously shown to compete with the surface bound DNA (Perrie and Gregoriadis, 2000). In this instance, the loading buffer used contains an anionic component, bromophenol blue. Figure 5.2a shows that in the absence of competing anionic components, the DNA for all preparations remain at the site of application, presumably bound to the cationic charges of the liposomes. However, in the presence of anionic components, when plasmid DNA is complexed to the surface of SUV or DRV, the DNA is more exposed and susceptible to displacement by competing anionic components, as Figure 5.2b, lanes 2 and 5 illustrate, with more released DNA migrating towards the cathode in contrast to when DNA is entrapped within DRV (Figure 5.2b, lane 6). Once again, RV(DNA) (Figure 5.2b, lane 4) demonstrated DNA displacement intermediate between DRV(DNA) and DRV-DNA, confirming that effective entrapment requires the plasmid DNA to be present during both dehydration and rehydration. Therefore, the liposome preparation DRV(DNA) promotes the highest entrapment of plasmid DNA with the least DNA being displaced by anionic competition. High levels of DNA incorporation measured for DRV(DNA) cationic vesicles have also previously been reported in other studies (Gregoriadis *et al.*, 1996; Perrie and Gregoriadis, 2000). Gel electrophoresis studies along with zeta potential measurements, show increasing DNA displacement and decreasing surface charge in the order of empty DRV > DRV(DNA) > RV(DNA) > DRV-DNA. This supports the concept that for the latter preparation, DNA is predominantly complexed to the vesicle surface causing neutralisation of the cationic charges and that for DRV(DNA), incorporation values are a reliable and a representative indication of genuine DNA entrapment within the liposomes aqueous spaces, rather than complexation to the vesicles surface. Figure 5.3 illustrates the possible set of events that may take place when the competing anionic components are introduced to the liposomes incorporating DNA (entrapped or complexed), demonstrating the difference between various liposomal preparations.

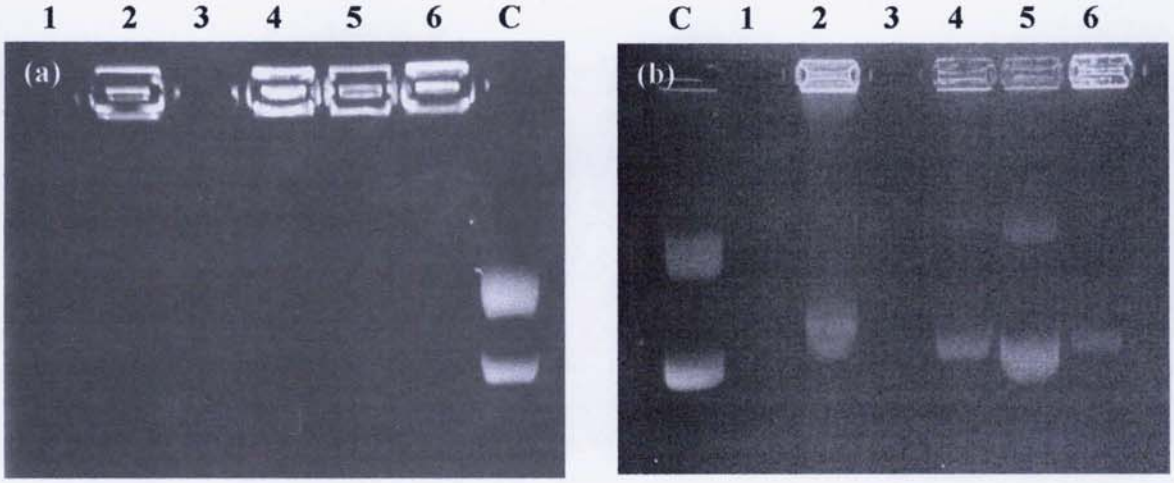


Figure 5.2 Gel electrophoresis of six different liposomal preparations composed of 16μmoles PC, 8μmoles DOPE and 4μmoles DC-Chol (lane C, control plasmid DNA only at 0.1 mg/ml; lane 1, SUV empty; lane 2, SUV-DNA, lane 3, DRV empty; lane 4, DRV-[DNA]; lane 5, DRV-DNA; lane 6, DRV(DNA)) comparing the amount of surface bound plasmid DNA (pRc/CMV HBS) displacement from the liposomal vesicles, when in the presence of anionic competitors. (a) in the absence of anionic components and (b) in the presence of anionic components.

The spatial location of DNA within each liposomal preparation was examined further by exposing each formulation to the digestive enzyme, deoxyribonuclease I, as detailed in section 2.6. As previously shown in chapter 3, Figures 5.4a and b, lane D show that plasmid DNA alone is readily degraded when exposed to deoxyribonuclease, once more revealing the imperative need for a delivery system, which provides protection of DNA from such harsh conditions. By incorporating DNA within liposomal vesicles, the DNA is protected against degradation upon exposure to deoxyribonuclease, as Figures 5.4a and b, lanes 1-8 reveal. In the absence of competing anionic components, the plasmid DNA along with lipid, remains at the site of application (Figure 5.4a, lanes 1-8) however, in the presence of anionic competitors, before or after digestion, plasmid DNA is displaced from liposomal vesicles and can be seen migrating towards the cathode (Figure 5.4b, lanes 1-8), revealing that the DNA remains intact and functional within all liposomal preparations even after exposure to degrading enzymes. As expected, with DNA entrapped within liposome bilayers (DRV(DNA)), the DNA is protected from enzyme digestion and remains intact (Figures 5.4a and b, lanes 1 and 2), as the enzyme is unable to attain access and reach its substrate. Due to the fact that the DNA is entrapped, the band of DNA is not visible on these gels as the plasmid along with lipid remains at the site of application. Though, when the lipid bilayers of DRV(DNA) liposomes are lysed with a solution of 2.5% SDS, the lipid bilayers are broken down and the

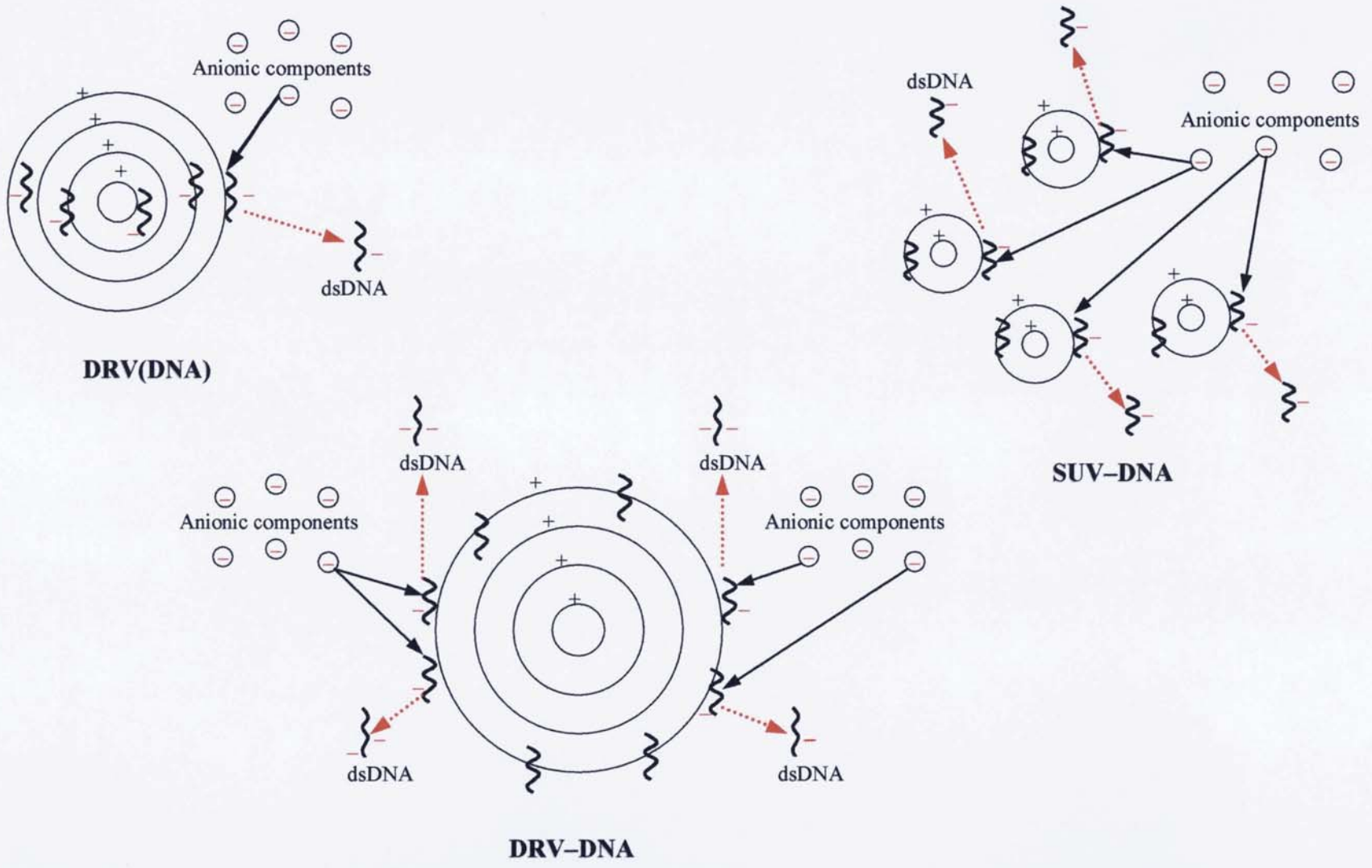


Figure 5.3. A schematic illustration of DNA displacement from various liposomal preparations. Anionic components compete with and knock off the anionic DNA bound to the cationic surface of the vesicles, with each liposomal preparation releasing different amounts of DNA, in relation to plasmid DNA spatial location.

vesicle contents is released, with Figure 5.5, lane 1 confirming that after enzyme digestion the entrapped plasmid DNA remains undamaged and protected from degradation within the liposome bilayers. Although, for surface complexed DNA where the DNA is readily accessible, Figures 5.4a and b (lanes 3-8) and Figure 5.5 (lanes 2-4) show that enzyme digestion is impeded due to the electrostatic interactions at the vesicles surface condensing the plasmid DNA thus preventing the enzyme attaining access (Gregoriadis *et al.*, 2002). In spite of this, when these preparations are applied *in vivo*, although complexed and condensed DNA is protected from enzyme digestion, any DNA bound to the surface of liposome-DNA complexes are presumably readily available for displacement by the negatively charged serum components present within the plasma, causing these formulations to lose a substantial amount of its DNA load. Furthermore, any displaced and free DNA within the biological milieu is susceptible to rapid degradation by intracellular nucleases before it can be effectively delivered to the required site, consequently reducing levels of transfection efficiency *in vivo* (Perrie *et al.*, 2001). Alternatively, entrapping DNA within closed bilayers of DRV reduces the level of DNA displacement, increasing the prospect of effective DNA delivery.

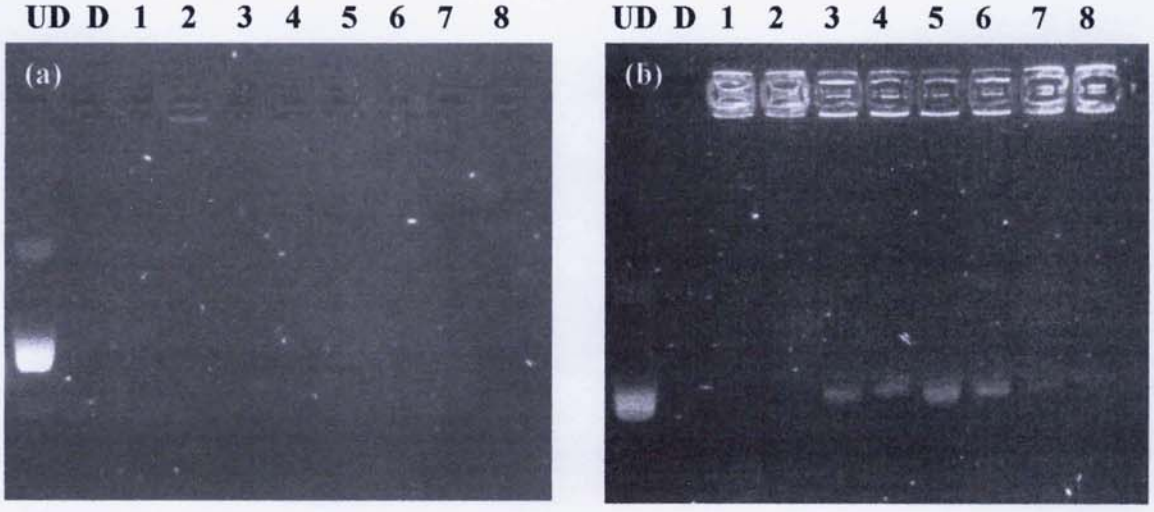


Figure 5.4 Agarose gel electrophoresis of various liposome preparations before and after digestion by digestive enzyme, DNase I. Liposomes composed of 16μmoles PC, 8μmoles DOPE and 4μmoles DC-Chol with 100μg plasmid DNA (pRc/CMV HBS) added. (Lanes UD and D, naked DNA before and after digestion at 0.1mg/ml; Lanes 1 and 2, DRV(DNA) before and after digestion; Lanes 3 and 4 , SUV-DNA before and after digestion; Lanes 5 and 6, DRV-DNA before and after digestion; Lanes 7 and 8, RV(DNA) before and after digestion). a, in the absence of competing anionic components and b, in the presence of anionic competitors.

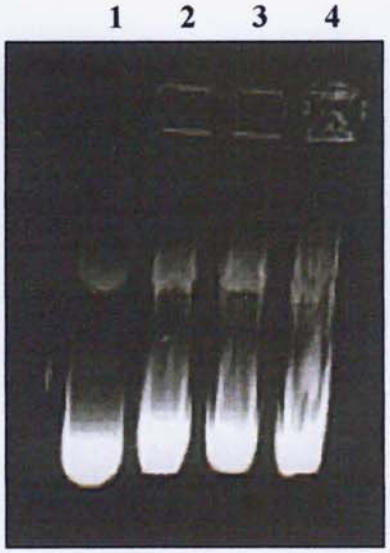


Figure 5.5 Agarose gel electrophoresis of various liposome preparations which have been lysed to confirm the presence of intact DNA after enzyme digestion. Liposomes composed of 16μmoles PC, 8μmoles DOPE and 4μmoles DC-Chol with 100μg plasmid DNA (pRc/CMV HBS) added. Liposome bilayers lysed with 2.5% SDS to confirm the presence of intact DNA (lane 1 DRV(DNA); lane 2, SUV-DNA; lane 3, DRV-DNA; lane 4, RV(DNA)).

5.3.3 The effect of helper lipid in DRV formulations

To assess the effect of helper lipid within liposomal systems, each liposomal preparation was tested, however this time the previous formulation of PC:DOPE:DC-Chol (16:8:4 μmoles/ml) was replaced with PC:Chol:DC-Chol (16:8:4μmoles/ml). By substitution of the helper lipid DOPE with Chol, there are moderate alterations in liposome physiochemical characteristics, as shown in Table 5.2. Replacement of DOPE with Chol, within these formulations had no influence on the association/entrapment values of SUV-DNA or DRV(DNA), which is agreement with previous studies reported with different plasmids and liposome compositions (Gregoriadis *et al.*, 1999; Perrie *et al.*, 2001; 2002). However, when Chol is present in the liposome bilayer and plasmid DNA is added at a later stage of the DRV procedure, during rehydration, surface complexation of plasmid DNA with preformed cationic DRV (DRV-DNA and RV(DNA)) resulted in lower DNA incorporation values than DRV entrapping DNA (DRV(DNA)) (83% and 71% vs 95%; Tables 5.2, respectively). Presumably, this results from the reduced bilayer and aqueous space availability and overall surface area for the DNA to bind to. However, this observed decrease in DNA incorporation to preformed DRV appears to only occur when Chol is present within the bilayer, as DOPE-containing liposomes (Table 5.1), exhibited consistently high levels of entrapment for all preparations. By inclusion of Chol, the liposomal bilayers become rigid and stabilise due to an increase in the order of orientation of the lipids within the bilayer, additionally causing an increase in the packing of the lipids hydrophobic chains (Bhattacharya & Haldar, 1996). As a consequence, before the addition of plasmid DNA, these preformed rigid Chol-containing

liposomal vesicles form multilayers, sealing off from the external environment with the majority of the lipids never coming into contact with the plasmid DNA. Preformed liposomes containing Chol are likely to be very structurally solid and stable, therefore impeding the capacity of DNA to become incorporated within the lipid bilayer. Therefore, the plasmid DNA is forced to resort only to surface binding, where as the presence of DOPE in the bilayer may allow bilayer re-organisation and liposome fusion thereby promoting additional complexing.

With respect to vesicle size, there were no significant differences ($P>0.05$) found between the vesicles formed from the two different helper lipids. For both, DOPE-containing DRV-DNA complexes and Chol DRV-DNA complexes (2700 nm and 2517 nm, respectively) are significantly larger than empty DRV and DRV(DNA) (for DOPE, 884 nm and 889 nm, respectively and for Chol, 805 nm and 900 nm, respectively), once again suggesting the presence of entrapped DNA in the latter liposomal preparation, as the presence of entrapped DNA does not significantly influence vesicle size of these Chol containing liposome systems (Table 5.2) (Perrie & Gregoriadis, 2000). However, in terms of zeta potential, there were significant difference ($P<0.05$) between the two lipids, showing that both empty DRV and DRV(DNA) liposome formulations composed of Chol produced higher zeta potential values (54-55 mV; Table 5.2) than those formulations composed of DOPE (41-42mV; Table 5.1). Therefore, in this instance, the variations in surface charge results from interactions occurring between lipids within the bilayer rather than lipid-DNA interactions. It has been suggested (Perrie & Gregoriadis, 2000) that some of the cationic charges may be made more available for measurement by the presence of Chol or even that the zwitterionic nature of the DOPE may be concealing some of the cationic charges of the cationic lipid, due to the formation of salt bridges between the charged head-groups of cationic lipids and zwitterionic head-group of such PE lipids (Zuidam & Barenholz, 1999). However, although the measured surface charges of Chol-containing preparations were higher than DOPE, the same trend in surface neutralisation is observed, where the liposome-DNA complexes, such as SUV-DNA and DRV-DNA, produce significantly lower zeta potential values in comparison to their empty counterparts (empty SUV and empty DRV, respectively), whereas with the DRV entrapping DNA the surface of charge of DRV liposomes is unaltered, as shown when comparing Tables 5.1 and 5.2.

Taken together, these results (Tables 5.1 and 5.2) indicate that the type of helper lipid incorporated within liposome bilayers, either DOPE or Chol, in various liposomal preparations, show significant variation with regard to zeta potential measurements and DNA incorporation values regarding preformed DRV. Although interestingly, the type helper lipid used does not seem to affect vesicle size of any of the liposomal preparations.

Liposome preparation	DNA incorporation (% of used ± SD)	Size (nm ± SD)	Zeta potential (mV ± SD)
SUV	–	86 ± 4	44.3 ± 9.0
SUV–DNA	94.1 ± 1.6	252 ± 10 *	37.6 ± 7.1 *
DRV	–	805 ± 104	54.4 ± 3.5
DRV(DNA)	94.7 ± 2.3	900 ± 72	54.5 ± 3.8
RV(DNA)	70.6 ± 8.2	1630 ± 121 **	47.2 ± 5.8 **
DRV–DNA	82.6 ± 2.4	2517 ± 864 **	39.2 ± 12.5 **

Table 5.2 Effect of liposome preparation procedure on DNA incorporation, size and zeta potential. Of liposomes composed of 16μmoles of PC, 8μmoles of Chol, and 4μmoles of the cationic lipid DC-Chol. Dehydration and rehydration of SUV in the absence of DNA resulted in the formation of "empty" water containing DRV(DNA). Incubation of these preformed DRV with a solution of plasmid DNA (100μg) for 30 min resulted in the formation of DRV with surface complexed DNA (DRV–DNA). Dehydration and rehydration of SUV (freeze-dried in the absence of DNA) with a solution of 100μg DNA produced RV(DNA). Incorporation values were based on PicoGreen analysis. The vesicle z-average diameter and zeta potential of each liposomal preparation was measured in double-distilled water or 0.001 M PBS, respectively, at 25°C using a Brookhaven ZetaPlus. * signifies that the characteristics (i.e. size and surface charge) of SUV-DNA complexes are significantly ($P<0.05$, ANOVA) different from empty SUV. ** signifies that the characteristics (i.e. size and surface charge) of DRV-DNA and RV(DNA) are significantly ($P<0.05$, ANOVA) different from empty DRV. Results denote mean ± SD, $n = 3$.

5.3.4 Effect of cationic lipid within DRV formulations

Previous reports (Gregoriadis *et al.*, 1997) have shown that cationic liposomes entrap DNA more efficiently than neutral or anionic liposomes due to electrostatic interactions with the anionic plasmid DNA. Table 5.3 illustrates that the presence of the cationic lipid DC-Chol, a derivative of natural cholesterol (Fang *et al.*, 2003), consisting of a tertiary amine covalently linked to two methyl groups within its terminal, increases entrapment levels by almost two-fold to 95%, in contrast to 52% for liposome formulations excluding this cationic lipid (i.e. PC:Chol). Upon dehydration the lipid and solute are brought into close contact in an

anhydrous state, and subsequently under controlled rehydration the vesicles lose functionality due to a loss of stability causing vesicles to fuse into larger multilamellar liposomes entrapping solutes as they form (Perrie *et al.*, 2000). By addition of a cationic lipid within the liposomal formulation and rendering vesicles in a more positive state, as shown by the measured zeta potential of 54.5 ± 3.8mV (Table 5.3), the anionic DNA efficiently binds and condenses to the cationic charges of vesicles, greatly enhancing lipid-DNA interactions, thereby promoting an increase in solute entrapment than neutral liposomes. DRV omitting DC-Chol had a neutral zeta potential of 0.2 ± 0.1mV, which may explain lower incorporation values for this formulation, due to the reduced availability for anionic DNA to bind electrostatically (Table 5.3).

Formulation	DNA incorporation (% ± SD)	Size (nm ± SD)	Zeta potential (mV ± SD)
PC:Chol	51.5 ± 2.7	1281 ± 56	0.19 ± 0.1
PC:Chol:DC-Chol	94.7 ± 2.3	900 ± 72	54.5 ± 3.8
PC:Chol:DOTAP	90.0 ± 3.0	932 ± 123	53.9 ± 1.5

Table 5.3 Physiochemical characteristics and percentage plasmid DNA entrapment of DRV entrapping DNA comparing neutral and cationic liposomes. Neutral liposomes composed of 16μmoles PC and 16μmoles Chol and cationic liposomes composed of 16μmoles PC, 8μmoles Chol and 4μmoles DC-Chol or DOTAP were prepared by the DRV method with 100μg plasmid DNA (pRc/CMV HBS) was added to each formulation. Percentage entrapment was estimated based on the PicoGreen assay reagent. Vesicle z-average diameter and zeta potential of each liposomal preparation was measured in double-distilled water or 0.001 M PBS, respectively, at 25°C using a Brookhaven ZetaPlus. Results denote mean ± SD, n = 3.

The cationic DRV containing PC:Chol:DC-Chol (16:8:4 μmole/ml) produced vesicle sizes of around 900 ± 72nm, which can be attributed to the inclusion of DC-Chol within the formulation (Table 5.3). Figure 5.6 shows a diagrammatic illustration of events that may occur in vesicle formation during the freeze-drying and rehydration stages, comparing PC:Chol and PC:Chol:DC-Chol liposome formulations. The cationic nature of vesicles composed of DC-Chol promotes bilayer repulsion consequently reducing vesicle fusion between individual liposomes during the rehydration process, thus leading to a reduction in DRV size (Figure 5.6). Conversely, vesicle fusion is enhanced within PC:Chol (16:16

μmole/ml) liposomes, due to the lack of electrostatic repulsive forces acting between these neutral liposomes, causing the formation of large aggregates with vesicle sizes of 1281 ± 56nm. The inclusion of DOTAP as the cationic lipid in replacement of DC-Chol had no influence on DNA entrapment values or vesicle size and there were no differences in zeta potential values between liposomes formulated with either DC-Chol or DOTAP, as would be expected due to their similar protonation.

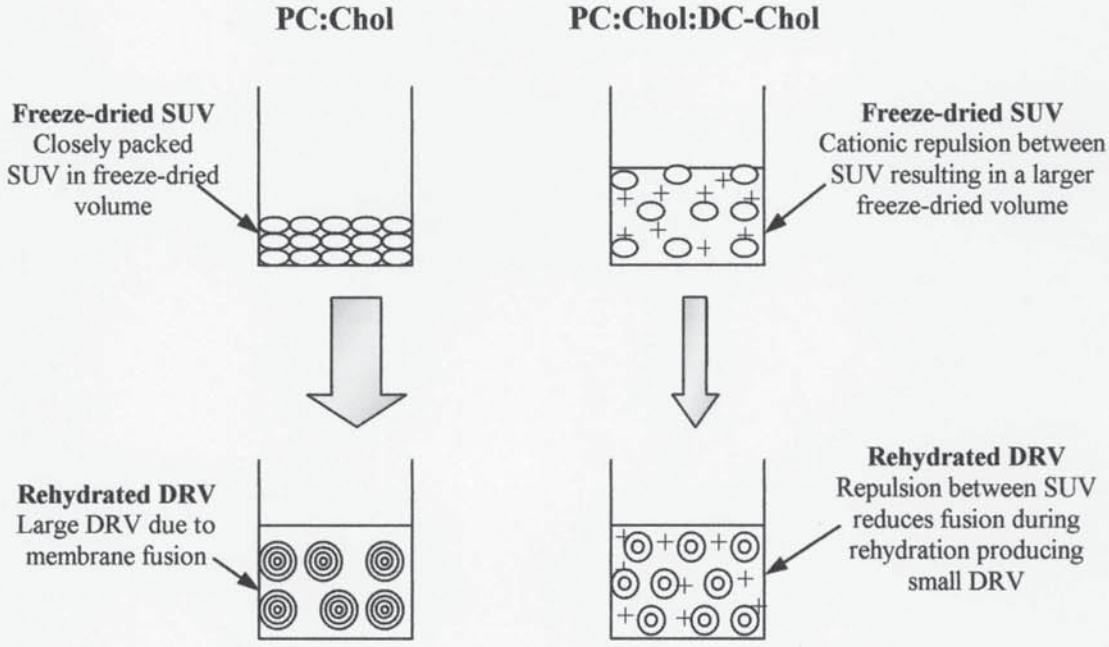


Figure 5.6 Diagrammatic illustration of events that may occur during controlled dehydration and rehydration. Liposome formulations composed of either PC:Chol (16:16 μmoles) to give 'neutral' vesicles or PC:Chol:DC-Chol (16:8:4 μmoles) to give 'cationic' vesicles.

5.3.5 Effect of lipid alkyl chain length on DRV formulations

To study the physiochemical characteristics of liposome vesicles containing lipids of various alkyl chain lengths, liposomes were prepared where PC was either included or substituted by lipids consisting of longer alkyl chains, DMPC (C_{14}) and DSPC (C_{18}). Further formulations were prepared where DOPE was replaced with Chol. Table 5.4 shows DNA incorporation values as a percentage of total plasmid DNA added (pRc/CMV HBS; 100 μg) and zeta potential values of all formulations tested. DNA incorporation values for all liposomal

formulations remained consistently high, ranging from 93-99%, again demonstrating the high efficiency of the DRV procedure at entrapping plasmid DNA, irrespective of the lipids alkyl chain length or the helper lipid included within the liposomal formulation. Once more, Table 5.4 illustrates that for all formulations tested DOPE-containing liposomes exhibit lower zeta potential values than those containing Chol. In addition, it seems that when DSPC is included within the formulation zeta potential values are lower than PC or DMPC, for both DOPE and Chol-containing DRV.

Formulation	DNA incorporation (% ± SD)	Zeta potential (mV ± SD)
PC:DOPE:DC-Chol (16:8:4 μmoles)	93.3 ± 3.3	41.4 ± 2.8
PC:Chol:DC-Chol (16:8:4 μmoles)	94.7 ± 2.3	54.5 ± 3.8
PC:DOPE:Chol:DC-Chol (16:8:8:4 μmoles)	99.2 ± 2.1	43.9 ± 2.7
DMPC:DOPE:DC-Chol (16:8:4 μmoles)	99.8 ± 1.6	47.1 ± 6.0
DMPC:Chol:DC-Chol (16:8:4 μmoles)	99.7 ± 1.3	53.2 ± 2.8
DSPC:DOPE:DC-Chol (16:8:4 μmoles)	98.9 ± 1.1	38.8 ± 4.9
DSPC:Chol:DC-Chol (16:8:4 μmoles)	99.3 ± 1.3	45.5 ± 1.9

Table 5.4 The effect of alkyl chain length and helper lipid on incorporation of plasmid DNA and zeta potential of DRV(DNA) formulations. Plasmid DNA (pRc/CMV HBS; 100μg) was incorporated into DRV of various formulations. DNA incorporation values were determined by PicoGreen analysis and zeta potential values of each formulation were measured in 0.001 M PBS, at 25°C using a Brookhaven ZetaPlus. Results denote mean ± SD, $n = 3$.

5.4 Addition of a cyroprotectant and its role in bilayer stability: a modified DRV procedure for the formation of small liposomes

In some cases, DRV(DNA) formulations are required to remain stable within the circulating blood for prolonged periods of time, in order to reach their target cells or to equally provide optimum biodistribution throughout the body. Liposome-entrapped DNA vesicles prepared by the dehydration-rehydration (DRV) procedure (Gregoriadis *et al.*, 1999) produce liposomes of relatively large size (>200nm). *In vivo*, these large liposomes can be rapidly cleared from blood circulation and as a result may not reach the desired site to elicit

appropriate immunity to the encoded antigen (Zadi and Gregoriadis, 2000). Vesicles of a small size have shown to prolong the presence of liposomes *in vivo* and accordingly deliver the plasmid DNA directly and efficiently to the APC (Zadi and Gregoriadis, 2000). It was discovered that after intravenous injection, those vesicles that possess a larger size than 200nm, were cleared from the blood circulation very rapidly and digested by enzymes, when they are redirected towards the reticuloendothelial system (Gregoriadis, 2003). Therefore, in such instances, the production of small liposomes (<200nm) may be advantageous. With regard to subcutaneous and intramuscular injection, larger liposomes are more likely to remain at the site of injection forming a depot system, therefore smaller liposomes are more likely to deliver the DNA efficiently to the target cell and thus may also be advantageous for these routes of administration.

There are various ways of producing small vesicles, including extraction, microfluidization and sonication. However, these procedures are carried out in the presence of the entrapped material and are therefore, due to the disruptive effects of these methods, are only reliable for certain solutes. Plasmid DNA is very susceptible to the destructive and detrimental effects of these procedures, and are therefore most likely to shear and cleave under these conditions. In 2000, Zadi and Gregoriadis developed a novel and non-destructive method of producing small liposomal vesicles, which are still able to retain a high percentage of entrapped solutes. It is a modification of the DRV procedure, where a disaccharide is utilised as a cryoprotectant during freeze-drying. The method is an extension of Crowe and Clegg's (1973) suggestion that disaccharides added to the vesicles could replace the water that normally surrounds the polar groups of the lipids. When liposomes are subjected to the DRV procedure, the freeze-drying process removes the water content of the vesicles. With water removal, the vesicles fuse due to the physical changes in the bilayer structure, with a transition from the gel to liquid phase, creating larger liposomes than those prior to the process (i.e. SUV). The disaccharides interact with the polar groups of the phospholipids by hydrogen bonds and as a consequence stabilise the vesicles during freeze-drying (Crowe *et al.*, 1988). This hypothesis is known as the 'water replacement hypothesis' as proposed by Oliver *et al.*, (1998).

To study the physiochemical characteristics of DRV with the addition of a cyroprotectant during the freeze-drying stage, SUV composed of PC:DOPE:DC-Chol (16:8:4μmoles) were

formulated and increasing amounts of the disaccharide sucrose was added in terms of sucrose to lipid weight ratio, in order to determine the optimum sucrose concentration in which size decreases to below 200nm however, while maintaining a high plasmid DNA load. As shown in Figure 5.7 and as demonstrated in previous studies (Zadi and Gregoriadis, 2000), increasing sucrose to lipid ratio causes a substantial decrease in vesicle size.

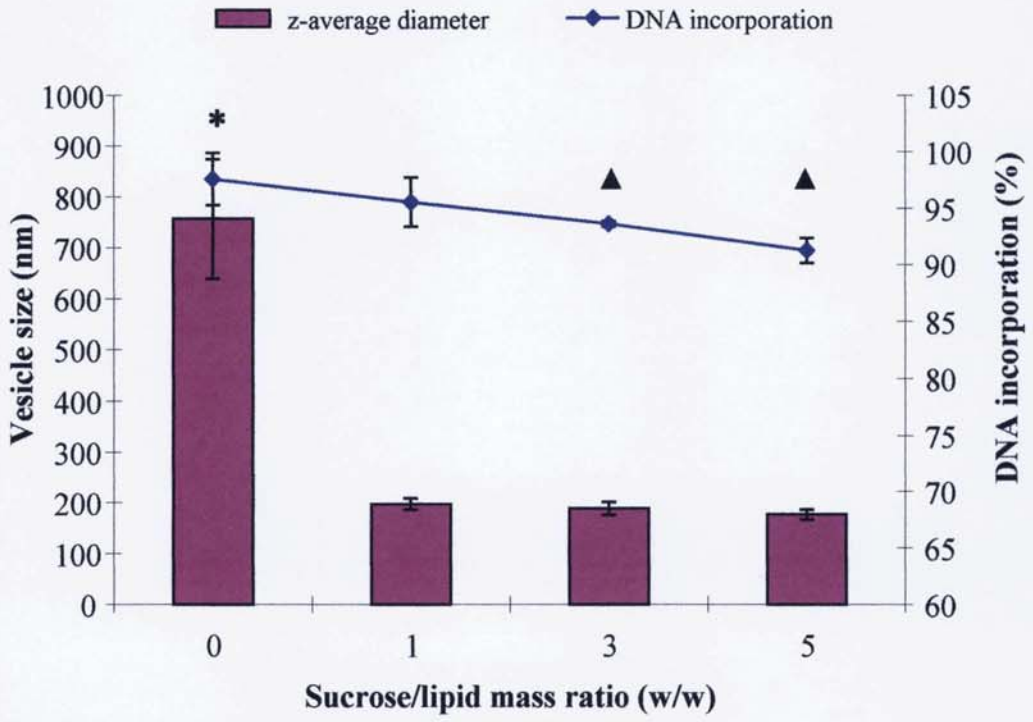


Figure 5.7 Effect of increasing sucrose/lipid ratio on vesicle size and percentage entrapment of plasmid DNA within DRV liposomes. Increasing amounts of sucrose was added to PC:DOPE:DC-Chol (16:8:4μmoles) liposome suspensions containing 10.2mg/ml total lipid to generate sucrose/lipid mass ratios of 0, 1, 3 and 5. The vesicle z-average diameter of each liposomal preparation was measured in double-distilled water at 25°C using a Brookhaven ZetaPlus and incorporation values were based on PicoGreen analysis. * signifies that liposomes containing a sucrose/lipid weight ratio of 0 are significantly ($P<0.05$) larger than those containing weight ratios of 1, 3 and 5. ▲ signifies that sucrose/lipid weight ratios of 3 and 5 significantly ($P<0.05$) reduce DNA incorporation values. Results denote mean ± SD, $n = 3$.

Vesicle sizes were shown (Figure 5.7) to reduce considerably when sucrose is added to the liposome suspension, demonstrating that even with sucrose to lipid mass ratio of 1:1 the vesicle size reduces considerably from 757 nm (i.e. liposomes without sucrose) to 198 nm. During the DRV procedure, the liposomes endure two distinct stresses; freezing and drying (Allison, *et al.*, 2000). Upon rehydration, the liposome bilayer membranes lose functionality and become disrupted, causing the membranes to fuse and aggregate, resulting in the

production of large vesicles (757 nm) in which the antigen becomes entrapped. However, in the presence of sugars this disruption does not occur (Crowe *et* al, 1985), although, it still remains relatively unclear to exactly how disaccharides interact and protect lipid bilayers. Previously, Levine & Slade (1988 & 1992) proposed that in the presence of disaccharides, the lipid membrane is maintained in a glassy excipient matrix, acting as a protective barrier and immobilising the lipid membrane, consequently reducing membrane-membrane fusion (Crowe and Crowe, 1995) and plasmid DNA leakage. This theory, known as the 'vitrification hypothesis', suggests that the formation of a glassy matrix is sufficient enough to prevent aggregation and maintain vesicle size (Levine & Slade, 1992). Oliver *et al.*, (1998) highlights the important role vitrification of carbohydrates plays in the protection of lipid bilayers during dehydration-rehydration, however, it is not the only important factor involved, as the liquid crystalline state needs to be maintained in order to prevent physical changes of the lipid bilayer reducing leakage of plasmid DNA from the vesicles. This is accomplished by the presence of the carbohydrate, which depresses the gel to liquid crystalline phase transition temperature. Disaccharides are said to maintain the lipid bilayer membranes in a liquid crystalline state upon rehydration and prevents phase transitions occurring from gel to liquid phase (Crowe *et al*, 1988). The disaccharide replaces the water molecules situated between the polar components, and therefore it stabilises the membrane bilayer. With the presence of disaccharides, during the freeze-drying process, the vesicles original structure is relatively maintained. By increasing the sucrose to lipid mass ratio further, the reduction in size is minimal. Therefore, a sucrose/lipid mass ratio of 1 fulfils the criteria for producing small liposomes (198 nm) with high entrapment (96%).

Effective cryoprotectants, which immobilise the lipids and stabilises liposome membranes during freeze-drying, should possess a balance between two distinct properties; firstly, the sugar molecules should efficiently associate and interact with the phospholipid head groups, arranging themselves within the bilayer, so impeding the leakage of solute content. Furthermore, the T_g of the sugar should be sufficiently high to prevent vesicle fusion (Wolkers *et al.*, 2004). There are many stabilisers to select, but all differ in efficiency. Earlier work (Wolkers *et al.*, 2004) has shown that most disaccharides are able to protect lipid membranes, as these sugar molecules are small enough to associate with the phospholipid and the high T_g of these molecules enable stabilisation of the liposomes in a dry lipid state.

Figure 5.7 shows that ratios 1, 3 and 5 all produce vesicle sizes at approximately 200nm or lower, but simultaneously maintain a relatively high DNA load (between 97-89%). Although DNA incorporation is shown to significantly decrease ($p<0.05$, ANOVA) at the higher weight ratios (i.e. 3 and 5), incorporation of 89% plasmid DNA is still relatively high and sufficient. Conversely, Zadi & Gregoriadis (2002) found that the lower ratio of 1:1 was the ideal sucrose/lipid mass ratio when entrapping water-soluble solutes such as penicillin, riboflavin, doxorubicin, etc, with entrapment reducing considerably with the addition and increase of sucrose. Therefore, it is thought that the solutes applied by Zadi & Gregoriadis are more reliant on the destabilisation of the vesicles in order to become encapsulated within the lipid bilayers. In this instance, when the dry lipid is rehydrated in the absence of sugars, the lipid bilayer re-arrange and fuse to form larger, aggregated vesicles and as the DNA is in very close contact with the cationic liposomes, a high percentage of DNA becomes entrapped in the aqueous spaces within bilayers. Even though reduced liposome fusion occurs in the presence of disaccharides, it could be considered that upon rehydration these vesicles may be destabilised to the extent where DNA can become entrapped within the bilayers, still producing a high percentage of DNA entrapment (Figure 5.7). However, with further reduction in liposome size it is highly likely that the majority of the plasmid DNA is associated to the liposome surface due to electrostatic interactions, rather than true entrapment. Therefore, upon gel electrophoresis in the presence of anionic competitors, Figure 5.8 shows that as the sucrose/lipid increases, the amount of DNA displaced increases. In contrast, when the samples were mixed with a sucrose solution in replacement of loading buffer, where there are no anionic competitors present to displace the surface bound DNA, plasmid DNA remains at the site of application (Figure 5.9).

This implies that as vesicle size decreases, as a result of membrane stabilisation by sucrose, plasmid DNA has to revert to surface complexation, as the DNA is unable to obtain entry into the lipid bilayers in the absence of vesicle fusion. For this reason, it is thought that the electrostatic interactions that occur between the cationic lipid and the anionic DNA will also assist in retaining high incorporation levels. The anionic plasmid DNA binds to the cationic charges of the liposomal surface thus preventing dissociation during rehydration and maintaining the majority of its DNA load, despite the insufficiency of vesicle fusion. In this instance the incorporation of DNA into lipid bilayers is less reliant on vesicle destabilisation as the DNA can bind to the cationic surface of liposomes. Furthermore, as shown in Figure

5.10, surface zeta potential decreases with increasing sucrose content, verifying that due to sucrose stabilised bilayers and the reduction in vesicle fusion the DNA is reverting to surface complexation causing surface neutralisation.

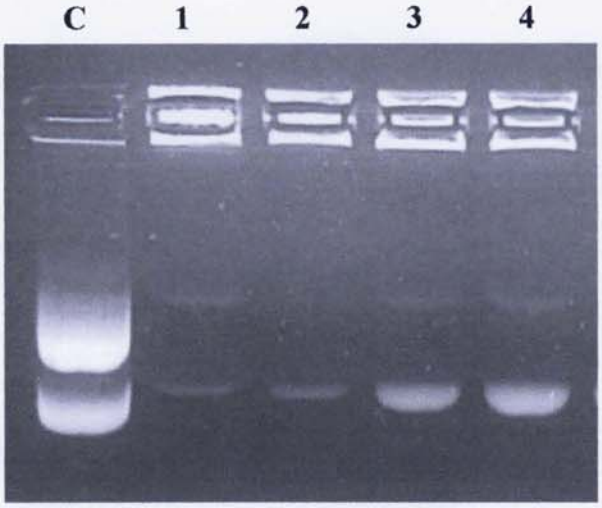


Figure 5.8 Agarose gel electrophoresis of plasmid DNA, pRc/CMV HBS, entrapped within liposomes with different sucrose/lipid mass ratios in the presence of competing anionic components. Samples were applied to 0.8% agarose gel. (Lane C, 0.1mg/ml plasmid DNA; Lane 1, sucrose/lipid mass ratio of 0; Lane 2, sucrose/lipid mass ratio of 1; Lane 3, sucrose/lipid mass ratio of 3; Lane 4, sucrose/lipid mass ratio of 5; Lane 5, sucrose/lipid ratio of 10).

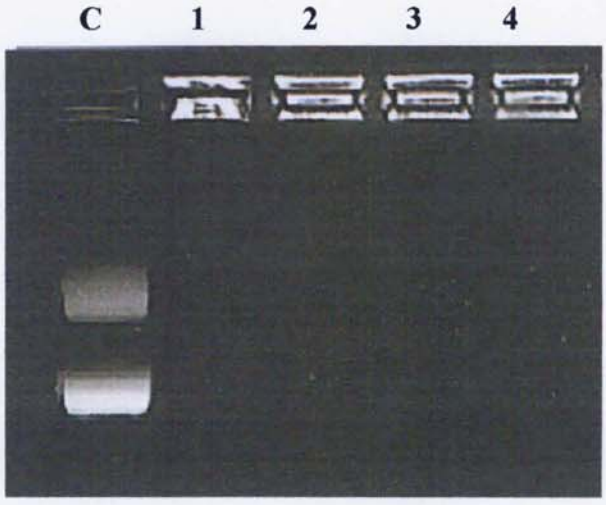


Figure 5.9 Agarose gel electrophoresis of plasmid DNA, pRc/CMV HBS, entrapped within liposomes with different sucrose/lipid mass ratios in the absence of competing anionic components. Samples were applied to 0.8% agarose gel. (Lane C, 0.1mg/ml plasmid DNA; Lane 1, sucrose/lipid mass ratio of 0; Lane 2, sucrose/lipid mass ratio of 1; Lane 3, sucrose/lipid mass ratio of 3; Lane 4, sucrose/lipid mass ratio of 5; Lane 5, sucrose/lipid ratio of 10).

A balance between percentage entrapment and size needs to be determined, as the ideal is to produce liposomes of a small size (200 nm) with DNA content within the bilayers remaining relatively high. This is achieved with a sucrose to lipid mass ratio of 1:1.

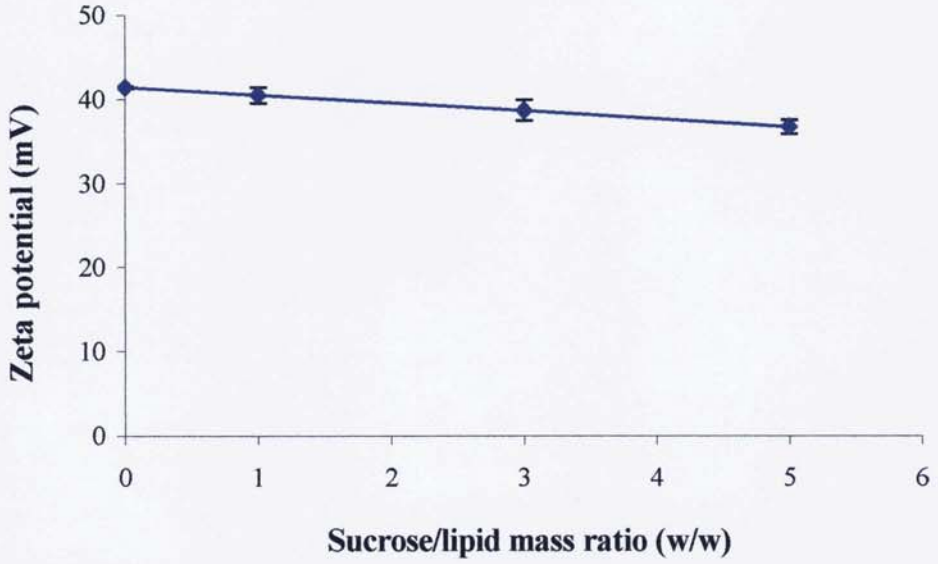


Figure 5.10 Effect of increasing sucrose/lipid ratio on vesicle surface charge in the presence of 100μg plasmid DNA. Increasing amounts of sucrose was added to liposome suspensions containing 10.2mg/ml total lipid to generate sucrose/lipid mass ratios of 0, 1, 3 and 5. The zeta potential of each liposomal preparation was measured in 0.001 M PBS, at 25°C using a Brookhaven ZetaPlus. Results denote mean ± SD, $n = 3$.

5.5 Transfection efficiency of DRV liposome formulations *in vitro*

Further to the transfection results obtained in chapter 4, a select few liposome formulations were elected which were then prepared using the DRV procedure. Previous studies report that DRV entrapping DNA (DRV(DNA)), elicits greater levels of immunity than surface complexed (Gregoriadis *et al.*, 1996). In addition, results presented previously in this chapter reveal that entrapping plasmid DNA provides better protection against anionic components within serum and the biological milieu, therefore, presumably DRV(DNA) *in vivo* should transfect cells and deliver DNA more efficiently, however very few reports have shown how well these liposomes-entrapping DNA transfect cells *in vitro*.

Figure 5.11 shows that Chol:DC-Chol (1:1 molar ratio), prepared by the DRV procedure, produced significantly higher values of transfection efficiency than either DOPE:DC-Chol (1:1 molar ratio) or DOPE:Chol:DC-Chol (0.5:0.5:1 molar ratio). However, results in Figure 5.11 also show that the luciferase activity of DRV(DNA) preparations are considerably

lower when compared to SUV-DNA complexes formulated with the same composition (as shown in chapter 4), showing to be approximately 10-20 times lower in efficiency. Within *in vitro* conditions, formulations are only incubated with COS-7 cells for a time period of 5 hours, after which the formulations are removed and the cells are incubated for a further 48 hours before luciferase assay is carried out. Within this small period of time the liposomal vesicles and its plasmid DNA contents need to interact with the cultured cells and gain entry into the cells presumably by endocytosis (Zhdanov *et al.*, 2002). Following this, the plasmid DNA needs to dissociate from the liposomal vesicle and subsequently be released from the endosome, after which the key stage follows, where the DNA must gain entry to the nucleus followed by gene expression (Tomlinson & Rolland, 1996; Zhdanov *et al.*, 2002). This highlights the major obstacles and numerous pathways that the plasmid DNA needs to progress through in order to generate gene expression. With the plasmid DNA entrapped within membrane bilayers, DNA dissociation and release from DRV requires much more energy and force than SUV-DNA complexes. As not only does the DNA need to dissociate from the liposomes cationic charges, it is essential that the lipid bilayer breaks down in order to expose the entrapped DNA. Previous reports (Perrie *et al.*, 2001) have shown that some of the liposomes may act as a depot, where the vesicles release their DNA content slowly, therefore DNA release from such DRV may not be fast enough for gene expression to occur *in vitro*.

However, as Figure 5.11 shows, despite the fact that the DRV formulations entrapping DNA produce lower values of transfection than SUV-DNA complexes *in vitro*, these DRV formulations nonetheless produce significantly higher levels of activity when compared to naked DNA, confirming the ability of DRV liposomes to deliver DNA effectively to COS-7 cells.

The above theory was confirmed by further *in vitro* transfection studies, in which COS-7 cells were transfected with liposome-DNA complexes composed of DOPE:DC-Chol, at a 1:1 molar ratio, prepared by four different methods; DNA complexed to MLV, SUV and DRV (i.e. MLV-DNA, SUV-DNA and DRV-DNA) and also DNA entrapped within DRV (i.e. DRV(DNA)). Interestingly, results in Figure 5.12 show that DRV-DNA complexes produced the highest value of luciferase activity (1747% of lipofectin control) proving to be significantly ($P<0.05$) better than MLV-DNA or SUV-DNA complexes.

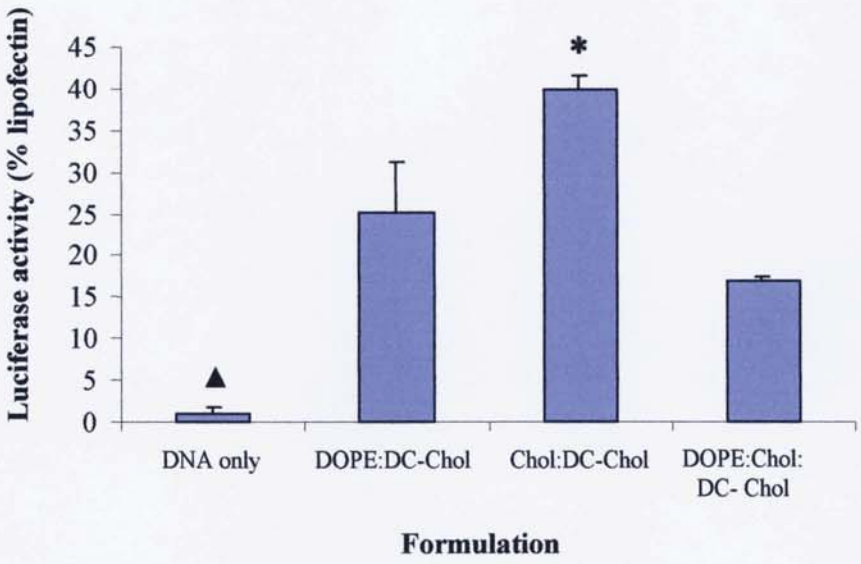


Figure 5.11 Transfection efficiency of various DRV(DNA) formulations *in vitro*. Liposomes were formulated with either DOPE:DC-Chol (1:1molar ratio), Chol:DC-Chol (1:1 molar ratio) and DOPE:Chol:DC-Chol (0.5:0.5:1 molar ratio), which were mixed with plasmid DNA to give a lipid:DNA charge ratio of 1:1. Liposomes were prepared by the DRV procedure. * signifies that Chol:DC-Chol prepared by the DRV procedure transfects COS-7 cells significantly better ($P<0.05$, ANOVA) than those liposomes containing DOPE or a combination of the two helper lipids. ▲ signifies that all DRV(DNA) formulations produce significantly ($P<0.05$, ANOVA) higher levels of transfection efficiency than DNA only. Results denote mean ± SD, $n = 3$.

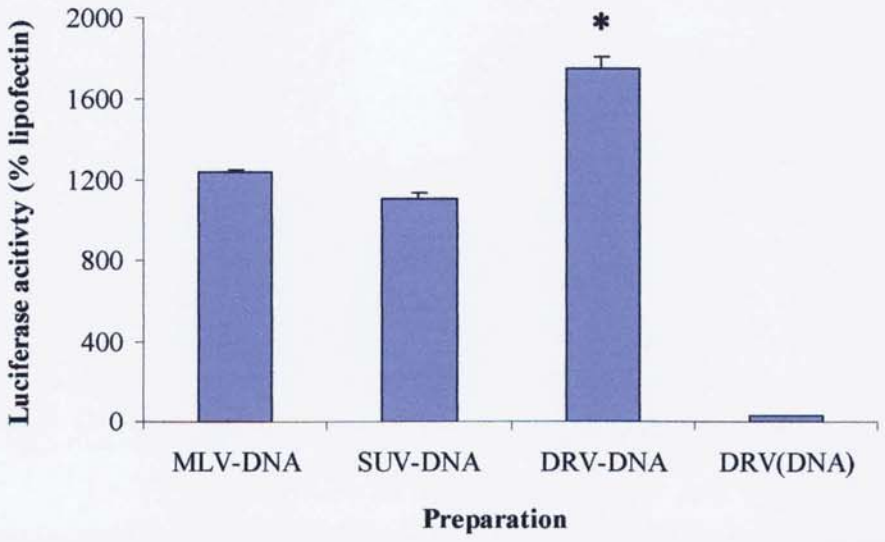


Figure 5.12 Effect of preparation on transfection efficiency of liposomes composed of DOPE:DC-Chol (1:1 molar ratio). Liposomes composed of DOPE:DC-Chol (1:1 molar ratio) were prepared by four different methods. Plasmid DNA was complexed to the surface of liposomes of various morphologies; MLV-DNA, SUV-DNA, DRV-DNA or either entrapped within DRV as prepared by the DRV procedure; DRV(DNA). Total lipid concentration of 0.0078 μmole/ml containing 1 μg/ml plasmid DNA was added to COS-7 cells. Transfection efficiency was determined by luciferase assay and results are expressed as percentage of positive control (i.e. lipofectin). * signifies that DNA complexed to the surface of DRV liposomes (i.e. DRV-DNA) produce significantly ($P<0.05$, ANOVA) higher values of transfection efficiency than any other preparation. Results denote mean ± SD, $n = 3$.

Preparation	Size (nm ± SD)	Zeta potential (mV ± SD)
MLV	579.4 ± 35.9	43.9 ± 5.8
MLV-DNA	1024 ± 183	-33.8 ± 12.2
SUV	78.7 ± 4.5	44.0 ± 14.5
SUV-DNA	363.4 ± 23.2	-43.3 ± 14.0
DRV	393.1 ± 15.2	49.1 ± 8.5
DRV-DNA	614.8 ± 59.6	-28.9 ± 8.0
DRV(DNA)	421.6 ± 19.5	-22.3 ± 9.7

Table 5.5 Physiochemical characteristics of various liposomal preparations used in transfection studies. Liposomes composed of DOPE:DC-Chol (1:1 molar ratio, at a total lipid concentration of 1.56 μmole/ml) were prepared by various methods. Size and zeta potential of each empty SUV and SUV-DNA complexes was measured on a ZetaPlus (Brookhaven Instruments), in ddH_2O and 0.001 M PBS, respectively, at 25°C.

However, presumably transfection efficiency of these different preparations is due to the morphological differences between each system (i.e. number of bilayers) and the localisation of the plasmid DNA, as there is no apparent trend between transfection efficiency and the physiochemical characteristics of each liposomal preparation (Table 5.5).

To study the transfection efficiency of DRV formulations further, formulations were made in which PC was included in combination with either DOPE or Chol and the cationic lipid DC-Chol, formulated with higher lipid molar ratios (as used within section 5.3). In addition, formulations omitting PC were also tested. The transfection efficiency of all these DRV(DNA) formulations were compared against their SUV-DNA counterparts. In a further preparation, sucrose (at a 1:1 sucrose to lipid mass ratio) was added prior to the freeze-drying stage, in order to study how DRV size affected transfection efficiency *in vitro*.

As shown in Figure 5.13, when plasmid DNA was complexed to SUV composed of DOPE:DC-Chol (8:4 μmoles), transfection efficiency was approximately 10 times higher than DRV-entrapping DNA of the same composition, with significantly ($P<0.05$) greater values of luciferase activity. Once again, this demonstrates the enhanced ability of liposome-DNA complexes to transfect cells *in vitro*. However, with this DOPE:DC-Chol formulation, the μmoles of DOPE used was double that of the cationic lipid, DC-Chol, whereas within previous formulations, the molar ratio of DOPE:DC-Chol was 1:1. By increasing the ratio of

DOPE, transfection efficiency decreases from 498% (of lipofectin control) for DOPE:DC-Chol at a 1:1 molar ratio to 47% for DOPE:DC-Chol at a 2:1 molar ratio, as shown previously within chapter 4, section 4.4.

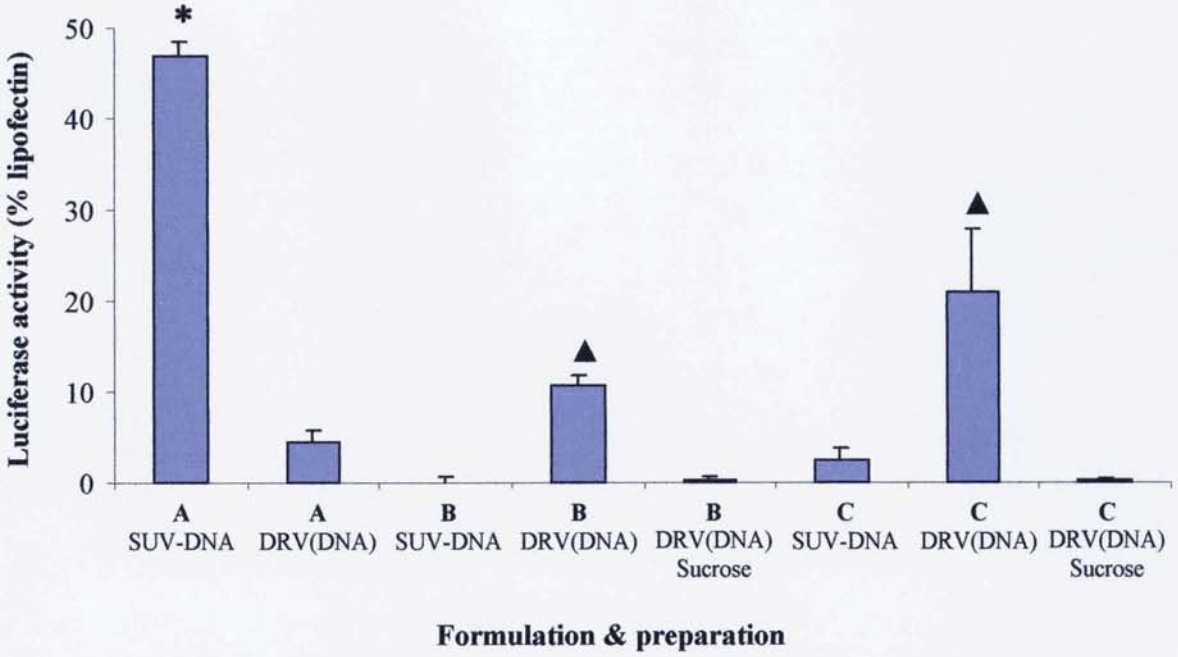


Figure 5.13 Effect of liposome preparation and composition on transfection activity *in vitro*. Liposomes were formulated as follows: A, DOPE:DC-Chol (8:4 μmoles); B, PC:DOPE:DC-Chol (16:8:4 μmoles); C, PC:Chol:DC-Chol (16:8:4 μmoles) and were prepared by different methods. * signifies that activity is greater for SUV-DNA complexes than DRV(DNA), ▲signifies that freeze-drying in the absence of a cryoprotectant produces enhanced levels of activity than liposomes freeze-dried in the presence of a cyroprotectant (i.e. sucrose), for both formulations. Results denote mean ± SD, $n = 3$.

Results presented in chapter 4, demonstrated that SUV-DNA complexes omitting PC, produced significantly higher levels of transfection efficiency than complexes including this lipid. However, contrary to this, Figure 5.13 show there is a remarkable reversal in terms of transfection efficiency where activity increases with the addition of PC within DRV-entrapping DNA preparations. It appears that for DRV(DNA), stabilisation is the key to increasing transfection efficiency, as the addition of PC or Chol or both, is known to stabilise lipid bilayers of liposomal vesicles and it is likely that bilayer stability (Bhattacharya & Halder, 1996; Liu *et al.*, 2000) is essential for these vesicles to maintain its DNA load. So therefore, when Chol is substituted with DOPE, effectively reducing bilayer rigidity and stability, Figure 5.13 shows that there is a significant ($P<0.05$) reduction in values of transfection activity (21% vs. 10%, respectively; Figure 5.13). In the case of SUV, instability

is beneficial in order for these vesicles to interact with and fuse around the anionic DNA (Farhood *et al.*, 1995). Overall, however, for optimum transfection *in vitro* it appears that it is more favourable for liposomal vesicle to possess an innate instability, in order for effective endocytosis and entry into the cultured cells to generate gene expression. By the addition of a cyroprotectant (i.e. sucrose) to the formulations PC:DOPE:DC-Chol (16:8:4 μmole/ml) and PC:Chol:DC-Chol (16:8:4 μmole/ml) at a 1:1 sucrose to lipid mass ratio prior to freeze-drying, there was a significant reduction in transfection efficiency. This could be attributed to the addition of sucrose causing a decrease in DRV size, as previous results in chapter 4 show it seems that the smaller the liposomes are less effective transfecting agents.

5.6 Conclusion

DRV liposomes when produced by the DRV procedure generate multilamellar DRV liposomes, which possess very different physiochemical characteristics and retain more of its DNA load than the comparative cationic liposomal complexes. Freeze-drying SUV liposomes in the presence of plasmid DNA generates liposomal structures where the DNA is predominantly entrapped within the bilayers (DRV(DNA)). It appears that this is the preferable procedure as freeze-drying in the absence of DNA but rehydrating in the presence of DNA results in a mixture of both entrapped and complexed DNA (RV(DNA)). In this instance the vesicle size increases, zeta potential decreases and n increased DNA displacement in the presence of competitive anions. By the addition of the cyroprotectant, sucrose, liposomal vesicle size decreases while still maintaining high levels of DNA encapsulation, although it appears that more of DNA becomes surface complexed as the sucrose to lipid mass ratio increases.

It is well known that the requirements for efficient and successful transfection *in vitro* and *in vivo* are different (Felgner, 1996; Sternberg *et al.*, 1998), as the biological barriers and obstacles associated with *in vivo* delivery, are not effectively portrayed within *in vitro* conditions (Escriou *et al.*, 1998). For *in vitro* transfection, liposomes are more favoured for an unstable structure, whilst for efficient plasmid DNA delivery *in vivo*, it is preferential to use stable delivery systems, which can endure the harsh environment of the biological milieu, such as anionic competitors and endonucleases. Unstable SUV-DNA complexes may perform better *in vitro*, whereas stable DRV(DNA) may produce higher levels of immunity to the encoded-antigen *in vivo*.

6. Liposome-Mediated DNA Delivery: *In vivo* Studies

6.1 Introduction

From the *in vitro* studies carried out in chapters 4 and 5, a select few liposome formulations were chosen for further investigation, studying their immunological characterisation within *in vivo*. In relation to transfection efficiency *in vitro*, the cationic lipid DC-Chol generated the highest levels of luciferase activity than any other cationic lipid tested within these experiments (chapter 3). Furthermore, previous reports have shown that DC-Chol is a strong Th1/Th2 adjuvant for the HBS antigen, whilst also eliciting consistently high levels of IgG antibody responses in the murine model (Brunel *et al.*, 1999). Therefore, taking all these into consideration DC-Chol was the cationic lipid chosen and used throughout these *in vivo* studies. SUV-DNA complexes, in previous reports (Farhood *et al.*, 1995; Okayama *et al.*, 1997; Birchall *et al.*, 1999) and results presented in chapter 4, have shown to be effective at transfection plasmid DNA *in vitro*, although when applied *in vivo*, results appear to vary (Niven *et al.*, 1998). So far naked DNA has failed to elicit appropriate levels of immune responses when administered by the subcutaneous route, therefore liposome-mediated DNA delivery may offer the possible advantage of promoting and enhancing gene transfer and expression (Gregoriadis, 1998). Subcutaneous administration is a useful means of delivering antigens to the lymphatic system and also, it is a much easier and less painful route for injection, compared to intramuscular. Therefore, the subcutaneous route was chosen as the mode of administration within these following *in vivo* studies.

6.2 Optimisation of Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a widely used immunological technique, in which enzymes (e.g. peroxidase) covalently bind to specific antibodies present within sera. This is a highly sensitive assay, thereby antibody titres can be detected even at low amounts (Madigan *et al.*, 2000). There are many variables within the ELISA protocol that can have a profound effect on the final outcome. Firstly, the amount of washes at various stages during the ELISA protocol could influence the final absorbance readings. It is essential to wash the wells thoroughly, to ensure complete removal of any contaminants that may interfere with the final absorbance readings and produce high background levels, thereby allowing greater distinctions between groups, although it is also crucial to verify that an increase the amount of washing does not displace any bound antigen. Optimisation of the washing protocol was investigated, in which ELSIA

plates, at specified stages (full detail in section 2.13.2), were either washed three or five times with PBST buffer.

Figures 6.1a and b show that washing the plates five rather than three times allows for greater discrimination between groups, especially at the higher dilutions and additionally reduces the background readings as shown by the negative controls (in the absence of Hep B serum sample, –ve Hep B). Hep B surface antigen used to coat the wells of the 96-well plates will efficiently absorb to the plastic surface of the ELISA plates, due to high interactions between the hydrophobic region of the protein and the non-polar plastic surface (Madigan *et al.*, 2000). After sufficient incubation time (i.e. overnight at 4°C), the Hep B antigen will be tightly bound to the bottom surface of the well, therefore, an increase in the amount of washes during the protocol will only reduce the background values without displacing bound Hep B antigen. The ABTS concentration also appears to effect final absorbance readings, with higher concentrations producing the greater absorbance readings (Figure 6.1c). However, all protocols were standardised with 20 minutes incubation period, after addition of colouring agent (i.e. ABTS). Therefore it was noted that although results were enhanced with higher ABTS concentrations (Figures 6.1a and b) compared to lower concentrations (Figure 6.1c), the ABST concentration only appears to alter the rate of the reaction. Further to the results shown in Figure 6.1, it additionally seems that the type of blocking agent added to the wells, to prevent non-specific binding, effects absorbance readings. 1% Marvel appears to generate lower background readings therefore this will allow for better discrimination between experimental groups.

6.3 Optimisation of cell proliferation studies

Within these set of *in vivo* experiments, the level of proliferation of the splenocytes was examined. In brief, after immunisation with the plasmid DNA encoded for a specific antigen and after successful delivery and gene expression, the T cells were isolated, stimulated and activated. The activated T cell then increases in size and subsequently divides by mitosis into two separate cells leading to the production of a clone of competent T cells, which then differentiate into various different T cell types (i.e. helper T cells, Cytotoxic T cells and memory T cells). Therefore, measuring cell proliferation will provide information on whether the cells have efficiently been stimulated and activated by immunisation with various liposome formulations and preparations.

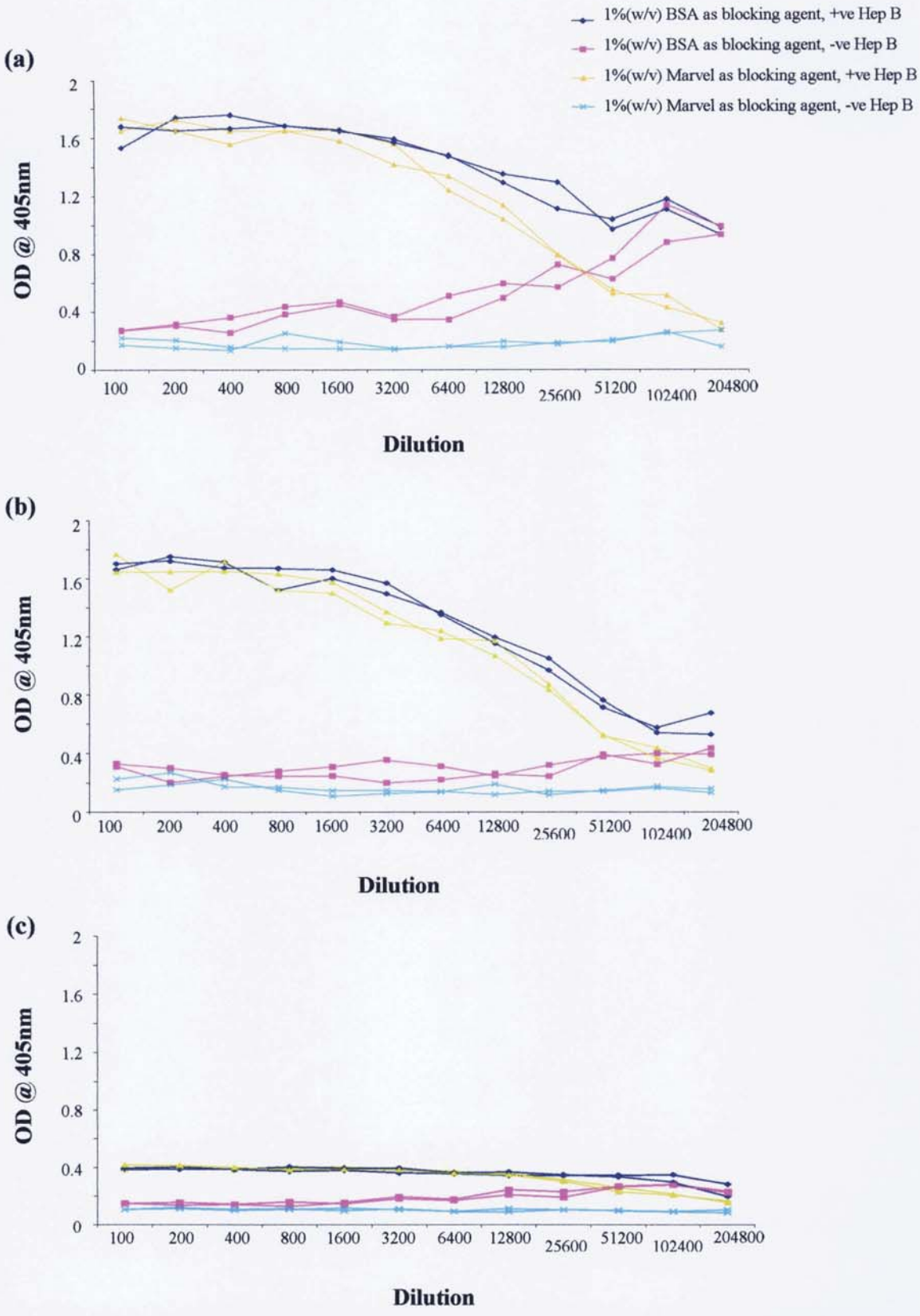


Figure 6.1 Optimisation of Enzyme-Linked Immunosorbent Assay (ELISA). Serum hepatitis B surface antigen-specific total IgG titres following intramuscular administration of 3 μg hepatitis B surface antigen mixed with incomplete Freund's adjuvant in a volume of 50 μl. (a) ELISA plates washed three times with 3 x ABTS (colouring reagent) diluted in a total volume of 50 ml, (b) ELISA plates washed five times with 3 x ABTS diluted in a total volume of 50 ml and (c) ELISA plates washed five times with 1 x ABTS diluted in a total volume of diluted in a total volume of 100 ml.

To this end, the antigen specific cell proliferation experimental protocol was optimised in order to gain maximal information from this study.

The isolated and cultured spleen cells from mice intramuscularly immunised with the hepatitis B surface antigen (3 μg/ml), administered by Freund's incomplete adjuvant in a volume of 50 μl, were stimulated with various concentrations of the hepatitis B surface antigen. By doing so, upon exposure to the antigen the optimum hepatitis B antigen concentration, which induced the most effective proliferation, would be utilised within the forthcoming *in vivo* studies.

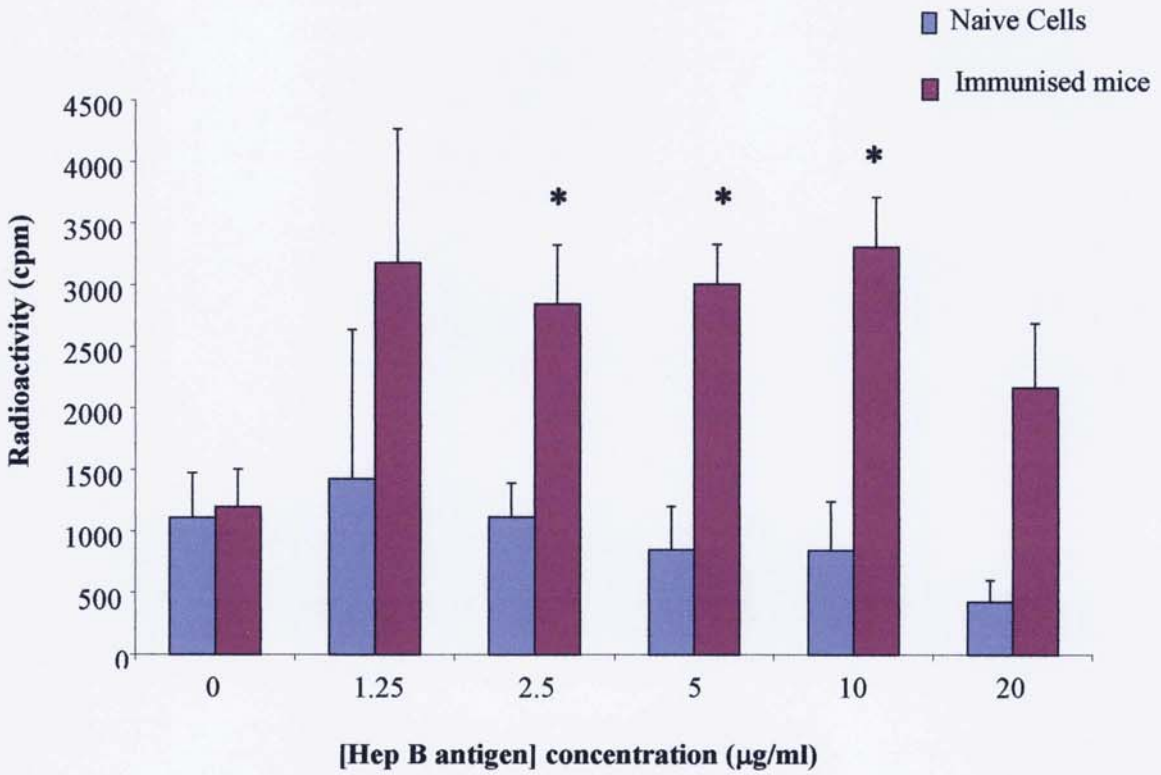


Figure 6.2 Effect of hepatitis B surface antigen concentration on cell proliferation within naïve and immunised Balb/c mice. Mice were intramuscularly immunised with 20 μg hepatitis B surface antigen (awy) mixed with incomplete Freund's adjuvant. * signifies that cell proliferation in the presence of 2.5, 5 and 10 μg/ml of Hep B antigen is significantly higher ($P<0.05$, ANOVA) than cell proliferation in the presence of either 0 or 20 μg/ml of Hep B antigen.

Results in Figure 6.2 shows that by stimulating the cultured cells with an antigen concentration of 1.25 μg/ml, there is an immense variation of proliferation between

individual cultures. However, splenocyte proliferation was most effective upon exposure to a hepatitis B antigen concentration between 2.5-10 μg/ml, with results showing to be significantly higher than both antigen concentrations of 0 and 20 μg/ml. The hepatitis B surface antigen is considered to be relatively cytotoxic to the cells, which may account for the reduction in cell proliferation at a concentration of 20 μg/ml. Therefore, to prevent the possibility of losing the discrimination between groups as a result of cytotoxicity, a concentration of 5 μg/ml hepatitis B surface antigen will be utilised within the subsequent experimental studies.

6.4 Immunological characterisation of liposome-mediated DNA delivery *in vivo*

The ability of liposomes to deliver plasmid DNA effectively and induce specific immune responses was tested. Mice, in groups of 8, were subcutaneously immunised with 4 injections (on days 0, 14, 35 and 74) of 20 μg plasmid DNA either naked or incorporated within various liposomal formulations and preparations as detailed within Table 6.1. Prior to immunological characterisation, each group from 1-8, received an additional mock challenge dose of 1 μg hepatitis B protein antigen, administered intramuscularly (day 127).

Group	Formulation	Preparation
1	PC:Chol:DC-Chol (16:8:4 μmole/ml)	DRV(DNA)
2	PC:Chol:DC-Chol (16:8:4 μmole/ml)	DRV(DNA)+sucrose
3	PC:Chol:DC-Chol (16:8:4 μmole/ml)	SUV-DNA
4	PC:Chol:DC-Chol (0.39:0.39:0.39 μmole/ml)	SUV-DNA
5	Chol:DC-Chol (0.39:0.39 μmole/ml)	SUV-DNA
6	DOPE:DC-Chol (0.39:0.39 μmole/ml)	SUV-DNA
7	Naked DNA	-
8	Naïve (with challenge)	-
9	Naïve (without challenge)	-
10	Positive control (DNA with incomplete Freund's)	-

Table 6.1 Immunisation protocol for liposome-mediated DNA delivery.

Within group 10, the positive control, the mice received 2 intramuscular immunisations with 3 μg hepatitis B protein antigen mixed with incomplete Freund's adjuvant, at 3 week intervals.

6.4.1 Physiochemical characteristics

For each liposomal formulation and preparation administered *in vivo*, the physiochemical characteristics were measured. As expected, liposomes composed of PC:Chol:DC-Chol at a lipid molar of 16:8:4 μmole/ml generated positively charged vesicles, with the liposome preparation DRV(DNA) producing the highest value at 54 mV (Table 6.2). The size of these DRV liposomes-entrapping DNA was measured at 791 nm, matching sizes previously found (chapter 5). Due to the method of preparation (i.e. DRV procedure) liposomes destabilise and form into larger vesicles, with the plasmid DNA becoming entrapped within and between the lipid bilayers. When sucrose is added as a cyroprotectant, at a 1:1 sucrose to lipid mass ratio, the liposomal vesicles remain stable during the dehydration-rehydration cycle thereby in the absence of vesicle fusion, the liposome size remains relatively low (174 nm; Table 6.2). However, due to the immense reduction in vesicle size and inhibition in vesicle fusion, as previously discussed in chapter 5, these smaller DRV(DNA) will have a much lower capacity for solute entrapment, therefore the majority of the plasmid DNA will be presumably complexed to the liposomal surface. In contrast, addition of DNA to SUV liposomes composed of PC:Chol:DC-Chol (16:8:4 μmole/ml) also produce relatively small structures, which are presumably a result of the formation of SUV-DNA complexes (136 nm; Table 6.2), in which the DNA is also complexed to the surface of these liposomal preparations. This is further supported by the zeta potential studies, whereby zeta potential values of liposomes indirectly reflect the vesicle surface charge and can therefore be utilised to evaluate the extent of association and interactions between the mixture of anionic DNA and cationic liposomes (Perrie *et al.*, 2003). Indeed, as expected the DRV entrapping DNA produced the highest zeta potential value (54 mV; Table 6.2) with SUV-DNA producing the lowest (41 mV; Table 6.2), supporting the proposal that in the latter case there is more DNA on the liposome surface, thus neutralising more of the cationic charges. DRV(DNA) with the addition of sucrose produces an intermediate zeta potential value, presumably indicating a combination of both entrapped and surface complexed DNA (46 mV; Table 6.2).

Results presented in Table 6.2, reveal that the lipid molar ratio has a huge impact on the physiochemical characteristics when formulating SUV-DNA complexes. When the lipids are at equal molar ratios, as in PC:Chol;DC-Chol with an equal molar ratio of 0.39:0.39:0.39 μmole/ml, the zeta potential values become highly negatively charged. This is presumably a consequence of the low lipid content within these formulations thus inefficiently neutralising the high DNA content. Additionally, as the cationic lipid, DC-Chol, is in equal proportions to the various other neutral lipids there may be extremely high and intense electrostatic interactions occurring between the anionic plasmid DNA and cationic SUV, resulting in aggregated vesicles, as shown by the large sizes (Table 6.2). DOPE:DC-Chol (0.39:0.39 μmole/ml) produced the largest complexes upon mixing with DNA (1295 nm; Table 6.2), confirming the instability of these complexes due to the presence of the helper lipid DOPE. In contrast, the more stable formulations produced smaller liposome-DNA complexes in the order of Chol:DC-Chol (0.39:0.39 μmole/ml) and PC:Chol:DC-Chol (0.39:0.39:0.39 μmole/ml), generating sizes of 231 nm and 500 nm, respectively (Table 6.2).

Group	Liposome formulation (μmole/ml)	Liposome preparation	Size (nm ± SD)	Zeta potential (mV ± SD)
1	PC:Chol:DC-Chol (16:8:4)	DRV(DNA)	791 ± 77.6	54.2 ± 6.3
2	PC:Chol:DC-Chol (16:8:4)	DRV(DNA)+sucrose	174 ± 9.7	46.2 ± 7.2
3	PC:Chol:DC-Chol (16:8:4)	SUV-DNA	136 ± 13.0	40.7 ± 5.5
4	PC:Chol:DC-Chol (0.39:0.39:0.39)	SUV-DNA	500 ± 47.3	-50.2 ± 7.9
5	Chol:DC-Chol (0.39:0.39)	SUV-DNA	231 ± 21.1	-54.9 ± 6.1
6	DOPE:DC-Chol (0.39:0.39)	SUV-DNA	1295 ± 70.6	-50.6 ± 8.5

Table 6.2 Size and zeta potential of various liposome formulations and preparations administered *in vivo*. Each liposome system contains 100 μg/ml. The vesicle z-average diameter and zeta potential measured in 0.001M PBS of each liposomal preparation was measured in double-distilled water or 0.001 M PBS, respectively, at 25°C using a Brookhaven ZetaPlus. Results denote mean ± SD, $n = 3$.

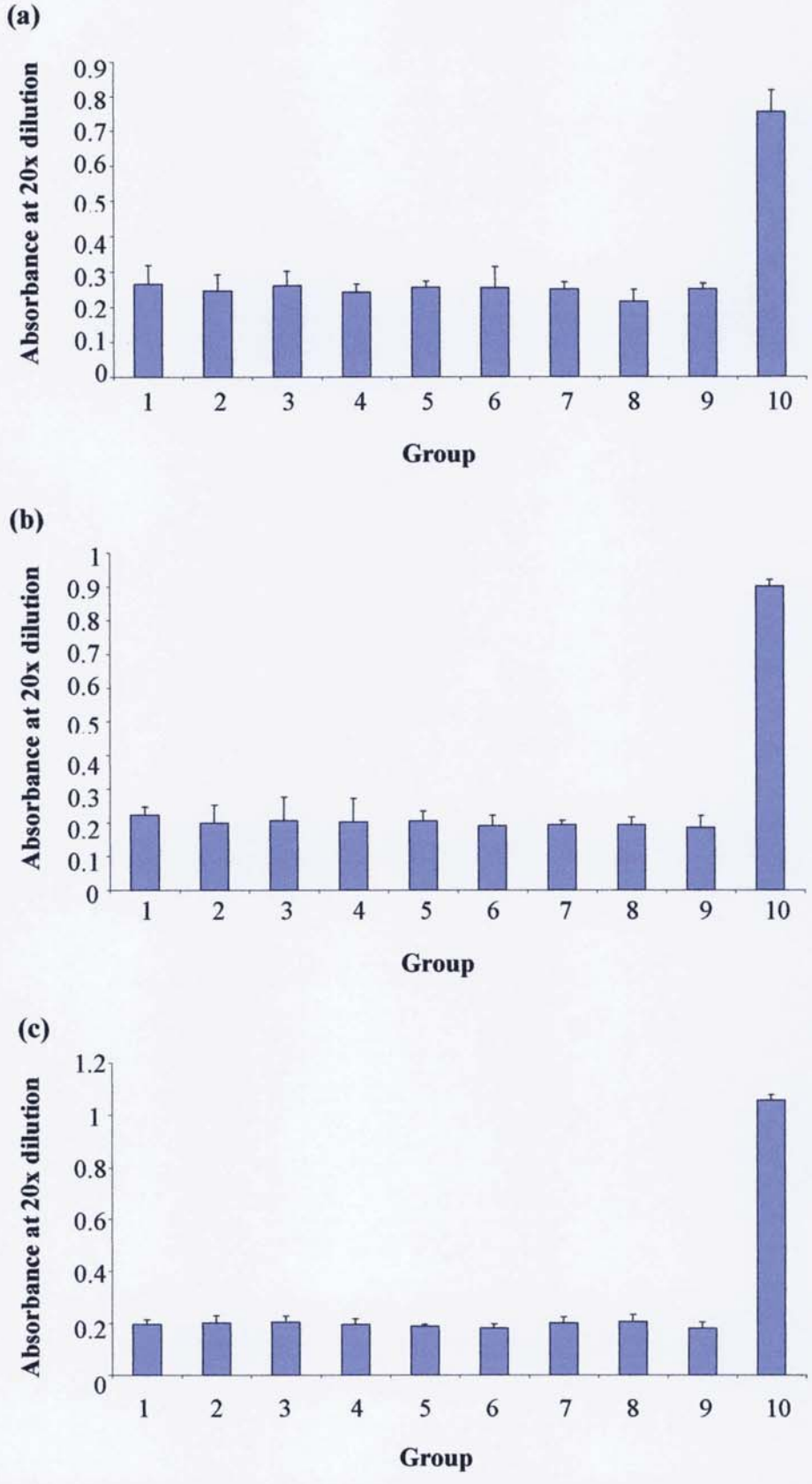


Figure 6.3 Serum hepatitis B surface antigen specific IgG and IgG subclass titres following subcutaneous administration of 20 μg DNA. Groups 1-10 represent liposome formulations and preparations as detailed in Table 6.1. Positive control was intramuscular administration of 3 μg hepatitis B surface antigen mixed with incomplete Freund's adjuvant. (a) IgG, (b) IgG1 and (c) IgG2a. Values are represented as the mean absorbance at 20x dilution. Results denote mean ± SD, $n = 5$.

6.4.2 ELISA

Results from Figure 6.3 show that none of the liposomal formulations tested were able to elicit increased levels of antibody titres in comparison to naked DNA or the naive group. Therefore, none of these formulations were capable of inducing an effective humoral response. However, previous studies have shown that liposomes entrapping Hep B DNA (Gregoriadis *et al.*, 2002) and influenza DNA (Perrie *et al.*, 2003) are fully capable of inducing effective levels of antibody responses when administered subcutaneously. Although, within these studies they applied a different liposomal systems consisting of PC:DOPE:DOTAP and in additionally it is not stated whether the plasmid DNA was endotoxin free, which may explain the relatively high readings for naked DNA. Throughout the following experiments, the plasmid DNA used was produced within endotoxin-free conditions.

It has previously been reported that one of the associated problems with subcutaneous administration is that the vectors for gene delivery are substantially retained near or at the site of injection. Therefore, the injected material may only exert a local effect (Pouton and Seymour, 2001) and thus may not reach the APC for effective DNA transfection, gene expression and finally antigen presentation.

6.4.3 Splenocyte proliferation studies and cytokine production

The level of splenocyte proliferation was also investigated, in which splenocyte preparations isolated from mice subcutaneously immunised with the plasmid DNA, pRc/CMV HBS, were re-stimulated with the recombinant Hep B antigen. Figure 6.4 shows that the SUV-DNA formulation consisting of PC:Chol:DC-Chol (16:8:4 μmole/ml) significantly induced higher levels of proliferation upon exposure to antigen when compared to control spleens (group 9) and any other liposomal formulation. Although, these liposmal preparations and formulations were inefficient at inducing a humoral response, the ability of these liposomes to elicit a cell-mediated response was evaluted by measuring endogenous cytokine levels (IL-2, IL-6 and IFN-γ) within spleen homogenates.

The level of cell-mediated immunity induced by each formulation was also measured in terms of IL-2, IL-6 and IFN-γ content within the spleens of mice immunised with pRc/CMV HBS and injected intramuscularly with 1 μg hepatitis B antigen. Figure 6.5 shows that

overall, there were greater levels of cytokines within the spleen of mice immunised with the liposome-DNA complex PC:Chol:DC-Chol (16:8:4 μmole/ml; group 3). This formulation induced significantly higher levels of IL-2 production (20 pg/ml; Figure 6.5) than any other group and interestingly values were also shown to be much higher than the positive control (group 10 with 3 pg/ml; Figure 6.5). In response to an antigen, the CD4+ T cells bind to the APC and consequently the helper T cells begin to produce IL-2. IL-2 is an important cytokine for the activation of both CD4+ and CD8+ T cells and natural killer cells. Thus, under the influence of IL-2, the infection-fighting cells begin to divide and multiply, making clonal copies by inducing proliferation (Madigan *et al.*, 2000; www.thebody.com). Therefore, the presence of high levels of IL-2 within group 3, positively corresponds with the cell proliferation data shown in Figure 6.4, where there is an increase in cell proliferation presumably due to an increase in T-cell (CD4 cell) counts. This increase in IL-2 and cell proliferation indicates that this liposomal formulation is effective at inducing Th1 type T-cell immune responses.

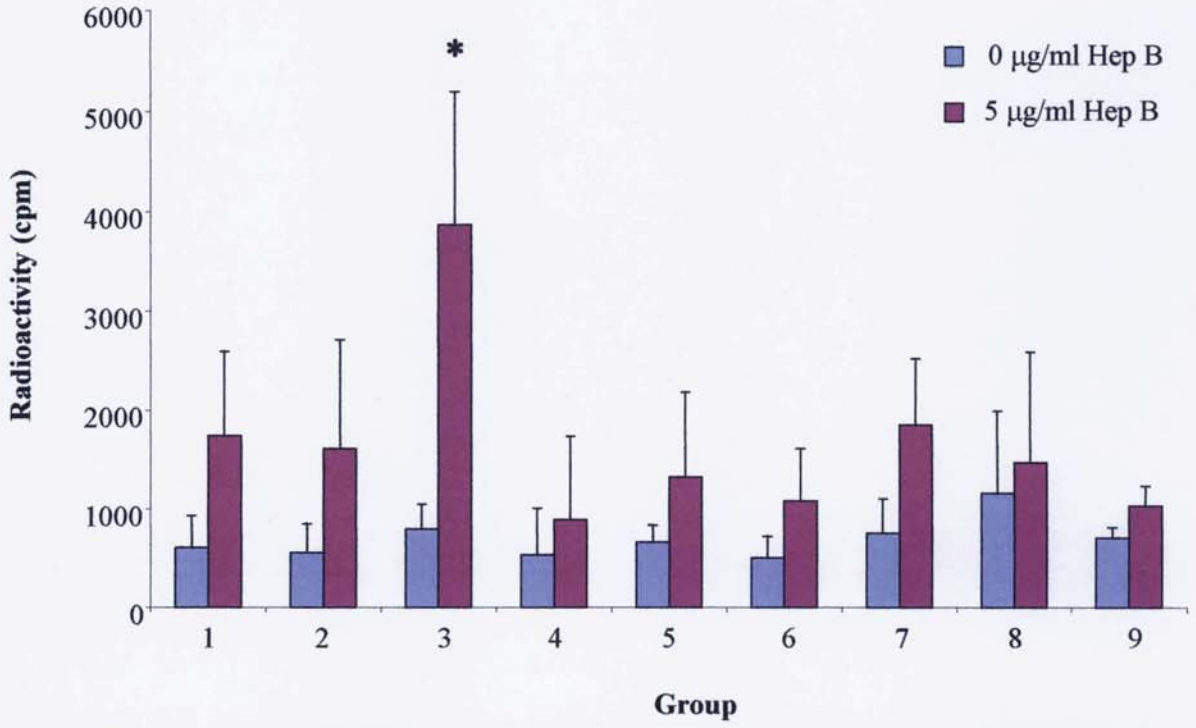


Figure 6.4 Analysis of cell proliferation following stimulation of splenocytes with recombinant hepatitis B surface antigen. Groups 1-9 represent liposome formulations and preparations as detailed in Table 6.1, when stimulated with either 0 or 5 μg/ml Hep B. Samples not pooled (n = 5). Cpm = counts per minute. * signifies that group 3 (SUV-DNA complexes composed of PC:Chol:DC-Chol 16:8:4 μmole/m) significantly ($P<0.05$, ANOVA) induced higher levels of splenocyte proliferation than the control spleens (group 9) and any other liposomal formulation.

IL-6 is a pro-inflammatory cytokine, which is reported to mediate an immune response to the acute phase of an infection. It is an important cytokine for the proliferation of B-cells and thus the release of antibodies (Madigan *et al.*, 2000). Interestingly, although Figure 6.3 indicated that there were no antibody titres present within serum, relatively high levels of IL-6 was detected within the spleen of mice immunised with liposome formulations from groups 2, 3 and 5 (see Table 6.1) (58, 51 and 41 pg/ml, respectively; Figure 6.5), which were similar to the level of IL-6 release from the positive control (group 10, 58 pg/ml; Figure 6.5). However, the release of IL-6 also reinforces the effect of IL-2, thereby promoting the differentiation of CD4+ T cells into Th2 type cells and in addition activates and stimulates natural killer cells. The measured vesicle sizes for the liposomal formulations that generated high levels of IL-6, were all below 250 nm, for the modified DRV(DNA) freeze-dried in the presence of a cyroprotectant and SUV-DNA complexes. This indicates that smaller liposomes may generally be better at stimulating the release and production of IL-6.

With respect to IFN-γ production, Figure 6.5 shows that groups 2 and 3 induced higher levels of cytokine release (23 and 18 pg/ml, respectively; Figure 6.5) compared to the control groups (group 8 and 9) and also naked DNA (group 7). The presence of this cytokine indicates a Th1 type immune response.

In an additional study, as well as the control group that received 2 immunisations with 3 μg hepatitis B protein antigen in incomplete Freund's adjuvant, another group (group 11) received 2 intramuscular immunisations with 50 μg of DNA and 2 boosters of 3 μg hepB protein antigen in incomplete Freund's adjuvant at 3 week intervals. Therefore, the only difference between group 10 and 11 is that group 11 received an initial injection of naked DNA prior to protein antigen injection within incomplete Freund's. Results presented in Figure 6.5 show that by initially immunising the mice with free DNA, cytokine production more than doubles, indicating that intramuscular immunisation with naked DNA primes the immune response and significantly enhances the adjuvanticity and immunogenicity of the subsequent injections.

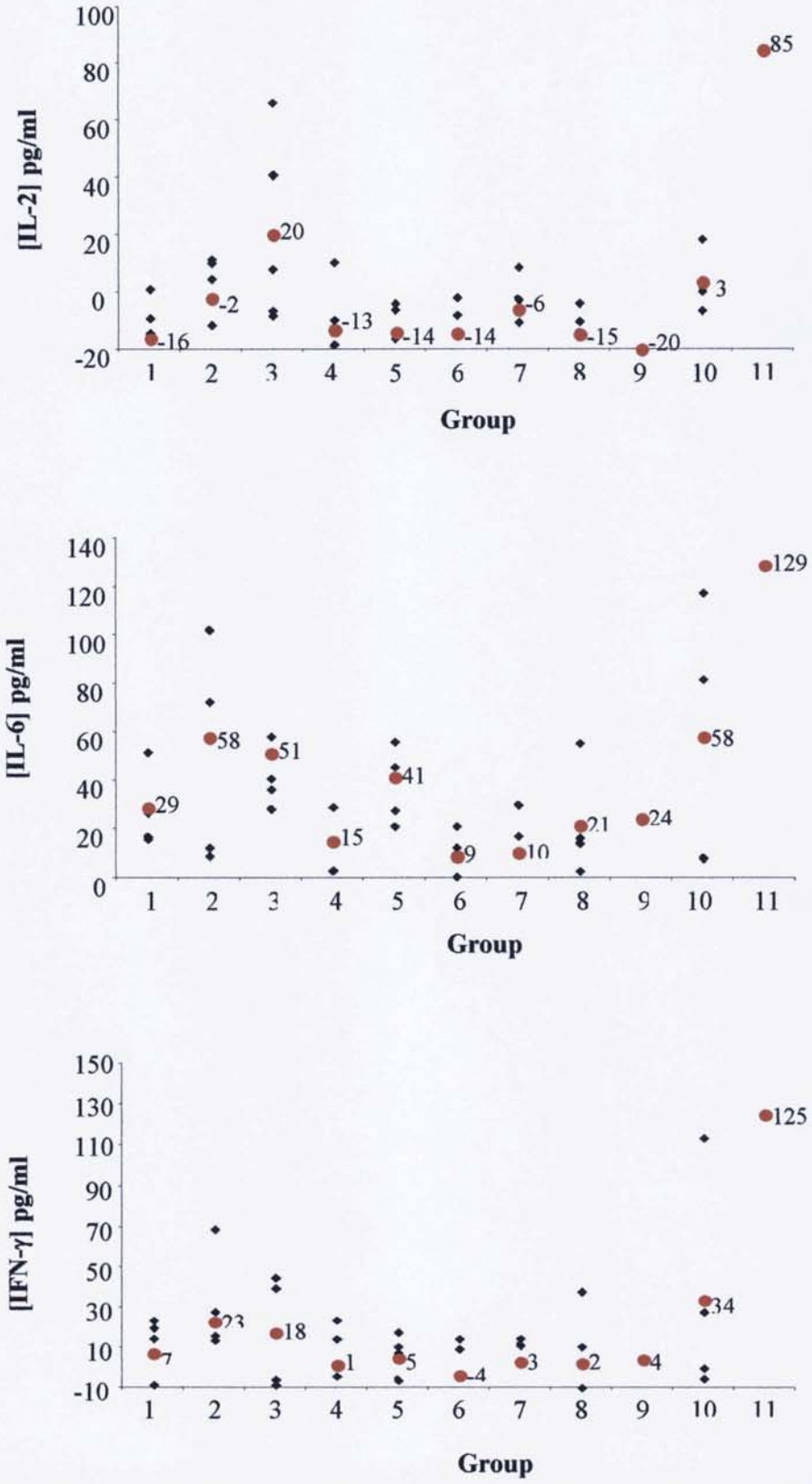


Figure 6.5 Cytokines (IL-2, IL-6 and IFN-γ) detected within the supernatants of stimulated splenocytes 40 hours post-stimulation. Groups 1-10 represent liposome formulations and preparations as detailed in Table 6.1. Group 11 received 2 intramuscular injections of 50 μg naked DNA prior to 2 boosters of 3 μg protein antigen administered by incomplete Freund's adjuvant. Red dots indicate the average level of cytokine (IL-2, IL-6 or IFN-γ) detected, with values in picogrammes per ml. The blue dots represent the average of five separate stimulations for each of the five animals tested.

It is well recognised that intramuscular administration of naked plasmid DNA (i.e. pRc/CMV HBS), induces a cytotoxic T cell immune response (Davis *et al.*, 1993; Chattergoon *et al.*, 1997; Manickan *et al.*, 1997). This may be achieved by the antigenic material transferring between the myocytes and the professional APC, to generate efficient antigen presentation to the CD8+ cells. Equally the plasmid DNA could be directly taken up by the APC infiltrating the site of injection (Gregoriadis, 1998). Therefore, with intramuscular injection of DNA, the immune cells will be stimulated and with further immunisation with the relatively potent adjuvant, incomplete Freund's, the immune cells presumably recognise the introduced DNA as foreign therefore, enhancing the overall immune response against the encoded antigen.

6.5 Conclusion

Previous reports have shown that DNA vaccination is potent at eliciting enhanced levels of T cells (Mancini-Bourgine *et al.*, 2005). Results presented here indicate that mice immunised with liposomes consisting of PC:Chol:DC-Chol either prepared by the modified DRV procedure, to produce small DRV(DNA) or SUV-DNA complexes which also generated small liposomal structures, elicit relatively high levels of cell-mediated immunity, yet do not induce any humoral immune response, as no antibody titres were detected for any formulation. Groups 2 and 5 appear to predominantly elicit a Th2 type immune response as indicated by the high levels of IL-6, although surprisingly, no antibody titres were detected within serum for this formulation even though Th2 emphasises the antibody response (Morein *et al.*, 1996). Group 3, on the other hand, generated relatively high levels of IFN-γ and IL-2, especially when compared to control groups and naked DNA, indicating a Th1 type response. By inducing the Th1 response, this characterises a strong cell-mediated immune response, which is equally as important at eliminating viruses from the infected cells (Constant and Bottomly, 1997). In addition, immunomodulation can act by superimposing a Th1 type of immune response on Th2 (Morein *et al.*, 1996), suggesting a mixed Th1/Th2 type response, which may explain why group 3 also induces the production of IL-6.

It is well recognised that *in vitro* transfection rarely correlates well with *in vivo* transfection and immune responses. Indeed, the immunogenic characterisation results presented within

this chapter, reveal that SUV-DNA complexes formulated with PC:Chol:DC-Chol at a 16:8:4 molar ratio produces the most promising immune responses *in vivo* than any other liposome formulation tested. However, in contrast, results presented within chapter 5, section 5.5, show that this formulation is poor at inducing efficient levels of transfection *in vitro*. Even though, entrapping the DNA within DRV liposomes was shown to protect the DNA from digestive enzymes and anionic component, these *in vivo* results demonstrate that this preparation was poorly immunogenic. This could be attributed to the highly stable bilayers with the incorporation of Chol within the formulation, inhibiting effective DNA release from the liposome and into the cytoplasm for efficient DNA delivery and expression. Indeed, previous reports have shown that replacement of PC with the high transition temperature lipid, DSPC, within liposomes composed PC:DOPE:DOTAP, renders the lipid bilayer more rigid and therefore is not able to effectively interact with the target cells endosomal membrane (Perrie *et al.*, 2001). Thus, the stable DRV liposomes consisting of Chol, may not be able to effectively release its DNA content due to the stable lipid bilayers, whereas the complexation of DNA onto SUV has reduced stability in which the DNA is readily available for more effective DNA release. Indeed, by incorporating DOPE rather than Chol within the formulation, which will render the lipid bilayers more fusogenic and less stable, previous studies have shown that immunisation of liposomes consisting PC:DOPE:DC-Chol entrapping the plasmid pI.18Sfi/NP against the influenza virus generates sufficiently high levels of antibody titres against the encoded antigen (Perrie *et al.*, 2003). Therefore, this formulation could be tested for immunisation against the hepatitis B plasmid DNA.

Additionally, the ability of these liposomes to induce effective levels of immunity could be attributed to the liposomal vesicle size, as cytokines were only detected within spleens of mice immunised with liposomal formulations possessing vesicle sizes lower than 250 nm. As detailed previously, small liposomes may prove more beneficial for *in vivo* transfection, as after intravenous injection small liposomes were shown to remain in circulation for prolonged periods of time in comparison to large liposomes, which have been reported to be rapidly cleared from circulation (Gregoriadis, 2002). After administration by various other routes (i.e. intramuscular, subcutaneous, intranasal) it seems that the majority of larger liposomes remain locally at the site of injection, whereas the smaller liposomes migrate

towards the draining lymph nodes and therefore have a greater chance of reaching their target cells and exerting their immunogenic effect (Gregoriadis, 2002).

Although, plasmid DNA within SUV-DNA complexes is not entrapped and thus not fully protected from the detrimental effects of the biological milieu as DRV(DNA), results presented within chapter 5 show that the complexed DNA is largely protected from degrading enzymes, such as DNase I. This was attributed to the DNA being highly condensed within these liposome-DNA complexes, therefore making the DNA inaccessible to digestion by intracellular enzymes (Gregoriadis *et al.*, 2002). Therefore, these stable SUV-DNA complexes composed of PC:Chol:DC-Chol (16:8:4 μmole/ml) appear to effectively protect the DNA from digestion and thus efficiently induce cell-mediated responses.

The SUV-DNA complexes formulated at a low lipid ratio (i.e. PC:Chol:DC-Chol, 0.39:0.39:0.39 μmole/ml; Chol:DC-Chol, 0.39:0.39 μmole/ml; DOPE:DC-Chol, 0.39:0.39 μmole/ml), which produced significantly high levels of transfection efficiency *in vitro*, as shown in chapter 4, did not elicit any significant immune responses against the hepatitis B antigen when applied *in vivo*. This may be due to the low level of lipid within these preparations, where there will be a substantial amount of free un-associated plasmid DNA, as confirmed by the highly negative zeta potential (Table 6.2) measured for these formulations.

7. Protein delivery: Utilising liposomes as a delivery system for TB vaccines

7.1 Introduction.

Tuberculosis is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*, which was first isolated and described by Robert Koch (1882) (Madigan *et al.*, 2000). There are approximately 3 million deaths per year worldwide is due to infections of tuberculosis, with approximately a third of the world's population having been infected (Madigan *et al.*, 2000). Therefore, it is evident and crucial that an effective vaccine candidate is developed and available for human use. At present, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only vaccine available against tuberculosis and has been around and used as a vaccine against tuberculosis since 1921 (Reed *et al.*, 2003). It is a live attenuated vaccine, which generates variable levels of efficacy in humans (Brandt *et al.*, 2000), in which estimates of protection imparted by BCG can vary from 0-80%. Therefore due to a lack of efficiency and safety of this vaccine, new efforts and progress have been made into the development of new and improved vaccines against tuberculosis. Protein subunit vaccines when delivered by an appropriate adjuvant have proven to be effective in protecting mice, when BCG vaccination fails (Brandt *et al.*, 2002). Subunit vaccines are derived from protein pools that have been isolated and obtained from *M. tuberculosis* culture filtrates. These subunit vaccines have previously shown to produce highly efficient levels of memory-T-cells (Orme *et al.*, 2001) and at least 50% of candidate vaccines that have been tested and evaluated so far are subunit vaccines, as these provide a promising development to a successful vaccine against tuberculosis (Orme *et al.*, 2001).

7.2 Liposomes as efficient protein carriers

The identification of vital mycobacterial proteins, from the sequenced genome of *M. tuberculosis* (Cole *et al.*, 1998), is essential for the development of new subunit vaccines, with many protein antigens having been developed from the culture filtrates of *M. tuberculosis* (Olsen *et al.*, 2001). Throughout these studies, the fusion protein ESAT-6, a subunit vaccine that has been isolated from *M. tuberculosis*, was used as the subunit vaccine. The ESAT-6 antigen was the protein of choice as it is strongly recognised within various hosts (Brandt *et al.*, 2000) due to the abundance of T-cell epitopes, thus eliminating the possibility of variation of protection within a population, as seen with the BCG vaccine. In addition, studies have shown that, although the ESAT-6 antigen alone shows high levels of activity, when this antigen is fused with another isolated epitope, Ag85B, the immune

response is amplified (Harboe *et al.*, 1998; Olsen *et al.*, 2001; Olsen *et al.*, 2004), so for that reason, the fusion protein of ESAT-6 and Ag85B, was utilised throughout these studies. However, protein antigens alone are unable to efficiently induce protective immunity against tuberculosis (Lima *et al.*, 2004) and even though the ESAT-6 protein contains the appropriate epitopes on its own it possess very low immunogenicity. Studies have shown that the ESAT-6 protein in combination with adjuvants elicits high levels of protection in mice against tuberculosis (Olsen *et al.*, 2001; Brandt *et al.*, 2002) and application with fusion proteins has previously proven to be effective at eliciting both protection and long-term survival (Orme *et al.*, 2001), demonstrating the immense potential of ESAT-6 as a vaccine candidate.

As originally described by Ramon (1926), adjuvants are 'substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone'. Therefore, in order to enhance and increase the immune response to an antigen, vaccine adjuvants should possess a number of mechanisms, which will induce high levels of protective immunity (Gupta and Siber, 1995), such as;

(a) Acting as a delivery vehicle.
(b) Acting as a depot at the site of injection.
(c) Acting as an immunostimulator to enhance immune response.

The immunological role and adjuvant properties of liposomes were first identified by Alison and Gregoriadis (1974) for diphtheria toxoid. Since then, the immunological adjuvanticity of liposomes is well recognised and has been extensively investigated as an efficient vaccine adjuvant for more than 20 years for a number of antigens, including; tetanus toxoid (Davis and Gregoriadis, 1987), *Leishmania major* antigen (Kahl *et al.*, 1989), hepatitis B surface antigen (Brunel *et al.*, 1999), with some liposomal based vaccines (i.e. virosomes) having been licensed for human use (i.e. Inflexal vaccine for influenza). As most synthetic peptide and protein vaccines are unable to promote efficient immune responses and protection from disease, the problems associated with protein delivery could be circumvented by using a delivery system that transports the protein from the site of administration to the target cell, while additionally providing protection for the protein from the harsh environment of the biological milieu (Gregoriadis, 1990; Gupta and Siber, 1995). Liposomes have been utilised as a delivery system for many years with some successful applications and due to their

ability to act as a carrier system for a broad spectrum of substances and immense structural and functional versatility, liposomes are the prominent delivery system and are a good candidate for a delivery vehicle for proteins and peptides. The manner in which liposomes elicit protective immunity for protein antigens it still relatively unclear, although, it appears that, as well as acting as an efficient delivery vehicle, liposomes may also act as a depot, in which the incorporated protein antigen is released at a slow and controlled rate. Due to the broad spectrum of diverse liposome formulations used as effective adjuvants, it seems that the adjuvanticity of liposomes is not associated to any definitive formulation (Gregoriadis, 1990; Bramwell and Perrie, 2005). When developing liposomal formulations as an efficient protein delivery system, as previous chapters have detailed, the same factors and variables need to be considered and investigated in order to determine the most effective formulation. The adjuvanticity of liposomes is affected by the resultant physiochemical characteristics of the vesicle, such as bilayer fluidity, lipid composition, vesicle size and surface charge (Gupta and Siber, 1995). Therefore efforts detailed within this chapter examine and study various liposomal formulations with regard to physiochemical characteristics, in which the following factors were considered;

(a) Vesicle surface charge, in which negative, neutral and positive liposomes were studied and compared. Although for effective plasmid DNA entrapment and delivery, cationic liposomes are the principal formulation, as discussed in chapters 3, 4 and 5, protein delivery may present a different story.
(b) Method of preparation, in terms of surface absorption versus entrapped protein. Within chapter 5, with regard to plasmid DNA, surface complexation resulted in better levels of transfection activity than entrapped within *in vitro*, however, in terms of stability and level of antigen protection, entrapping the antigen within DRV was superior.
(c) Lipid composition and bilayer rigidity, which involved identifying the key formulation parameters.

In order to achieve efficient delivery of protein to the target cell and initiate the appropriate immune response, the delivery vehicle needs to not only protect the protein from degradation but also needs to retain the antigen and have sustained release of the protein in order to prolong the response (Holten-Andersen *et al.*, 2004). Within these following studies preparations were formulated of lipids with increased bilayer packing and that generate strong and rigid bilayers, such as DSPC and DMPC, which will assist in protecting the

protein and may act as depot systems, which will enable a slow and long term release of protein when applied *in vivo*, resulting in a prolonged immune response. Previous studies have shown that strong immune responses are generated to the membrane protein antigen when incorporated within rigid liposomes composed of phospholipids of high transition temperature (Gregoriadis, 1990; Gregoriadis, 2002). With the inclusion of high transition temperature phospholipids, the stable lipid bilayers will breakdown down at a slow and steady rate, gradually releasing the associated antigen and enhancing antigen presentation to APC, while also consequently prolonging the adjuvant action and immune response (Gregoriadis, 1990), as has previously been shown for tetanus toxoid. The protective immune response to antigens can be enhanced considerably by the addition of other adjuvants within liposome formulations. Indeed, previous reports have demonstrated that Ag85B-ESAT-6 fusion protein delivered by the cationic lipid, DDA, in combination with an immunomodulators, such as, monophosphoryl lipid A (Brandt *et al.*, 2000), trehalose 6,6'-dibehenate (TDB) (Holten-Andersen *et al.*, 2004), produced a very high immune response, with sustained protection against subsequent challenge of *M. tuberculosis* infection (Holten-Andersen *et al.*, 2004). Therefore, by the addition of other phospholipids, such as DMPC and DSPC, this depot effect could be enhanced, possibly prolonging the immune response to the antigen and thus providing long-term protection from subsequent *M. tuberculosis* challenges. . In addition, the adjuvanticity of these liposome formulations was enhanced by the addition of TDB, which is an immunostimulatory component of the mycobacterial cell wall (Lemaire *et al.*, 1986; Davidsen *et al.*, 2005). It is a synthetic analogue of trehalose 6,6'-dimycolate (TDM) often referred to as cord factor, however, the toxicity associated with TDM as an adjuvant system is effectively reduced by replacement with TDB, due to the shorter fatty acid chains of TDB (Olds *et al.*, 1980).

This chapter examines the affect of liposomal formulation and composition on protein incorporation, vesicle size, zeta potential and also protein release from vesicles. With regard to vaccine develop and the intended application within *in vivo*, the intended purpose of these release studies was to provide an insight into the permeability and release characteristics of various liposome formulations and preparations, comparing between formulations. The rate of protein release from liposome formulations composed of various lipid components that were taken *in vivo* were determined by measuring the amount of ^{125}Iodine labelled protein retained within the liposomal vesicles and released into the surrounding medium (i.e. PBS at

37°C) at various time points. Continuing with this work, chapter 8 looks at the effect of these liposome formulations in generating immune responses when applied *in vivo* and also looks at the long-term affects of protection against subsequent challenge with *M. tuberculosis*.

7.3 Radiolabelling of fusion protein

For entrapment and release studies, fusion protein containing Ag85B and the ESAT-6 antigen was radiolabelled with the radioisotope ^{125}Iodine (^{125}I) as instructed by the manufacturers' protocol (section 2.5.2.2). The labelled protein was separated from the free, unincorporated ^{125}I by subjecting the sample to a Sephadex G-75 gel column. Fractions of 0.5 ml aliquots were collected and the radioactivity of ^{125}I within various aliquots and additionally the spectrophotometry (A_{280}) for protein content within each aliquot was measured.

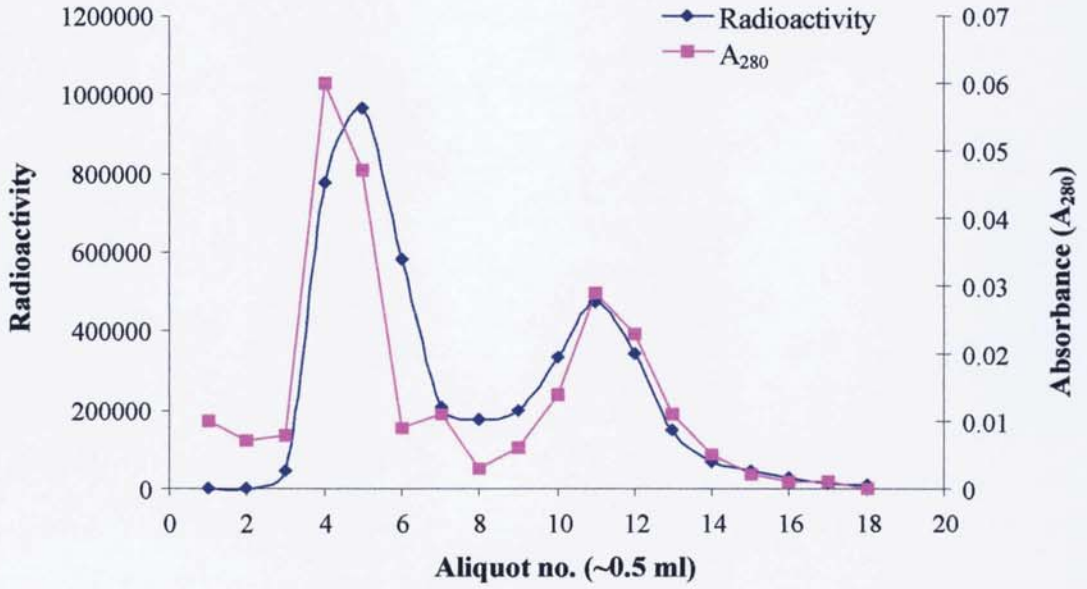


Figure 7.1 Elution profile for ^{125}I-labelling fusion protein. Radiolabelled protein was separated from unincorporated radioisotope and protein by gel chromatography (Sephadex G-75). Fractions (~0.5ml) were collected and spectrophotometrically (A_{280}) analysed and ^{125}I content measured.

The elution profile (Figure 7.1) shows two peaks for both the radioactivity for ^{125}I and the A_{280}, with the first peak between aliquot numbers 4 and 6 and the second peak between 10 and 12, with both radioactivity and absorbance values overlapping each other at both sets of peaks.

To ensure that the protein maintains its structure and functionality through out the radiolabelling process, unlabelled protein was subjected to a Sephadex G-75 column, with fractions collected as detailed previously. The protein content was again analysed spectrophotometrically (A_{280}) and results are presented in Figure 7.2. The elution profile for unlabelled protein only shows one peak, between aliquots 4 and 6, which is consistent with the first peak shown in Figure 7.1. This confirms the presence of intact protein antigen at the first peak, therefore, only fractions from the first peak were collected and used for protein incorporation and release studies.

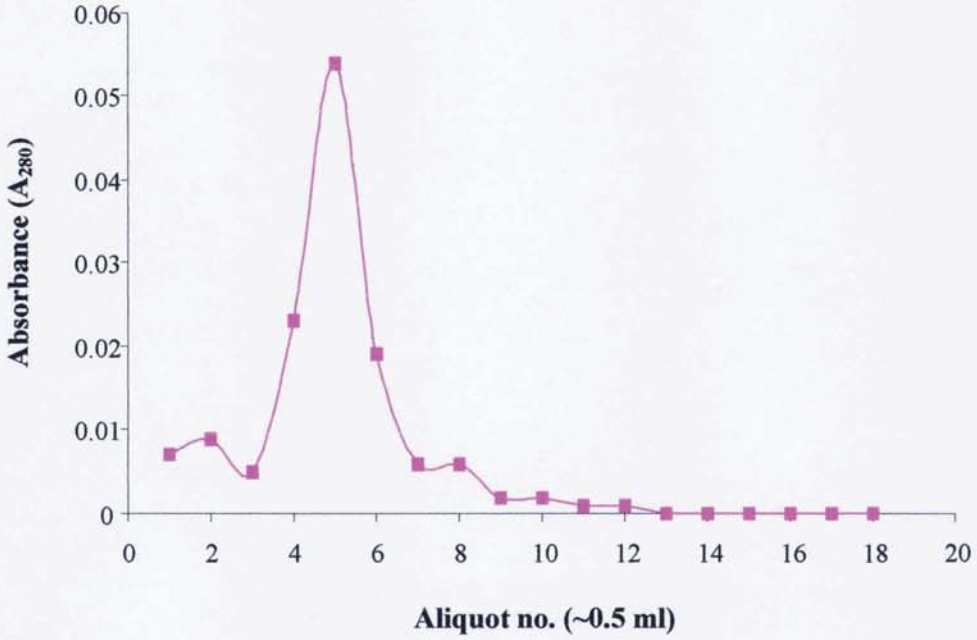


Figure 7.2 Elution profile for unlabelled fusion protein. Unlabelled protein was subjected to gel chromatography (Sephadex G-75). Fractions (~0.5ml) were collected and protein content within each aliquot was analysed by spectrophotometry (A_{280}).

7.4 Liposomal characteristics: effect of surface charge and liposome morphology

Liposomes have been extensively recognised as potent stimulators of the immune response, although, in order for liposomes to stimulate effective protective immunity and enhance the immunogenicity of the antigen, the protein antigen needs to be physically associated with the liposomal vesicles (Therien and Shahum, 1996). However, the physiochemical characteristics of liposome formulations and their adjuvanticity when applied *in vivo* alters,

depending on the manner in which the antigen is physically associated with these vesicles (Fortin *et al.*, 1996) and the way in which the antigen is presented may govern the type of the immune response (Leserman, 2004). It seems that the type of immune response induced alters depending on whether the protein antigen is surface-adsorbed or entrapped within liposome formulations (Shahum and Therien, 1994), thus, in order to activate the desirable immune response to the protein antigen, the antigen needs to be appropriately associated with the liposome vesicles. As previously discussed in chapter 5, the method of preparation has considerable effects on liposome structure, morphology and also on the physical association between liposomes and antigen. To study the effects of liposome morphology on antigen incorporation, vesicle size and zeta potential, liposomes of the same composition were prepared by two different methods (lipid hydration vs DRV method); therefore these formulations only differ in terms of structural morphology and antigen association.

7.4.1 Physiochemical characteristics

7.4.1.1 Effect of surface charge: anionic versus cationic liposomes

Anionic liposomes composed of DMPC:PG:TDB (1.8:0.2:0.3 μmole/ml), generating zeta potential values of -41 mV, produced significantly smaller vesicles (479 nm; Table 7.1) than cationic liposomes composed of DMPC:SA:TDB (2:1:0.3 μmole/ml) (924 nm; Table 7.1), with a zeta potential of 33 mV. The differences in vesicle size between formulations may result from the quantity of protein entrapped between lipid bilayers. The high value of protein entrapment (97 %; Table 7.1) determined for SA-containing liposomes could be attributed to additional electrostatic interactions between the cationic charges of the liposomes and the anionic charges of the protein. Indeed, previous studies have reported that the adsorption of this fusion protein antigen (i.e. Ag85B-ESAT-6) to cationic liposomes is most probably electrostatic in nature, as the antigen is highly negatively charged (Davidsen *et al.*, 2005). In addition, the incorporation of protein within anionic liposomes is significantly less (77 %; Table 7.1), presumably due to the lack of these electrostatic interactions. In this instance, the protein antigen could possibly be bound to the inner bilayers of the liposomes by hydrophobic interactions. However, overall protein incorporation was considerable within both anionic and cationic DRV liposomes.

Liposome formulation	Liposome preparation	Protein incorporation (% of used ± SD)	Size (nm ± SD)	Zeta potential (mV ± SD)
DMPC:PG:TDB	DRV(protein)	77.3 ± 3.0	479.0 ± 69.7	-40.8 ± 7.4
DMPC:SA:TDB	DRV(protein)	96.6 ± 1.0*	924.2 ± 87.4**	32.6 ± 5.8
DMPC:SA:TDB	MLV-protein	59.9 ± 9.2	594.8 ± 53.6	55.2 ± 8.9

Table 7.1 Effect of liposome preparation and surface charge on protein entrapment, vesicle size and zeta potential. Liposomes composed of DMPC, the anionic lipid PG or the cationic lipid SA and TDB, containing 10μg H1 fusion protein. Formulations were prepared by the DRV procedure entrapping the protein within liposome bilayers (DRV(H1)) and additionally for DMPC:SA:TDB liposomes protein antigen was adsorbed onto the surface of MLV (MLV-H1). Entrapment values were determined by ^{125}I-labeled protein. The vesicle z-average diameter and zeta potential of each liposomal preparation was measured in double-distilled water or 0.001 M PBS, respectively, at 25°C using a Brookhaven ZetaPlus. * signifies that DRV(protein) composed of DMPC:SA:TDB generates significantly ($P<0.05$, ANOVA) higher values of protein incorporation than DRV(protein) composed of DMPC:PG:TDB and MLV-protein composed of DMPC:SA:TDB. ** signifies that DRV(protein) composed of DMPC:SA:TDB produce significantly ($P<0.05$, ANOVA) larger vesicles than DRV(protein) composed of DMPC:PG:TDB and MLV-protein composed of DMPC:SA:TDB. Results denote mean ± SD, $n = 6$.

7.4.1.2 Liposome preparation: surface adsorbed versus entrapped antigen

Multi-lamellar vesicles were prepared by the rotary evaporation method, producing large vesicles with multi-lamellar bilayers. The antigen is added to preformed MLV, therefore, antigen, depending on interactions may be adsorbed onto the surface of the vesicles rather than entrapped. The second liposomal structure tested was the DRV entrapping the antigen within lipid bilayers, as detailed previously in chapter 5. MLV are preformed liposomal structures, in which the internal lamellar layers are inaccessible to protein antigen, therefore resulting in relatively low passive incorporation efficiencies of 60 % compared to the DRV systems (97 %; Table 7.2). This is likely a result of DRV actively entrapping solutes as these vesicles form in the presence of antigen upon controlled rehydration, enabling the protein antigen to come into close contact with lipid bilayers and reside in the liposomes aqueous spaces as the DRV form around the solute (Deamer and Barchfield, 1982; Perrie *et al.*, 2003). This once again emphasises the ability of the DRV procedure at entrapping large quantities of antigen.

7.4.2 Protein release *in vitro*

7.4.2.1 Effect of surface charge: anionic versus cationic liposomes

Figure 7.3 shows that within *in vitro* conditions, the rate at which the protein was released varies between the two different charged liposome formulations. Although for both anionic

and cationic preparations the total amount of protein released after 42 days was approximately 90% (of total protein entrapped). For anionic liposomes, consisting of PG, protein was released much more rapidly than cationic liposomes, as a significant amount of their protein content was released after 14 days (i.e. 336 hours) incubation at 37°C (86% of total entrapped protein; Figure 7.3), whereas SA-containing DRV liposomes exhibited a slower rate, releasing only 52% after the same time period.

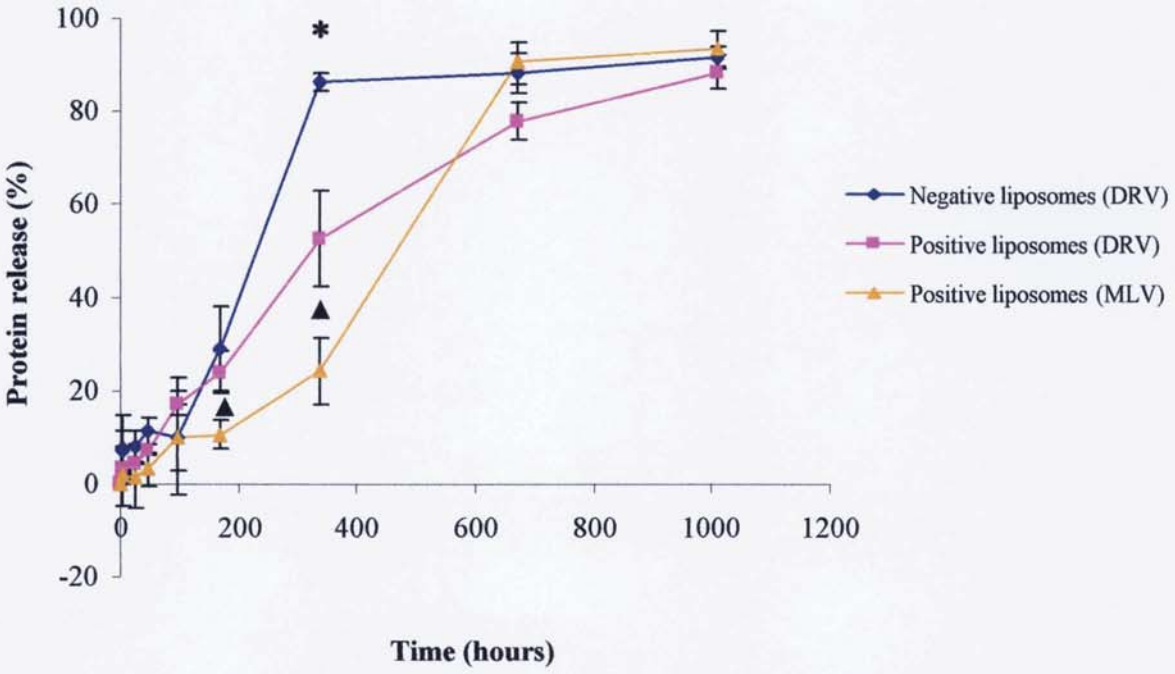


Figure 7.3 Effect of vesicle surface charge and liposome morphology on rate of protein release. Liposomes composed of DMPC, the cationic lipid SA or the anionic lipid PG and TDB, containing 10μg H1 fusion protein were prepared by the dehydration-rehydration procedure with DRV entrapping (DRV(protein)) and additionally for DMPC:SA:TDB liposomes protein antigen was adsorbed onto the surface of MLV (MLV-protein). Each formulation was incubated in a 37°C water bath, with continuous shaking. At various time intervals, 1ml samples were taken, measuring the level of radioactivity of I^{125} prior to centrifugation (to obtain total counts) and after centrifugation in the pellet and supernatant (to obtain percentage entrapment and recovery). The 1ml sample removed was replenished with 1ml PBS, to retain sink conditions. * signifies that negative liposomes composed of DMPC:PG:TDB release significantly ($P<0.05$, ANOVA) more protein compared to positive liposomes composed of DMC:SA:TDB, at 336 hours incubation. ▲ signifies that MLV-protein liposomes release significantly less protein than DRV(protein), at 168 and 336 hours incubation. Results denote mean ± SD, $n = 3$.

7.4.2.2 Liposome preparation: surface adsorbed versus entrapped antigen

Once again, regardless of the nature of liposome-antigen association, the release of 90-94% antigen after 1008 hours is equal between both liposomal preparations, however, the rate of

release over time varies to some extent (Figure 7.3). Protein release from DRV(protein) liposome formulations was slow and controlled over time. With sustained release, this liposome formulation may act as an effective depot (Storm and Crommelin, 1998) from which the protein antigen can slowly leak from the liposome and maintain therapeutic levels of antigen for presentation to APC. On the other hand, initially MLV-H1 exhibited slow release with only 24% released into the surrounding medium (PBS) after 336 hours, compared to 54% for DRV(protein) liposome preparation. Conversely, after 672 hours there was a sudden burst release of 90% protein antigen from MLV-protein, whereas DRV(protein) liposomes maintained its sustained release (77% of protein entrapped; Figure 7.4).

7.4.3 Immunological characterisation *in vivo*

A vital component of protection from *M. tuberculosis* is the production of IFN-γ from both CD4+ and CD8+ T cells (Jouanguy *et al.*, 1996; Reed *et al.*, 2003). This cytokine response activates bactericidal effector mechanisms within the mycobacterial host cell (i.e. the macrophage), to induce protective and effective immunity, by the Th1 response.

The adjuvant effect of anionic and cationic DRV and cationic MLV were analysed by immunising C57Bl/6j mice with the Ag85B-ESAT-6 fusion protein. The immune response of the blood cells was investigated by restimulating the isolated lymphocytes with Ag85B-ESAT-6 *in vitro*, both one week after second immunisation and one week after the third immunization and subsequently measuring IFN-γ as an indicator of the Th1 immune response.

Results in Figure 7.4a and b, show that initially liposomes composed of DMPC:PG:TDB prepared by the DRV procedure, produced higher levels of IFN-γ than either DRV or MLV liposomes composed of DMPC:SA:TDB. These anionic liposomes may release their protein content rapidly, as revealed by the *in vitro* release studies (Figure 7.3) showing a burst release of antigen from DMPC:PG:TDB liposomes, thereby promptly initiating high levels of immune response as indicated by high levels of IFN-γ release. Conversely, after the third immunisation, excluding DDA and DDA:TDB formulations, DRV liposomes consisting of DMPC:SA:TDB leads to the greatest release of IFN-γ, with anionic liposomes producing the least which shows to be almost equivalent to the naive mice (Figure 7.4), presumably due to

the slow release of protein from this cationic DRV formulation (Figure 7.3) initiating a long-term Th1 response. DRV liposomes formulated with DMPC:SA:TDB were relatively large in size (924 nm; Table 7.1), particularly in comparison to MLV of the same composition or DRV composed of DMPC:PG:TDB (595 nm and 479 nm, respectively; Table 7.1). Therefore, these larger cationic DRV liposomes are expected to be retained at the site of injection for longer, where the entrapped protein was slowly released as the lipid bilayers disintegrated, by tissue phospholipases over time (Velinova *et al.*, 1996; Perrie *et al.*, 2001; Gregoriadis, 2003) and additionally take longer to migrate to the regional lymph nodes to initiate an adjuvant action. Thus ultimately, an immense increase in IFN-γ production and release was observed for DRV DMPC:SA:TDB than the other two formulations, with efficacy in the order of cationic DRV > cationic MLV > negative DRV (Figure 7.4). Overall, the cationic liposomes induced higher immune responses than negative liposomes, which may be attributed to the presence of the cationic lipid SA. Although SA has previously been reported to inhibit PKC activation, it also appears to be an immunogenic lipid, therefore inclusion of SA within lipid formulations will enhance the immunological adjuvant properties of these liposomes (Bramwell and Perrie, 2005). Conversely, previous reports have found high levels of protection against challenge with *Candida albicans* after immunisation with anionic liposome formulations of DMPC:DMPG in combination with co-adjuvant lipid A (Eckstein *et al.*, 1997), at a 9:1 molar ratio. However, results presented here confirm findings from other studies (Nakanishi *et al.*, 1997) demonstrating that positively charged liposomes, consisting of SA, enhance antigen-specific immune responses by acting as more potent adjuvants to induce cytotoxic T lymphocyte (CTL) responses than negatively charged liposomes, consisting of PA. Once more illustrating that the adjuvant effect of liposomes for regulating immune responses is not associated with any specific formulation.

DRV liposomes, with the inclusion of a cationic lipid, such as SA, efficiently incorporate high values of the fusion protein, Ag85B-ESAT-6, while also inducing high levels of IFN-γ, compared to anionic DRV or cationic MLV. This could be attributed to the slow and controlled release of entrapped protein with the DRV formulation DMPC:SA:TDB acting as a depot at the site of injection.

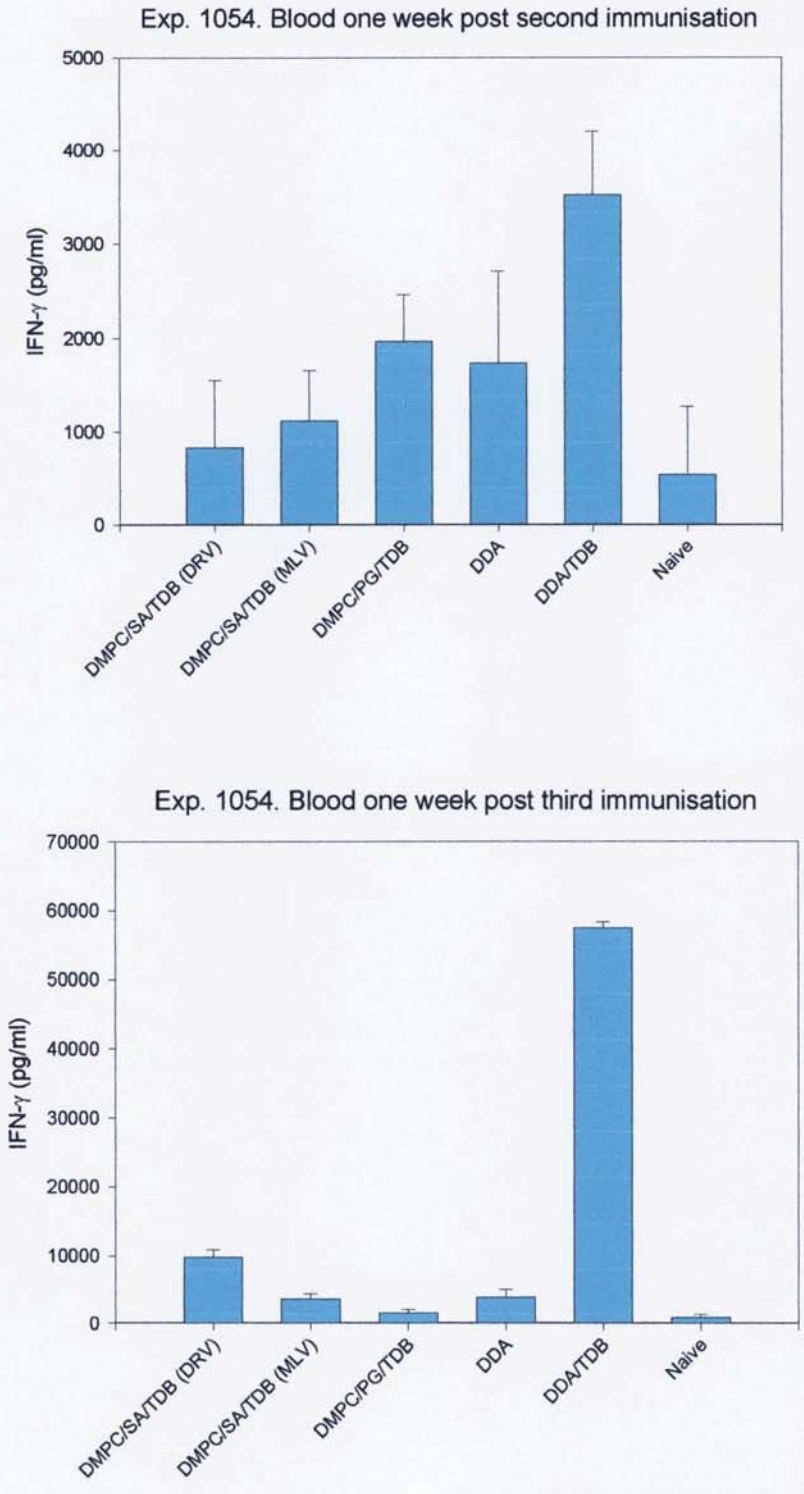


Figure 7.4 Effect of liposome preparation and surface charge on the induction of immune responses in mice immunised with DDA, DDA:TDB and DMPC based liposome-incorporated Ag85B-ESAT-6 fusion protein. Balb/C mice were injected subcutaneously with 2 μg of Ag85B-ESAT-6 fusion protein either entrapped within DRV liposomes composed of DMPC:PG:TDB or DMPC:SA:TDB, or surface adsorbed to MLV liposomes composed of DMPC:SA:TDB. Blood lymphocytes were isolated one week after second immunisation (a) and one week after third immunisation (b) and were subsequently re-stimulated *in vitro* with the Ag85B-ESAT-6 (5μg/ml). The amount of IFN-γ released was measured and analysed. (Immunological characterisation work carried out by SSI).

7.5 Liposome formulation: effect of lipid composition

Based on the *in vitro* release studies and immunological data obtained in section 7.4, it appears that the slow and steady release of protein antigen from DRV composed of DMPC:SA:TDB, induces high levels of IFN-γ. Liposomes composed of high transition temperature phospholipids are rigid and extremely stable in terms of permeability (Saarinen-Savolainen *et al.*, 1997), even when applied *in vivo* (Gregoriadis, 1990). It is now well detailed that increasing the rigidity and bilayer stability of liposomes can reduce clearance rates of liposomes from circulation (Gregoriadis, 1990), especially when formulated together with cholesterol, which is also known to enhance stability and reduce bilayer permeability, dependent on the optimum cholesterol content (Gregoriadis and Davis, 1979; Mohammed *et al*, 2004). The inclusion of DSPC, a phospholipid with a higher transition temperature than DMPC, will increase bilayer rigidity and stability even further, while conceivably generating slower antigen release from liposome formulations and potentially enhancing immune responses further. Cholesterol was included within all formulations, as previous studies have demonstrated the beneficial role of Chol inclusion within liposomal formulations (Papahadjopoulos *et al.*, 1973; Jonas and Maine, 1979; Semple *et al.*, 1996; Devaraj *et al.*, 2002), demonstrating that the inclusion of up to 50% Chol increases liposome stability and integrity, while equally reducing bilayer permeability (Gregoriadis and Davis, 1979; Gregoriadis, 1993).

Within this section, various factors related to lipid composition were investigated, in terms of characterisation, protein release and immunology. The role of liposome charge was once again analysed, alternatively comparing neutral and cationic formulations. Cationic liposomes formulated with the synthetic, amphiphilic cationic lipid compound DDA have previously been reported as effective adjuvant systems, greatly enhancing immune responses to the associated antigen against tuberculosis infections (Lindblad *et al.*, 1997; Holten-Andersen *et al.*, 2004). Therefore, DDA was utilised as the cationic lipid within the following studies. Furthermore, previous studies reported that the addition of TDB within these DDA liposomes induces a considerably high Th1 immune response against the Ag85B-ESAT-6 fusion protein antigen (Davidsen *et al.*, 2005).

7.5.1 Physiochemical characteristics of liposomes formulated with or without the cationic lipid DDA and the immunomodulator TDB

The inclusion of the cationic lipid DDA, within liposome formulations influenced protein entrapment, vesicle size and zeta potential values within DRV liposomes (Table 7.2). The addition of 2 μmole DDA to liposomes composed of 8 μmole DSPC, 8 μmole Chol and 125 μg/ml TDB per ml, significantly increased entrapment values by almost 20%, from 65% to 81%. By the addition of DDA, not only is the liposome surface rendered positively charged, as confirmed by an increase in zeta potential values from –14 mV to 50 mV, it is also known that the charged lipids cause an increase in the aqueous volume between lipid bilayers thus enabling more antigen incorporation within liposomal vesicles (Gregoriadis, 1990) and indeed results presented here confirm this. Furthermore, the high positive charge apposed on liposome vesicles consequently reduces vesicle size from 2066 nm to 782 nm, as a result of the charged surfaces repelling each other sufficiently to prevent membrane adhesion and liposome fusion upon rehydration during the DRV procedure (Perrie and Gregoriadis, 2000; Perrie *et al.*, 2001). Neutral liposomes are significantly larger due to the absence of DDA and vesicle repulsion, resulting in liposome fusion and the formation of larger vesicles (2066 nm; Table 7.2). However, the addition of the immunomodulator TDB did not affect the physiochemical characteristics of these DRV liposomes.

Liposome formulation	Protein incorporation (% of used ± SD)	Size (nm ± SD)	Zeta potential (mV ± SD)
DSPC:Chol:TDB	65.4 ± 5.3*	2066 ± 285**	-14.2 ± 10.6
DSPC:Chol:DDA	83.3 ± 2.0	811.3 ± 31.8	47.0 ± 6.7
DSPC:Chol:DDA:TDB	80.7 ± 3.3	782.4 ± 50.0	50.3 ± 6.2

Table 7.2 Effect of liposome composition on protein entrapment, vesicle size and zeta potential. Liposomes composed of 16μmoles of DSPC, 16μmoles of Chol and 4μmoles DDA and 25μg TDB, where applicable. All formulations were prepared by the DRV procedure entrapping the protein within liposome bilayers. Entrapment values were determined by ^{125}I-labeled protein. The vesicle z-average diameter and zeta potential of each liposomal preparation was measured in double-distilled water or 0.001 M PBS, respectively, at 25°C using a Brookhaven ZetaPlus. * signifies that protein antigen incorporation values of the liposome formulation omitting the cationic lipid DDA are significantly lower ($P<0.05$, ANOVA) than liposomes including DDA. ** signifies that formulations omitting the cationic lipid DDA, produce significantly larger ($P<0.05$, ANOVA) liposome vesicles than those including DDA. Results denote mean ± SD, $n = 6$.

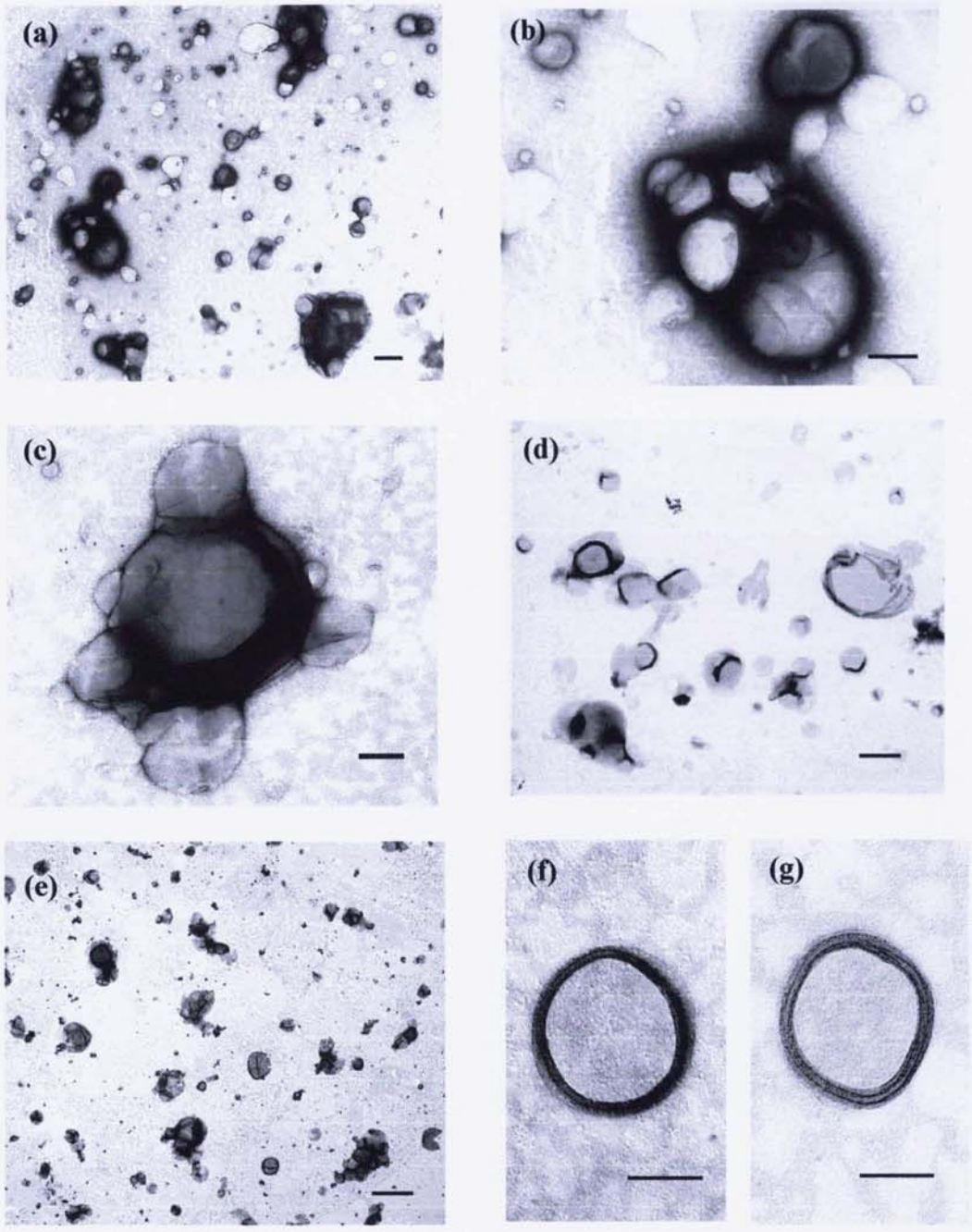


Figure 7.5 Transmission electron micrographs of empty DRV liposomes composed of DSPC:Chol:TDB and DSPC:Chol:DDA:TDB. Liposomes were prepared by the DRV procedure, in which SUV were freeze-dried in the absence of protein antigen, followed by controlled rehydration. Neutral liposomes (a,bar = 700 nm, b, bar = 300 nm and c, bar = 200 nm) composed of DSPC:Chol:TDB, appear as a heterogeneous population of individual liposomes and aggregates. The inclusion of the cationic lipid DDA within the liposome formulation, DSPC:Chol:DDA:TDB also produces a heterogeneous population of both aggregated and individual DRV liposomes (d, bar = 500 nm and e, bar = 1500 nm), however very small single liposomes are shown (f and g, bar = 100 nm). Samples were stained with 2% uranyl acetate and viewed with a JEOL 1200EX TEM.

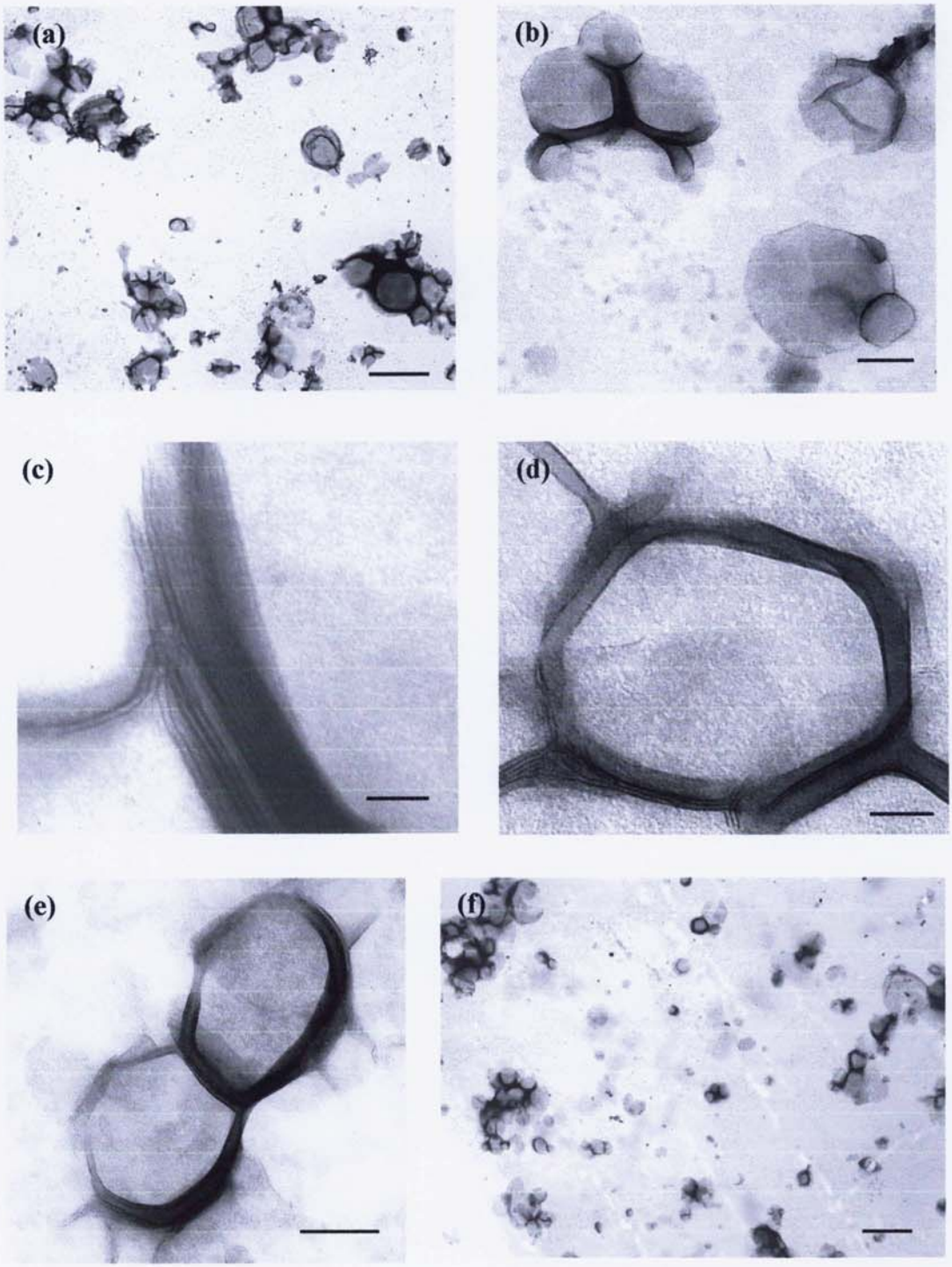


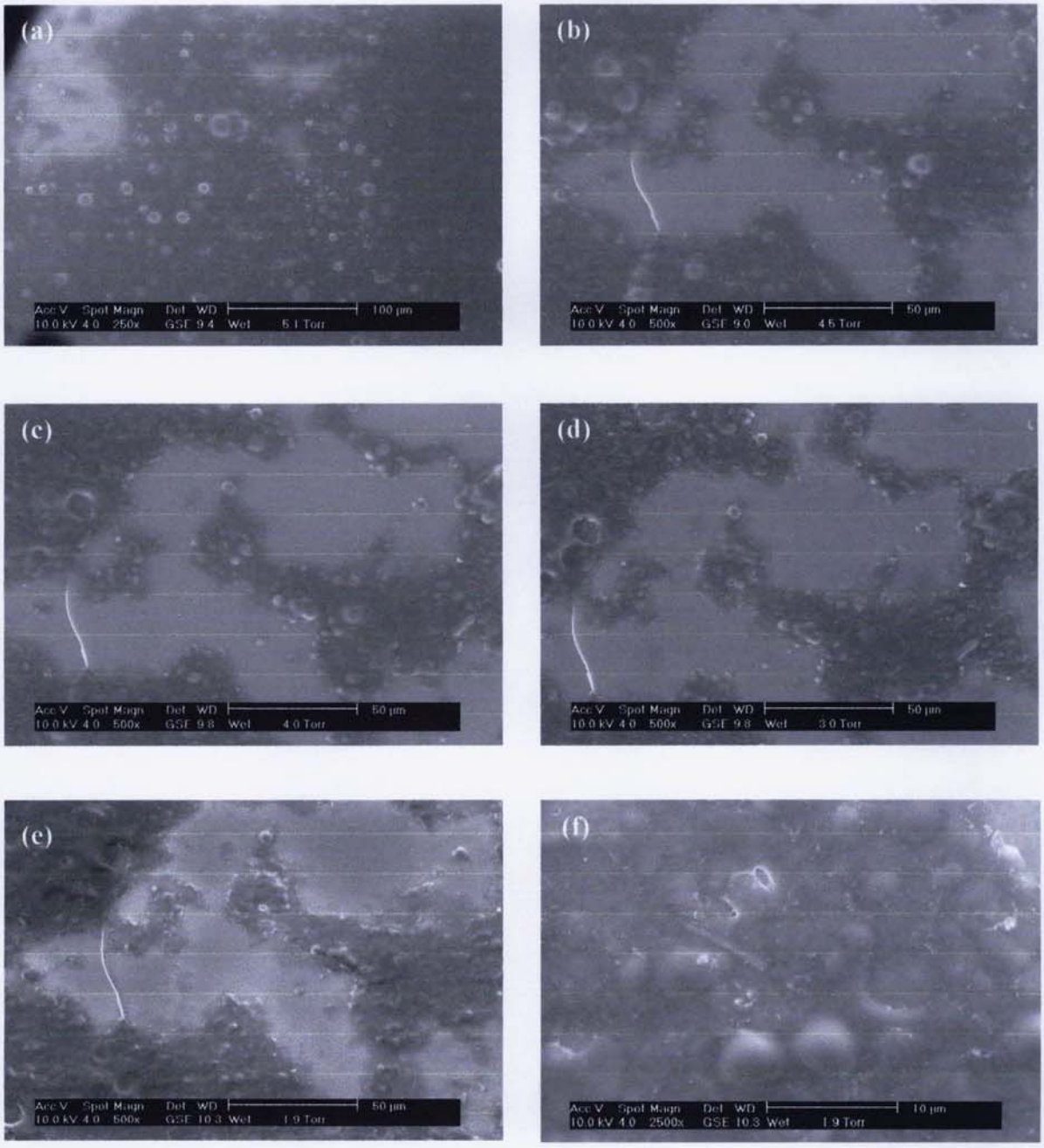
Figure 7.6 Transmission electron micrographs of DRV entrapping Ag85B-ESAT-6 fusion protein, with liposomes composed of DSPC:Chol:TDB and DSPC:Chol:DDA:TDB. Liposomes were prepared by the DRV procedure, in which SUV were freeze-dried in the presence of protein antigen, followed by controlled rehydration. Neutral liposomes (a, bar = 1500 nm and b, bar = 300nm, c = bar = 500 nm) composed of DSPC:Chol:TDB, appear as a heterogeneous population of individual liposomes and aggregates. The inclusion of the cationic lipid DDA within the liposome formulation, DSPC:Chol:DDA:TDB produces individual DRV liposomes (d, bar = 50 nm, e, bar = 200 nm and f, bar = 1500 nm). Samples were stained with 2% uranyl acetate and viewed with a JEOL 1200EX TEM.

Liposomes omitting or including the immunomodulator, TDB, did not exhibit any differences with regard to the liposome physiochemical characteristics, however, differences between neutral and cationic liposomes were evident. Therefore, liposomes composed of DSPC:Chol:TDB and DSPC:Chol:DDA:TDB were viewed by TEM, in order to observe the morphological differences between these formulations. In addition, both empty DRV (Figure 7.5a-g) and DRV(protein) (Figures 7.6 a-f) were viewed for both formulations, to further investigate whether the presence of entrapped protein antigen altered liposome morphology and structure.

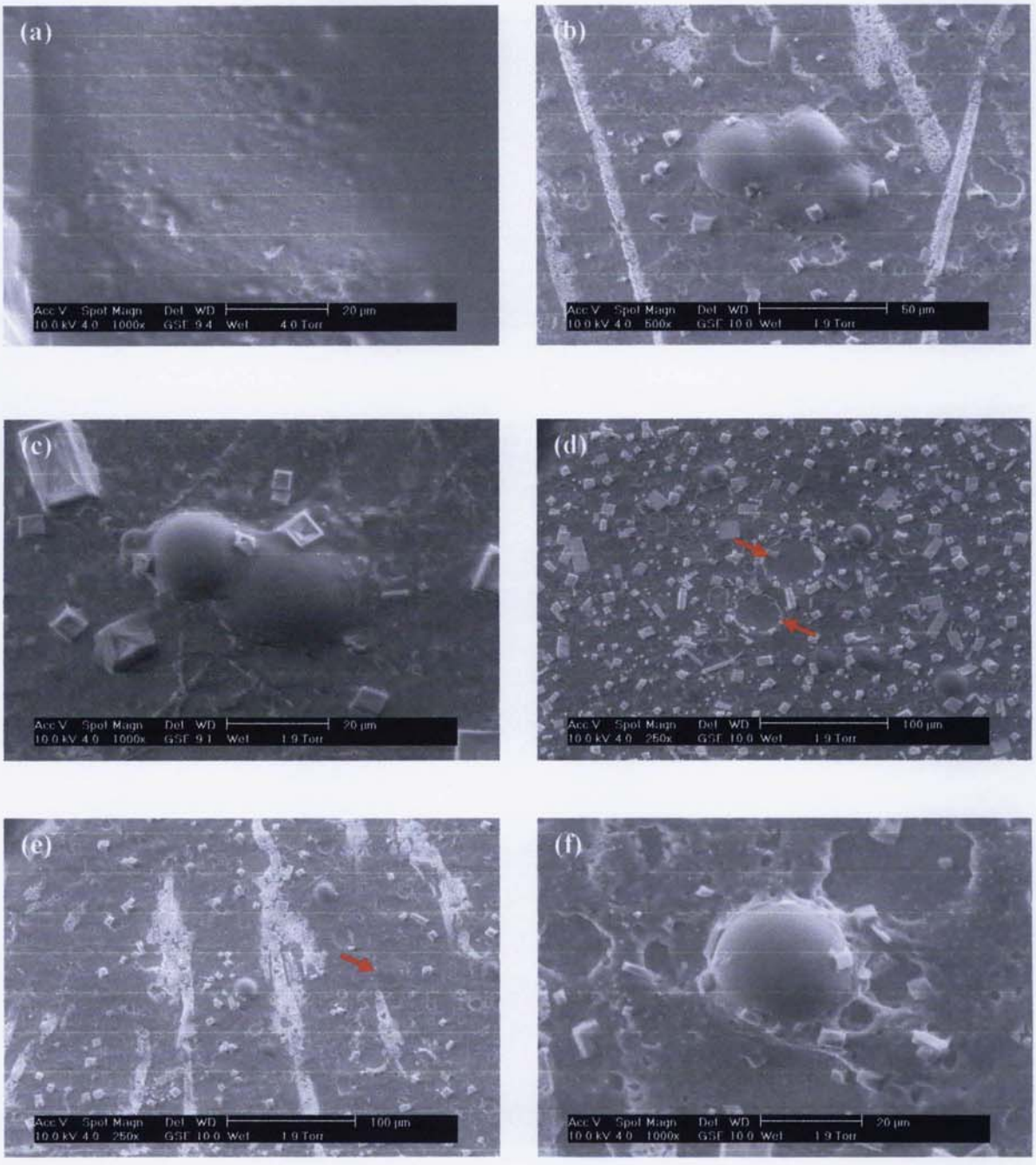
Neutral empty DRV liposomes composed of DSPC:Chol:TDB, appear as a heterogeneous population of both individual and aggregated liposomes, exhibiting immense variation in vesicle size (Figure 7.5a). It appears that these neutral liposomes have a tendency to collide and aggregate into larger structures (Figures 7.5b and c) due to the lack of electrostatic repulsion between individual liposomes. However, these aggregated liposomes appear to be loosely bound together, with each liposome remaining as individual vesicle structures. However, even with the addition of the cationic lipid DDA within this formulation (DSPC:Chol:DDA:TDB), it appears that these vesicles still exhibit aggregation of DRV liposomes, as shown in Figures 7.5d and e, although, to a lesser extent, presumably due to the existence of electrostatic repulsions. In addition to these larger DRV, smaller individual liposomes were seen (Figures 7.5f and g), which were only approximately 100-200 nm in size. These smaller vesicles could be small DRV liposomes that are that have not fully aggregated into larger DRV, after the freeze-drying process. This could be attributed to the strong electrostatic repulsions between these cationic vesicles, as these small liposomal structures were not found within the neutral formulation DRV suspension. In agreement with previous reports (Davidsen *et al.*, 2005), which analysed the morphology of DDA:TDB liposomes by TEM, the presence of protein antigen to both neutral and cationic liposome formulation did not effect DRV morphology, as shown by the TEM micrographs of DRV, neutral and cationic formulations, comparing empty DRV and DRV entrapping protein antigen (Figures 7.5a-g and Figures 7.6a-f, respectively). Once more, Figures 7.6a and b show aggregation within the neutral liposome suspension, with an extensive size range between 1200-2800 nm, showing the immense heterogeneity of this liposome formulation. The size ranges from the micrographs also fall within the size range as measured by photon correlation (between 1600-2400 nm; Table 7.2), although with a broader distribution. The

ZetaPlus particle sizer (Brookhaven) only sizes particles up to a maximum of 3000 nm, therefore, the larger aggregates observed by TEM may not be detected by the equipment used and may account the slight differences within size distributions. As expected, the micrographs reveal that these DRV liposomes exhibit bilayer structures, within both neutral and cationic formulations, as shown in Figures 7.6c and d. Within aggregated cationic structures, each individual vesicle appears to be in the size range 300-600 nm (Figures 7.6d and e), although the total aggregate measures approximately 2 or 3 times larger (Figures 7.6e and f), as expected. These cationic DRV entrapping protein antigen exhibit some aggregation, however, to a smaller extent when compared the neutral formulation (Figure 7.6f).

Further to the TEM micrographs, ESEM was also employed to investigate the morphology of these DRV liposomes, while additionally the stability of the sample preparation can be investigated by simple alterations of atmospheric pressure within the sample chamber as the ESEM allows variations to occur within the sample environment (Mohammed *et al.*, 2004). Figures 7.7 and 7.8 shows neutral liposomes composed of DSPC:Chol:TDB and cationic liposomes composed of DSPC:Chol:DDA:TDB, respectively, entrapping protein antigen within DRV. As shown in Figure 7.7a, neutral liposomes at a pressure of 5.1 Torr emerged as a heterogeneous population of spherical vesicles, although vesicle size was considerably larger than those sizes generated by photon correlation and TEM. These spherical liposomes appear to maintain shape and structure as the pressure is reduced to 4.5 or even 4.0 Torr, while the surrounding aqueous atmosphere is gradually evaporated with the dehydrated environment exemplified by the light grey background (Figures 7.7b and c, respectively). Decreasing the atmospheric pressure within the sample chamber even further to 3.0 Torr it is evident that surrounding environment dries out considerably, although spherical liposomes remain visible, demonstrating the stability of these neutral liposomes at this operating pressure (Figure 7.7d). However, at a pressure of 1.9 Torr and further evaporation of the aqueous environment, the liposomes begin to collapse and flatten as shown in Figures 7.7e and f, with the liposomes completely losing their spherical shape and forming a flattened lipid film on the sample holder (Figure 7.7f).



Figures 7.7a-f Environmental scanning electron micrographs of neutral liposomes composed of 16µmoles DSPC, 16µmoles Chol and 250µg TDB entrapping protein. Neutral liposomes appear as spherical vesicles when exposed to an operating pressure of 5.1 Torr (a). When the pressure is reduced to 4.5 (b) and even 4.0 (c) Torr, the liposomes remain spherical in shape. At an operating pressure of 3 Torr (d), it appears the liposomes begin to coalesce, losing their original form. With a further reduction to 1.9 Torr (e and f), liposomes begin to flatten and lose their spherical shape, although there are still a number of spherical liposomes visible.



Figures 7.8a-f Environmental scanning electron micrographs of cationic liposomes composed of 16 μmoles DSPC, 16 μmoles Chol, 4 μmoles DDA and 250 μg TDB entrapping protein. Cationic liposomes appear as spherical vesicles when exposed to an operating pressure of 4.0 Torr (a). When the pressure is dramatically reduced to 1.9 Torr (b and c), it appears the liposomes begin to coalesce, showing signs of aggregation with neighboring liposomes. The liposomes begin to fuse into larger structures (d-f) and may additionally flatten and lose spherical shape (as shown by arrows).

The liposome formulation DSPC:Chol:TDB maintains its spherical structure and appears to remain stable at quite low operating pressures. However, the ESEM micrographs of the cationic liposome formulation DSPC:Chol:DDA:TDB, reveals a completely different effect, as Figures 7.8a-f show. At an operating pressure of 4.0 Torr (Figure 7.8a), liposome vesicles appear to materialise as spherical liposomes. However, when the operating pressure is reduced to 1.9 Torr, Figures 7.8b-f reveal that rather than coalesce and flatten under low atmospheric pressure, these cationic liposome vesicles appear to aggregate and fuse into much larger structures, with some vesicles producing sizes of approximately 20-50 μm whereas the measured vesicle sizes, as confirmed by photon correlation and TEM micrographs, were around 800-1000 nm, exhibiting a considerable size difference. Presumably the electrostatic interactions between the individual cationic liposomes is highly dominated by the reduction in operating pressure, forcing the liposomes to interact and coalesce and as shown in Figures 7.8b and c inducing aggregation of vesicles. As a result, presumably to overcome instability at this low operating pressure, liposomes may fuse forming enlarged spherical individual liposomes as shown in Figures 7.8d-f. However, a few of these cationic liposomes do appear to lose their spherical shape and become flattened into a lipid film, as shown in Figures 7.8d and e (indicated by arrows).

7.5.2 Rate of protein release from liposomes formulated with or without the cationic lipid DDA and the immunomodulator TDB

Neutral liposomes, omitting the cationic lipid DDA, exhibited a slow rate of protein release from liposomal vesicles, as shown in Figure 7.9, and even after 1008 hours incubation at 37°C, approximately 20% protein still remained entrapped within neutral liposomes compared to 9% for both cationic formulations. Although for the first 336 hours of incubation, protein release from the cationic liposomes, DSPC:Chol:DDA and DSPC:Chol:DDA:TDB, was considerably slower than neutral liposomes, with only 19% of the total protein load released compared to 45% for DSPC:Chol:TDB. Alternatively, after 672 hours there is a reversal in terms of the amount of protein released from liposomes. Figure 7.9 shows that after 336 hours there is a burst release of protein from both liposome formulations consisting of DDA, with 86-91% total protein released after 672 hours, whereas on the other hand, only 61% of the total protein incorporated within neutral liposomes was released after 672 hours. Chol was included within the formulations to stabilise the lipid bilayer thereby decreasing bilayer permeability. It is now well recognised that the more rigid

and stable the liposomes are, the slower the rate of solute release and additionally clearance from circulation (Gregoriadis, 1990). However, by imposing a positive surface charge by the addition of a cationic lipid to the formulation, such as DDA, the permeability of the liposome bilayer increases (Mohammed *et al.*, 2004) while decreasing bilayer rigidity and stability, therefore the rate protein release is unrestrained, as is shown in Figure 7.9 when comparing neutral and cationic formulations. This occurs due to a depression of the phase transition temperature of the neutral phospholipid, DSPC, thereby lowering the temperature at which the lipids within the bilayer become mobile.

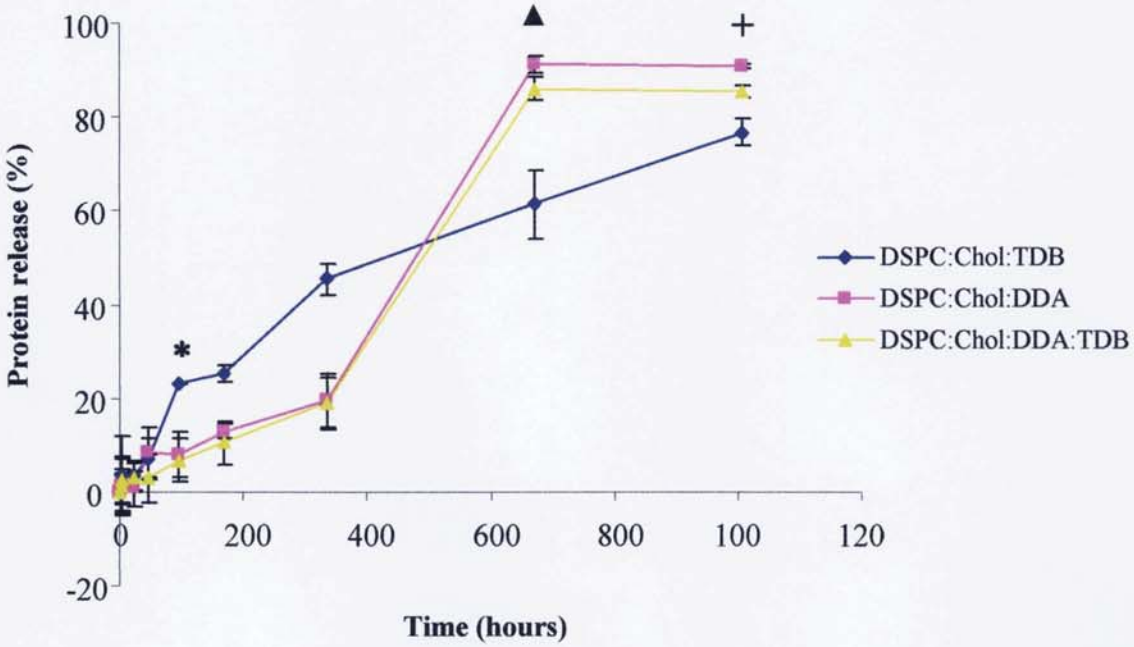


Figure 7.9 Rate of protein release from various liposomes formulated with or without the cationic lipid DDA and the immunomodulator TDB. Each formulation was incubated in a 37°C water bath, with continuous shaking. At various time intervals, 1ml samples were taken, measuring the level of radioactivity of I^{125} prior to centrifugation (to obtain total counts) and after centrifugation in the pellet and supernatant (to obtain percentage entrapment and recovery). The 1ml sample removed was replenished with 1ml PBS, to retain sink conditions. * signifies that DSPC:Chol:TDB significantly ($P<0.05$, ANOVA) releases more protein content than DSPC:Chol:DDA and DSPC:Chol:DDA:TDB, after 168 hours (i.e. 7 days). ▲ signifies that both DSPC:Chol:DDA and DSPC:Chol:DDA:TDB significantly ($P<0.05$, ANOVA) release more protein content than DSPC:Chol:TDB, after 672 hours (i.e. 28 days). + signifies that overall, DSPC:Chol:TDB releases significantly ($P<0.05$, ANOVA) more protein than DSPC:Chol:DDA and DSPC:Chol:DDA:TDB after the full 1008 hours (i.e. 42 hours) incubation. Results denote mean ± SD, $n = 3$.

Overall, liposomes composed of DSPC:Chol:TDB entrap reduced amounts of protein antigen (Table 7.3), although protein is released at a steady and controlled rate as a consequence of the lipid interactions. On the other hand, the cationic liposome formulations,

DSPC:Chol:DDA and DSPC:Chol:DDA:TDB, entrap a considerable amount of protein antigen due to the electrostatic interactions between the cationic lipids and the anionic charges of the protein antigen, yet protein release rates are not as steady and stable as anionic liposomes.

7.5.3 Immunological characterisation in vivo

C57L/6J mice were immunised with 2 μg of the fusion protein, Ag85BESAT-6, entrapped within liposomes composed of DSPC:Chol (1.6:1.6 μmole/mouse), DSPC:Chol:TDB (1.6:1.6 μmole:25 μg/mouse), DSPC:Chol:DDA (1.6:1.6:0.4 μmole/mouse) or DSPC:Chol:DDA:TDB (1.6:1.6:0.4 μmole:25 μg/mouse). Blood was taken week one and week three post last immunisation. From this, the level of the IFN-γ production was determined by and re-stimulating the isolated lymphocytes with Ag85B ESAT-6 fusion protein and measuring the amount of IFN-γ released into the cell cultures after 72 hours incubation. Liposomes composed of DSPC:Chol, which showed no difference in liposome characteristics than DSPC:Chol:TDB (data not shown), was also included within these *in vivo* studies as a control, to investigate the effect of TDB within neutral formulations.

Liposomes formulated with the cationic lipid DDA has previously been reported as an effective adjuvant for eliciting humoral and cell mediated immunity (Davidsen *et al.*, 2005). However, as DDA liposomes have shown to be physically unstable due to salt induced aggregation, various other compounds needed to be included within the formulation to form stable liposomal systems. With the addition of the immunomodulator, TDB, the protective immune response, as indicated by the amount of IFN-γ production, increases considerably, presumably resulting from a combination of enhanced immunogenicity due to the addition of TDB and enhanced vesicle stability (Figure 7.10a and b). The high melting phospholipid, DSPC was also added to the formulation, in order to enhance the stability of these liposomal systems even further. Results from Figure 7.10a and b, show that all four liposome formulations tested induced production and release of IFN-γ, one week and three weeks after the last immunisation. However, results show that the inclusion of TDB within these liposomal formulations enhanced IFN-γ production compared to those formulations omitting TDB. Immunisation with the liposome formulation DSPC:Chol:DDA:TDB entrapping the Ag85B-ESAT-6, induced the highest level of IFN-γ release than any of the other DSPC-containing liposome formulations, at both one week and three weeks after last immunisation

(Figures 7.10a and b). Additionally, one week after last immunisation the presence of DDA within both cationic formulations increased production of IFN-γ in comparison to the equivalent neutral formulations (Figure 7.10a), demonstrating that DDA is effective at eliciting high levels of cell-mediated immunity. Then again, after three weeks from the last immunisation, the amount of IFN-γ released for the cationic liposome formulation omitting TDB, decreased to values lower than the neutral counterpart and almost equivalent to the naïve group (Figure 7.10b), which could be attributed to a burst release of protein antigen, as shown within *in vitro* (Figure 7.9). While DSPC:Chol:DDA:TDB also exhibited burst release *in vitro* (Figure 7.9), the high levels of IFN-γ result from the inclusion of the immunomodulator, TDB. However, once applied *in vivo*, liposome characteristics of these cationic liposomes may alter due to the electrostatic interactions with anionic molecules in serum, therefore it would be interesting to carry out further analysis investigating the effect of serum on these liposome formulations physiochemical characteristics. This data confirms findings from previous reports (Davidsen *et al.*, 2005), showing that although DDA alone may possess immunological properties, immunogenicity is greatly enhanced by the incorporation of TDB.

The neutral formulations do not exhibit such a significant reduction in IFN-γ release in comparison to the cationic counterparts (Figure 7.10a and b), presumably due to the controlled and slow release of protein antigen from these neutral formulations, as shown within the *in vitro* release for DSPC:Chol:TDB (Figure 7.9). Additionally, some of these vesicles, due to their large size (Table 7.3), may break down locally to release their vaccine content slowly (Gregoriadis, 1990; Perrie *et al.*, 2001). Therefore, relatively maintaining therapeutic levels of protein antigen and prolonging the initial response (Figure 7.10a and b). However, despite this overall the cationic formulation, DSPC:Chol:DDA:TDB, produced the highest levels of IFN-γ. Once again, these neutral formulations show that the addition of TDB effectively enhances immune responses and increases the overall adjuvanticity of these liposome formulations.

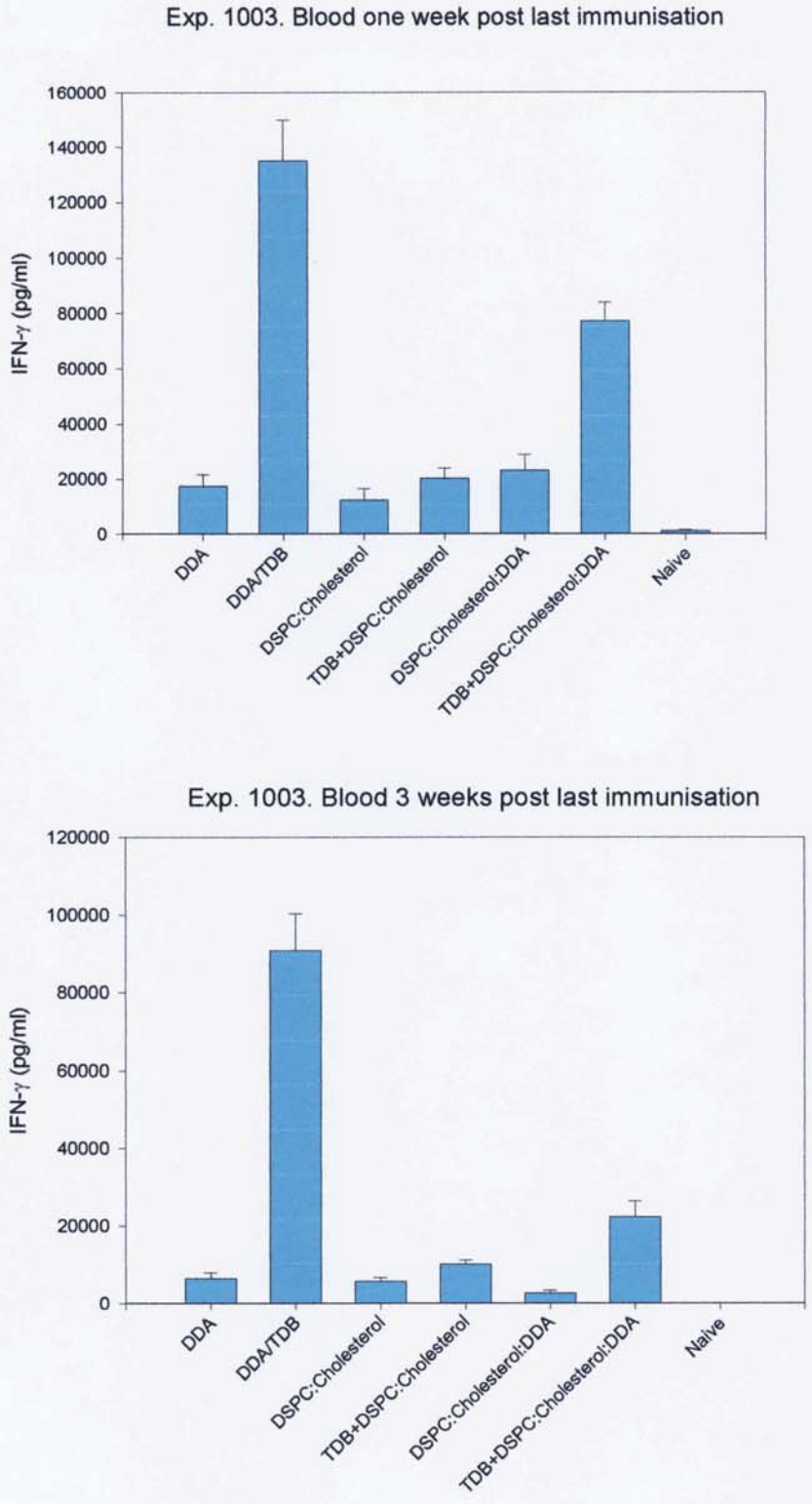


Figure 7.10 Effect of liposome composition on the induction of immune responses in mice immunised with DDA, DDA:TDB and DSPC based liposome-entrapped Ag85B-ESAT-6 fusion protein. C57L/6J mice were injected subcutaneously with 2 μg of Ag85B-ESAT-6 fusion protein, entrapped within DRV liposomes composed of DPSC:Chol:TDB, DSPC:Chol:DDA or DSPC:Chol:DDA:TDB. Blood lymphocytes were isolated one week after last immunisation (a) and three weeks after last immunisation (b) and were subsequently re-stimulated *in vitro* with the Ag85B-ESAT-6 (5μg/ml). The amount of IFN-γ released was measured and analysed. (Immunological characterisation work carried out by SSI).

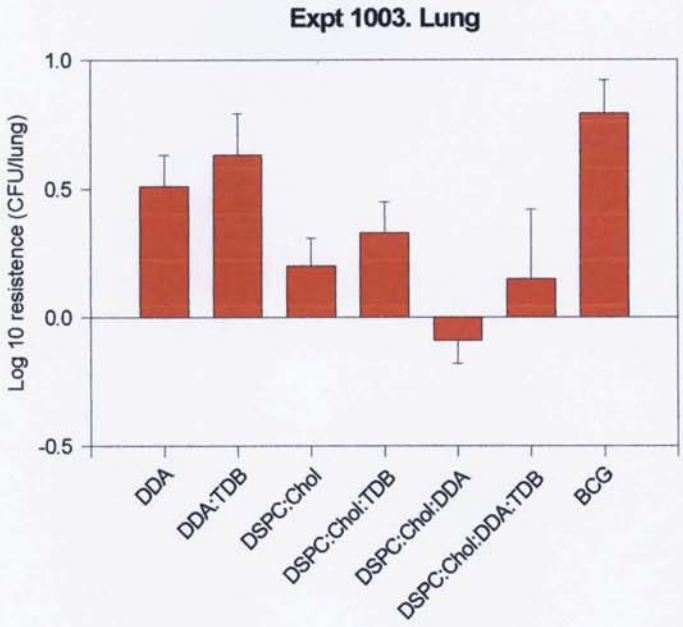


Figure 7.11 Resistance to challenge with an infection of tuberculosis after immunisation with the fusion protein Ag85B-ESAT-6 incorporated with various liposomal formulations. Immunised mice received an aerosol challenge with *M. tuberculosis* Erdman and the numbers of bacteria, stated as colony forming units (CFU), were quantified in the lungs 9 weeks after. All values are shown as 10 individual mice ± SD. (Immunological characterisation work carried out by SSI).

After immunisation, the mice received an aerosol challenge of *M. tuberculosis* to assess the level of long-term immunity for each DSPC-containing formulation. Lungs were removed 9 weeks after challenge and the resistance to bacteria within the lungs was measured and expressed as Log 10 resistance. As expected, results in Figure 7.11 show that the liposome formulations containing TDB produced the highest levels of protection, with both the cationic and neutral formulation producing comparable levels of resistance. This shows that although the presence of DDA within these DSPC-containing formulations induce good levels of IFN-γ, DDA does not enhance the level of overall protection when challenged with subsequent infections of *M. tuberculosis*, as shown in Figure 7.11.

Although the IFN-γ production and immune response is mostly dependent on TDB inclusion, the inclusion of Chol into the liposome formulations may also contribute slightly to the release of IFN-γ, as previous studies have previously shown (Batenjany *et al.*, 2001).

7.5.4 Pharmaceutical development: liposome stability

As a pharmaceutical product the liposome formulation should be stable over a long period of time and possess a relatively long shelf life. A stable product should; retain the entrapped protein efficiently, show no signs of aggregation and maintain its original characteristics over time. The ideal product should be in a freeze-dried form, however, my formulations at present are in liquid form and therefore stability is likely to be reduced. Aqueous formulations have a tendency to be less stable as the presence of water surrounding the lipid preparations causes hydrolytic degradation. However, the extent of this degradation process is governed by various factors; storage temperature, aggregation, phospholipid alkyl chain length and head group (www.avantilipids.com). Therefore, the long-term stability of liposomes is highly dependent on the lipid components used within liposome formulations. The stability of these liposomal formulations tested *in vivo* was measured by determining; the amount of protein retained within the liposomes (i.e. protein retention studies), vesicle aggregation and any changes in original physiochemical characteristics (i.e. size and surface charge).

7.5.4.1 Protein retention

DSPC:Chol liposomes in the presence or absence of the cationic lipid DDA or the immunomodulator TDB are stable for up to three months when stored at 4°C, as all formulations retained their protein load, with no significant differences between formulations (Figure 7.12a). This may be attributed to the rigid bilayers of these liposomal vesicles due to their lipid composition, as DSPC and Chol are know to stabilise and rigidify vesicle bilayers (Demel *et al.*, 1972; Gregoriadis and Davis, 1979; Gregoriadis, 1993; Bhattacharya and Halder, 1996; Mohammed *et al.*, 2004). The release of entrapped solutes from liposomes composed of DSPC has previously been shown to be slower than other phospholipids, such as PC or DMPC, due to the high transition temperature (T_c = 55°C) of this lipid (Mohammed *et al.*, 2004), as it is well known that solute release from liposomes is influenced and dependent on the phase transition temperature of the lipids within the bilayer (Senior and Gregoriadis, 1982; Saarinen-Savolainen *et al.*, 1997). Additionally, within liquid formulations, the inclusion of Chol impedes water diffusing deep into the lipid membranes, thus increasing liposome stability by reducing hydrolytic degradation (Simon and McIntosh, 1986). Therefore, overall these DSPC-containing liposome formulations should be relatively

stable during storage, even as liquid formulations, however, the temperature at which liposomes are stored could have a profound effect on stability (Small, 1986).

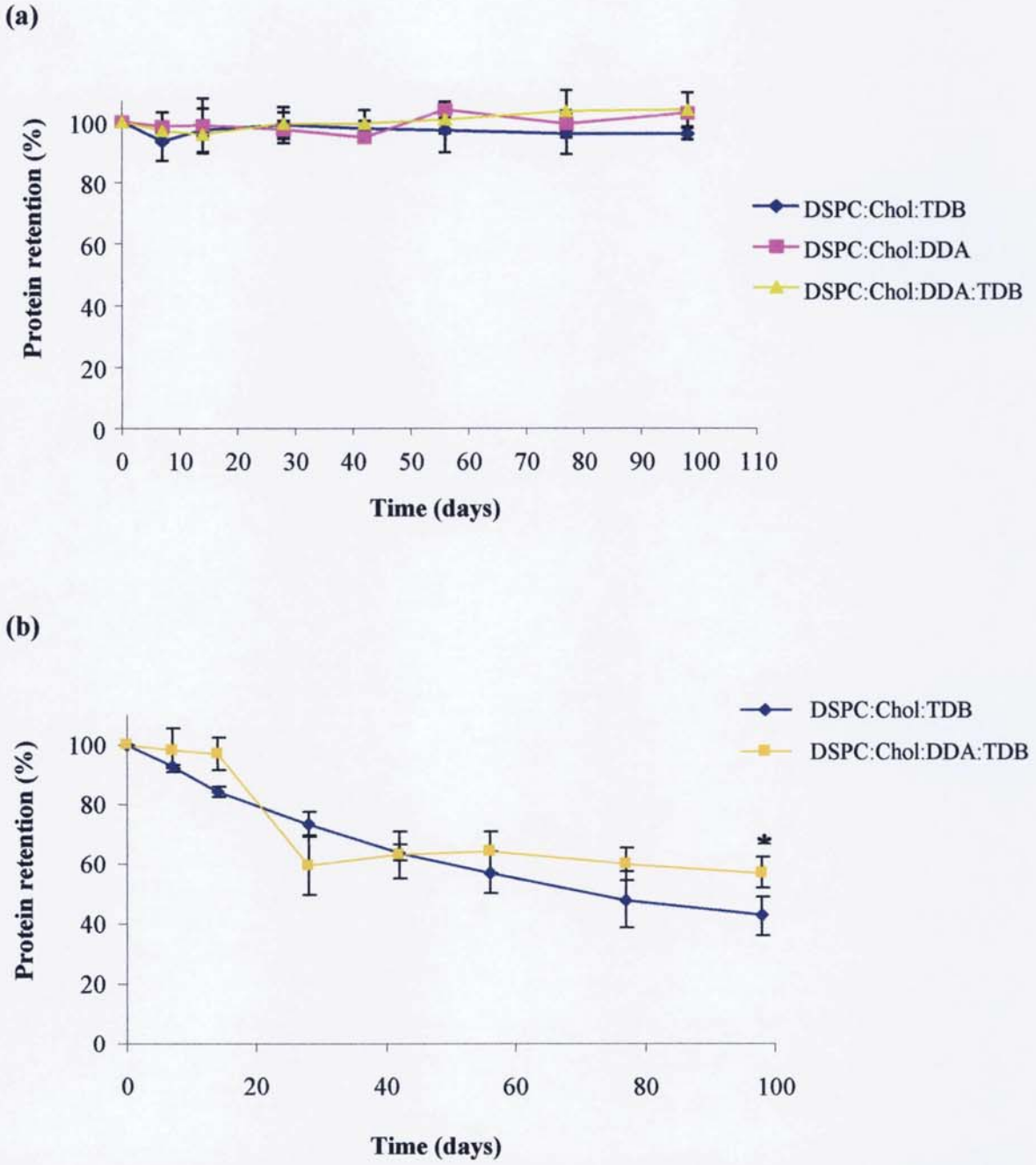


Figure 7.12 Effect of liposome composition on protein retention within liposome vesicles during storage at (a) 4°C and (b) room temperature (25°C). Liposomes composed of 16μmoles of DSPC, 16μmoles of Chol and 4μmoles DDA and 25μg TDB, where applicable All formulations were prepared by the DRV procedure entrapping the protein within liposome bilayers. Samples were stored at either (a) 4°C or (b) 25°C for approximately 3 months (i.e. 98 days). Samples were taken at set time intervals and the amount of protein retained within each liposomal formulation was measured by the level of radioactivity of I^{125} prior to centrifugation (to obtain total counts) and after centrifugation in the pellet and supernatant (to obtain percentage entrapment and recovery). * signifies that liposomes composed of DSPC:Chol:DDA:TDB retain significantly ($P<0.05$, ANOVA) more of its' protein load than DSPC:Chol:TDB, after 98 days stored at room temperature. Results denote mean ± SD, $n = 3$.

Although DSPC:Chol liposomes, in the presence or absence of DDA and TDB, are stable and retain their protein pay load when stored at 4°C (Figure 7.12a), conversely, when stored at room temperature (i.e. 25°C) the ability of these liposomes to retain protein is reduced (Figure 7.12b). Between day 14 and 28 there was a marked decrease in protein incorporation for liposomes composed of DSPC:Chol:DDA:TDB, releasing 37 ± 5% of its protein content (Figure 7.12b). Remarkably thereafter, protein incorporation within this formulation remains stable, retaining approximately 60% of its original payload throughout the remainder of the three month period (Figure 7.12b). The sudden leakage of protein at 25°C on day 28 may be attributed to aggregation of these liposomes.

7.5.4.2 Liposome stability: vesicle size and zeta potential studies

Cationic liposomes are relatively small in size, of z-average diameter between 780 nm and 810 nm, with and without the presence of TDB respectively. The presence of DDA within these formulations results in charged surfaces of the DRV, which sufficiently repel each other preventing aggregation or bilayer fusion (Perrie and Gregoriadis, 2000), thus DRV containing DDA remain relatively small in size. When stored at 4°C, as well as retaining their protein load (Figure 7.12a), these cationic liposomes additionally remain relatively stable with regard to size and zeta potential (Figure 7.13a and b), although the vesicle size of liposomes omitting TDB, does significantly increase at day 77 (998 nm; Figure 7.13a). However, neutral liposomes, immediately after preparation, are larger in size due to the lack of these electrostatic interactions between vesicles, therefore aggregation occurs with the vesicles fusing into significantly larger vesicles (2066 nm; Figure 7.13a). Interestingly, when these neutral liposomes are stored at 4°C, there is a considerable decrease in vesicle size to 1684 nm at day 42 (Figure 7.13a). As the TEM micrographs show (Figures 7.5a-c and 7.6a-c), these neutral liposomes consisting of DSPC:Chol:TDB, appear as clusters of particles held together in a loose arrangement, which are characterised as flocculates (Florence and Atwood, 1988). In addition, it was observed that this liposome formulation sediments faster than the cationic formulations, although re-dispersed easily once vortexed, further confirming that this neutral formulation may be a flocculated system. The cationic formulations, DSPC:Chol:DDA and DSPC:Chol:DDA:TDB, do not exhibit flocculates due to the existence of electrostatic repulsions between particles inhibiting close contact between vesicles. Thus, the measured decrease in liposome size during storage over time may occur due to the breakdown of these neutral aggregates or flocculates.

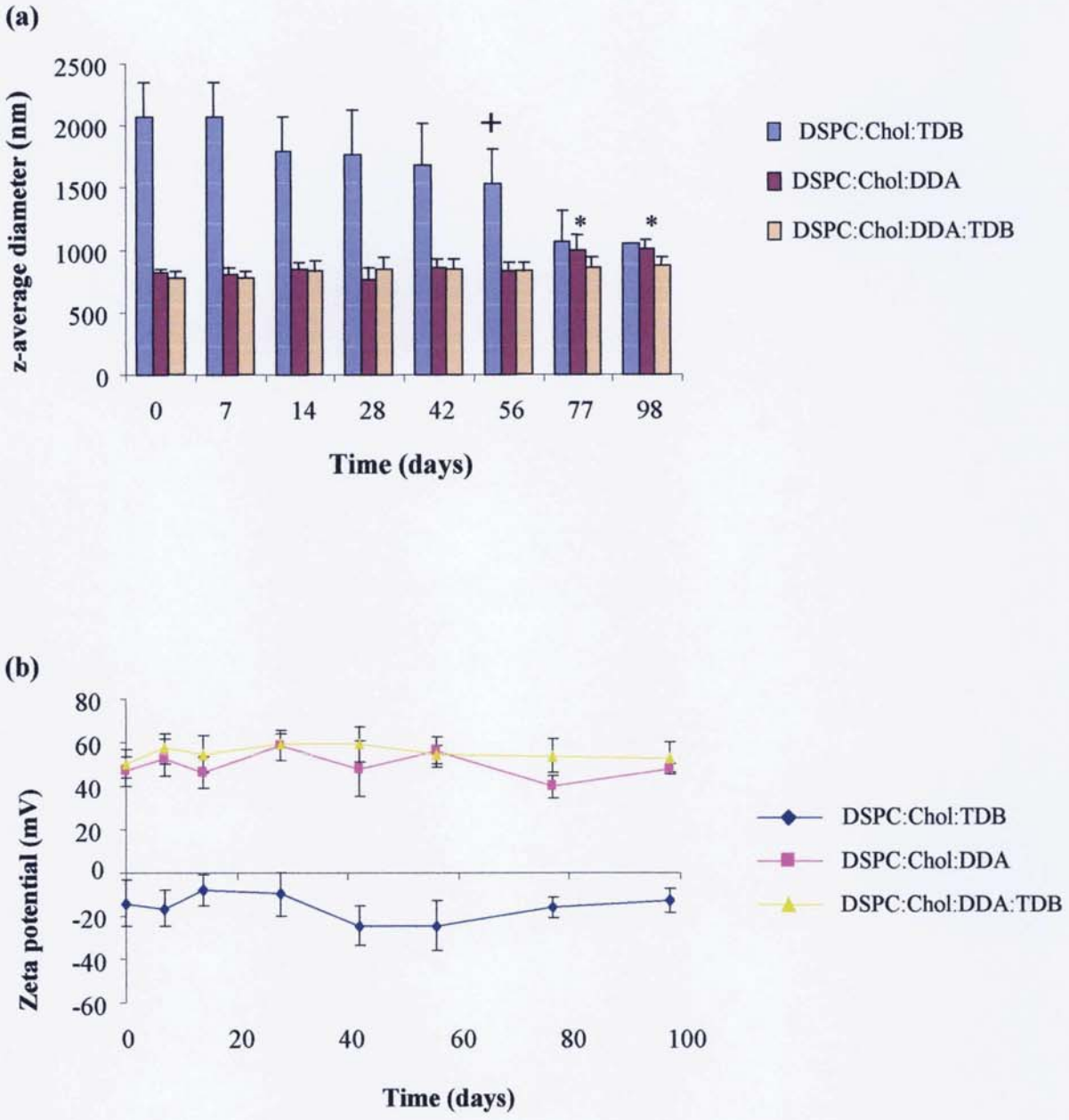


Figure 7.13 Effect of liposome composition on vesicle stability (size and zeta potential) of liposomes stored at 4°C. All formulations were prepared by the DRV procedure entrapping the protein within liposome bilayers. Samples were stored at 4°C for approximately 3 months (i.e. 98 days). Samples were taken at set time intervals in which vesicle size (a) and zeta potential (b) of liposomes stored at 4°C were measured in double-distilled water or 0.001 M PBS, respectively, at 25°C using a Brookhaven ZetaPlus. * signifies that vesicle size of liposomes composed of DSPC:Chol:DDA are significantly ($P<0.05$, ANOVA) larger at days 77 and 98 compared to day 0. + signifies that the vesicle size of DSPC:Chol:TDB significantly ($P<0.05$, ANOVA) reduces after 56 days incubation compared to day 0. Results denote mean ± SD, $n = 3$.

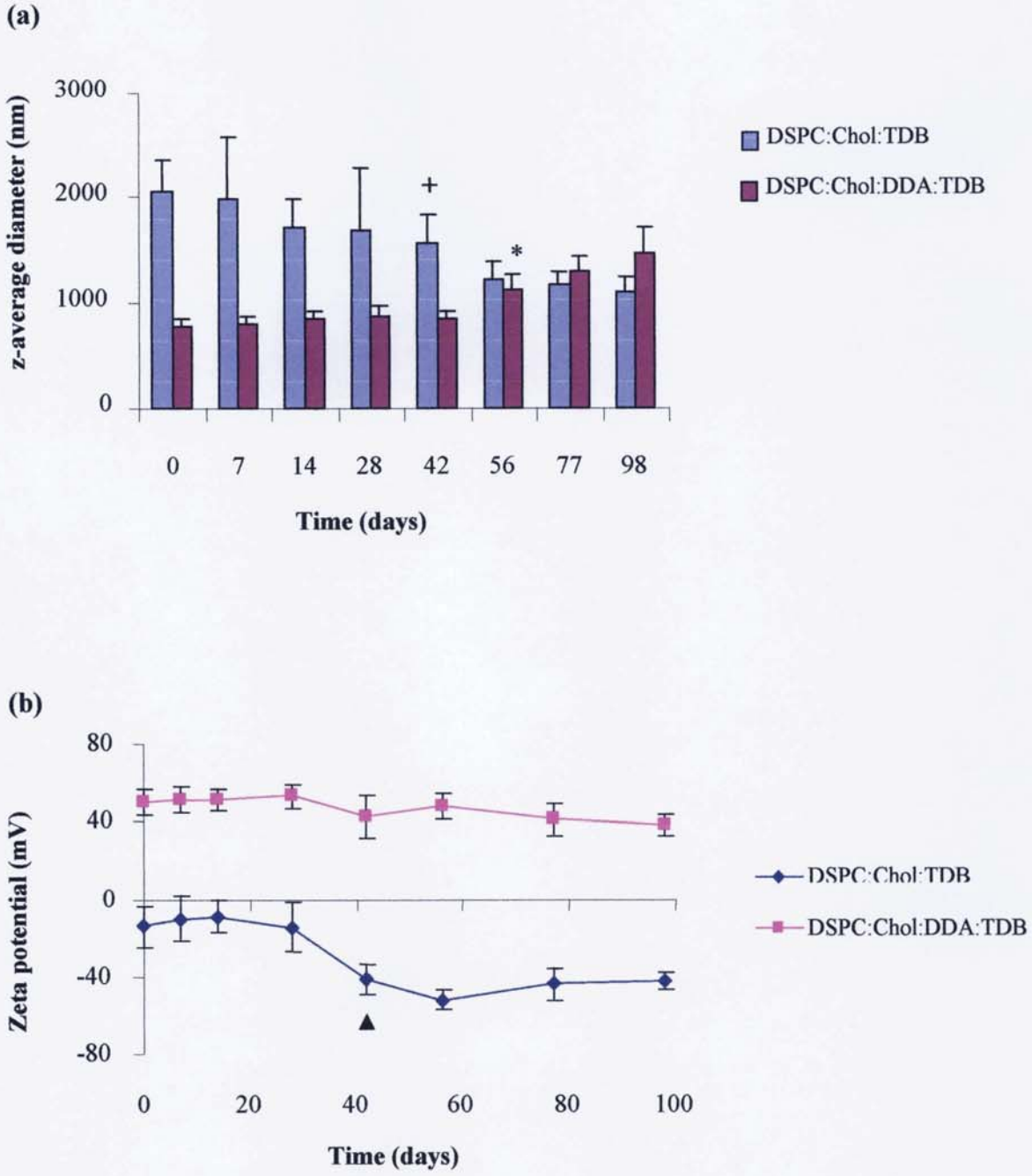


Figure 7.14 Vesicle stability (size and zeta potential) of liposomes composed DSPC:Chol:TDB and DSPC:Chol:DDA:TDB stored at room temperature. All formulations were prepared by the DRV procedure entrapping the protein within liposome bilayers. Samples were stored at room temperature (25°C) for approximately 3 months (i.e. 98 days). Samples were taken at set time intervals in which vesicle size (a) and zeta potential (b) of liposomes stored at room temperature (25°C) were measured in double-distilled water or 0.001 M PBS, respectively, at 25°C using a Brookhaven ZetaPlus. + signifies that the original vesicle size of DSPC:Chol:TDB liposomes decreases significantly ($P<0.05$, ANOVA) after 42 days. * signifies that after 56 days storage at room temperature the vesicle size of DSPC:Chol:DDA:TDB liposomes significantly ($P<0.05$, ANOVA) increases. ▲ signifies that zeta potential of DSPC:Chol:TDB liposomes significantly ($p<0.05$, ANOVA) becomes more negative after 42 days storage at room temperature. Results denote mean ± SD, $n = 3$.

Although protein is released from DSPC:Chol:DDA:TDB liposomes on day 28 of storage at room temperature (Figure 7.12b), a significant increase in liposome size (i.e. vesicle aggregation) was not measured until day 56 (Figure 7.14a). When stored at room temperature, liposomes composed of DSPC:Chol:TDB, also significantly ($P<0.05$) reduce in size from 2066 nm to 1554 nm after 42 days storage. However, in this instance, in addition to flocculation, this could be attributed to the existence of electrostatic repulsions between vesicles, as the zeta potential becomes significantly ($P<0.05$) more negative during storage (Figure 7.14b) from –14.2 mV at day 0 to –41.06 mV at day 42. The particles within these loose flocculates may repel each other, thereby forcing the large aggregates to disperse into single smaller aggregates/vesicles.

Alternatively, the measured reduction in vesicle size during storage at both 4°C and room temperature for liposomes consisting of DSPC:Chol:TDB, could simply be due to an increase in the existence of aggregated vesicles over time which have not been detected by the equipment used. To investigate this phenomenon further, liposome morphology of these neutral liposomes after 0 and 98 days storage, could be observed and compared by TEM.

7.6 Final challenge

Considering all the characterisation, *in vitro* release and *in vivo* immunological results from the previous studies, two liposomal formulations were chosen to be taken forward for further analysis and looked into more detail with regard to immunological markers. The chosen liposomal formulations were both DRV entrapping the fusion protein composed of DMPC:SA:TDB and DSPC:Chol:TDB. Although liposomes composed of DSPC:Chol:TDB did not produce the highest amount of IFN-γ, this formulation was chosen. Studies have previously found that the combination of only DDA:TDB, without any additional lipids induces a very strong specific immune response (Davidsen *et al.*, 2005), therefore, there is no advantage with the addition of DSPC and Chol. However, the liposome formulation, DSPC:Chol:TDB provides an effective neutral alternative to the cationic system. This may be beneficial and of importance if the cationic DDA liposomes prove to be toxic, as cytotoxicity to COS-7 cells *in vitro* was observed when DDA was used as the cationic lipid for DNA delivery (results presented in chapter 4).

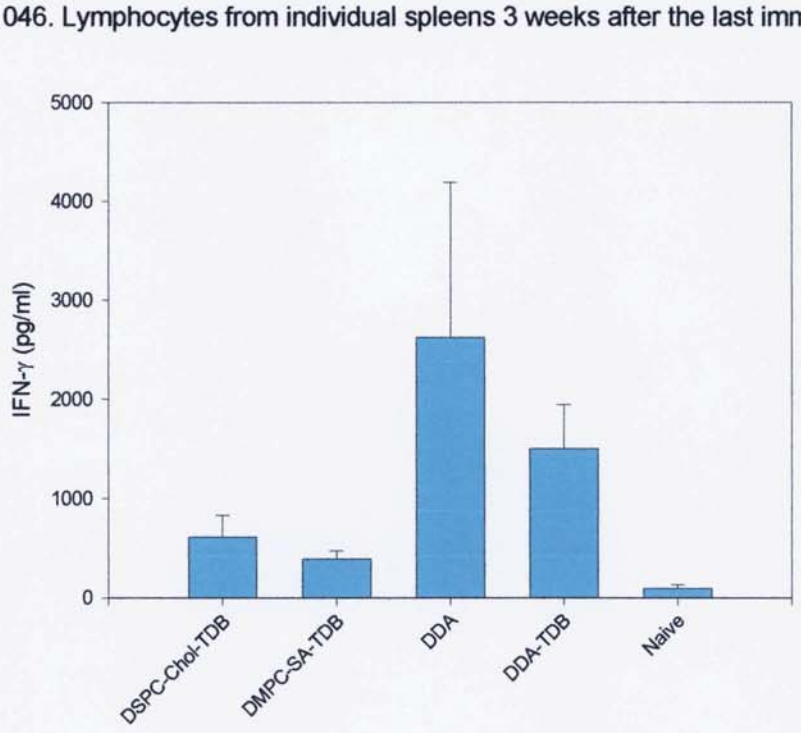
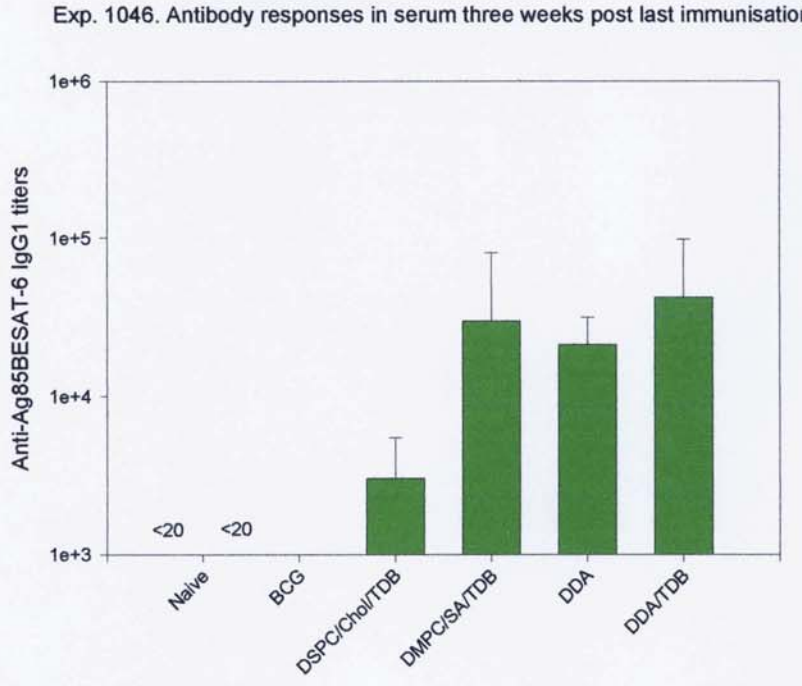


Figure 7.15 Effect of liposome composition on the induction of immune responses in mice immunised with Ag85B-ESAT-6 fusion protein. Balb/C mice were injected subcutaneously with 2 μg of Ag85B-ESAT-6 fusion protein, either entrapped within DRV liposomes composed of DPSC:Chol:TDB, DMPC:SA:TDB, DDA or DDA:TDB. Blood lymphocytes were isolated three weeks after last immunisation and were subsequently re-stimulated *in vitro* with the Ag85B-ESAT-6 (5μg/ml). The amount of IFN-γ released was measured and analysed. (Immunological characterisation work carried out by SSI).

Results in Figure 7.15 show that both liposome formulations induce production of IFN-γ greater than the naïve group, although DSPC:Chol:TDB induced higher levels of IFN-γ than DMPC:SA:TDB. In addition to IFNγ release, antibody responses after immunisation with DSPC:Chol:TDB or DMPC:SA:TDB was also investigated. The production of IgG1 was measured as an indicator of a Th2-type response, whereas IgG2a was measured as an indicator of a Th1-type of immune response. Interestingly, values for IgG1 from mice immunised with these liposomal formulations was higher than values for the IgG2a titres (Figures 7.16 and 7.16), indicating that these formulations predominantly initiate a Th2-type of immune response.



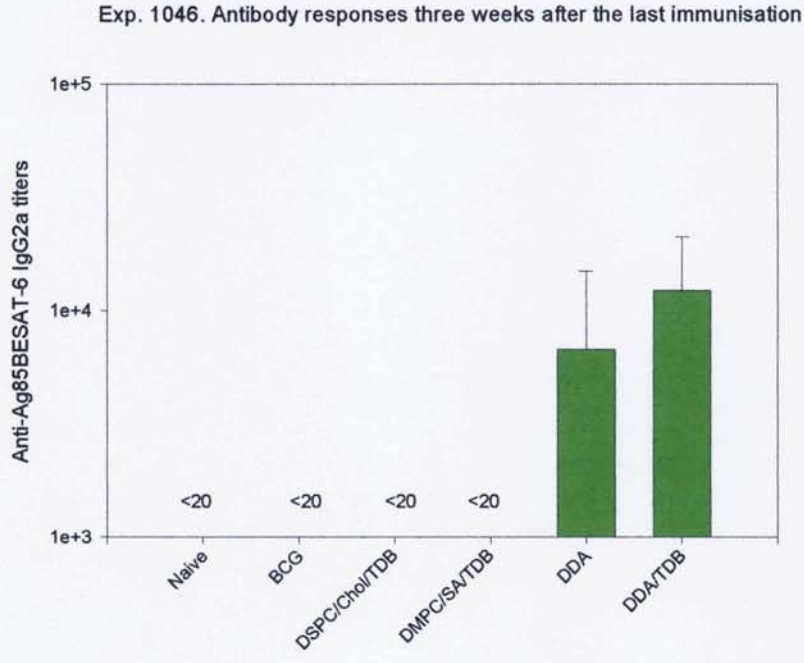


Figure7.16 Antibody responses generated by various liposome formulation incorporating the fusion protein, Ag85B-ESAT-6. BALB/c mice were immunized with 2 μg of Ag85B-ESAT-6 in DDA, DDA:TDB, DSPC:Chol:TDB and DMPC:SA:TDB. Antigen-specific antibody titres in serum from Ag85B-ESAT-6 immunized BALB/c mice were measured as IgG1 and IgG2a titers, respectively. (Immunological characterisation work carried out by SSI).

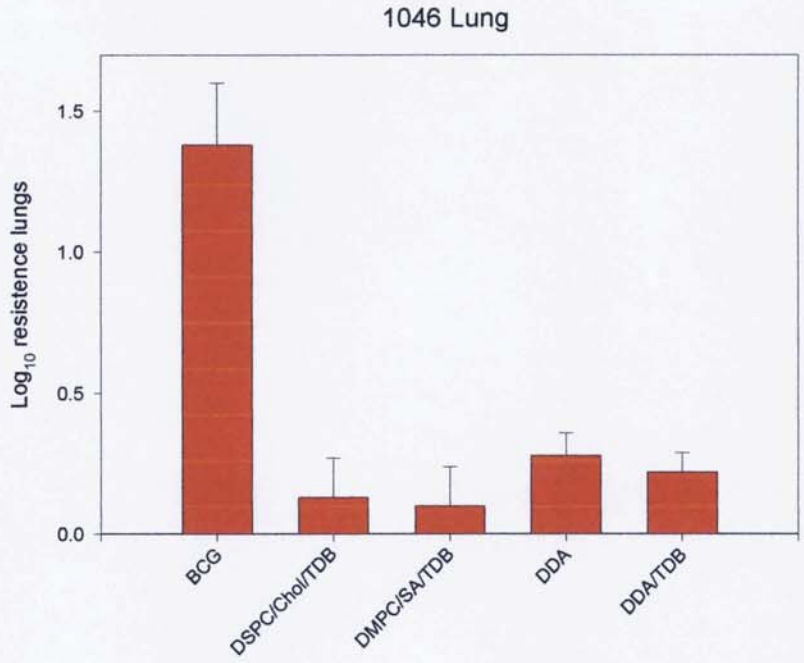


Figure 7.17 Efficacy of various liposomal formulations and resistance to challenge with an infection of tuberculosis after immunisation with the fusion protein Ag85B-ESAT-6. Immunised mice received an aerosol challenge with *M. tuberculosis* Erdman and the numbers of bacteria, stated as colony forming units (CFU), were quantified in the lungs 9 weeks after. All values are shown as 10 individual mice ± SD. (Immunological characterisation work carried out by SSI).

With regard to the overall resistance to infection with tuberculosis, results in Figure 7.17 show that both formulations induce resistance to infection and at the same level. However, on the whole, resistance is lower than BCG.

7.7 Conclusion

Association with liposome systems can significantly enhance the immunogenicity of subunit protein antigens. Results presented within this chapter show that cationic and neutral liposomes generate the highest level of immunity against tuberculosis with the fusion protein Ag85B-ESAT-6. Anionic liposomes, composed of DMPC:PG:TDB, show an initial enhancement in IFN-γ release, however, one week after the third immunisation, cationic liposomes composed of DMPC:SA:TDB produced considerably higher values of IFN-γ

production compared to anionic liposomes. This is presumably due to the slow and sustained rate of protein release from these cationic liposomes, compared to the anionic liposomes. In addition, the physical association between the liposome and protein antigen affects the level of immunity. Liposome-entrapped protein, prepared by the DRV procedure, generates considerably higher values of protein incorporation than surface adsorption. Therefore, with the protein entrapped within the lipid bilayers, the protein content is released at a slow and sustained rate, therefore maintaining therapeutic levels of antigen for presentation to APC.

Liposome formulations composed of DSPC and Chol were prepared to further enhance bilayer stability, thus enabling slow and sustained release of protein. Initially, cationic DSPC-based liposome systems composed of DSPC:Chol:DDA:TDB induced a substantial production of IFN-γ, however, the production of IFN-γ reduced over time. In contrast, neutral liposomes composed DSPC:Chol:TDB exhibited slow sustained release and therefore were able to relatively maintain a continuous release of IFN-γ from cells. In conclusion, within these stable liposome systems, consisting of DMPC or DSPC, it appears that sustained release of protein antigen (i.e. depot effect) enhances the overall immune responses against tuberculosis.

However, on the whole, cationic liposomes composed of DDA:TDB persistently exhibited high immune responses against tuberculosis than any other formulation, thus predominantly generating a strong specific Th1-type response as characterised by a high amount of IFN-γ release and elevated IgG1isotype antibodies. Whereas a promising bias towards a Th2-type response was generated by liposomes composed of DSPC:Chol:TDB and particularly DMPC:SA:TDB, as characterised by the relatively high production of IgG1 antibody titres. Therefore, these formulations may be promising delivery systems for various other protein antigens predominantly requiring humoral immunity.

8. General discussion

8.1 Antigen delivery: the role of liposomes

The results presented in this thesis provide further evidence of the effective and potential role of liposomal systems for antigen delivery and induction of specific immune responses. When antigens are administered alone, the resultant immunogenic responses are relatively low, due to a multitude of reasons, including digestion and degradation of the injected foreign vaccine antigen by nucleases, proteases and intracellular enzymes found within the body, insufficient targeting to the appropriate APC and with such vaccines they are not or only weakly immunogenic and therefore are inefficient at eliciting effective levels of immunity. Therefore, the development of safe and effective vaccines and delivery systems are predominantly required, in which liposomes are one of the leading candidates. Liposomes have been utilised as a delivery system for many years with successful applications. Their use as an effective adjuvant and/or delivery system, by enhancing the immunogenicity of potential vaccines, can be developed by essential and successful formulation and optimisation.

8.2 Liposome-mediated plasmid DNA delivery

With regard to plasmid DNA, previous studies have shown that with vaccination naked DNA is only effective at inducing specific immune responses to the encoded antigen when injected intramuscularly, providing only a local effect (Davis, 1996). However, when administered by any other route (i.e. intravenous, subcutaneous, intranasal) gene delivery and gene expression is extremely low. This has been mainly attributed to the degradation of the plasmid within the biological milieu. One of the major advantages of associating plasmid DNA with a delivery system, is the ability of such systems to protect the plasmid from external conditions, which might other wise lead to their rapid degradation, by the lipid membrane barrier. Indeed, gel electrophoresis of plasmid DNA without the protection of the lipid bilayer showed that the DNA had been completely degraded, with the plasmid DNA broken down to smaller nucleotide fragments, with the loss of all original structure and function, with no visible band after incubation with the digestive enzyme. In contrast, entrapment of the plasmid DNA, pRc/CMV HBS encoding the hepatitis B surface antigen, within liposomes show that the DNA was effectively protected from digestive enzymes, thus preventing degradation and damage of the dsDNA, with the plasmid remaining intact. This

was confirmed by gel electrophoresis on the basis of the intensity of the staining, in which staining was equal for both before and after treatment with DNase I.

While investigating influential factors effecting liposome-mediated gene delivery a multitude of studies were carried out. Comparison of the physiochemical characteristics of various liposome formulations and preparations reveal the interactions between different lipid components and additionally, the complex interactions that occur between liposomes and DNA upon mixing. The *in vitro* experiments reveal that gene transfer is highly dependent on a number of factors. The lipid composition, e.g. the cationic lipid, the 'helper lipid', the alkyl chain length and head-group of the phospholipid, liposome vesicle structure and physiochemical characteristics of liposome systems all play an important role in efficient plasmid DNA delivery to cells.

8.2.1 Effect of liposomes preparation, morphology and location of plasmid DNA

There are various ways in which to prepare and manufacture liposomes, with each preparation varying with regard to morphology and association with the plasmid DNA. Liposomes produced by the dehydration-rehydration procedure, as established by Kirby and Gregoriadis (1984) within the aqueous spaces of the liposome, resulted in high values of DNA entrapment within liposomal vesicles, with the highest entrapment values determined for cationic liposomes (96-100%).

The physiochemical characteristics of these DRV liposomes composed of PC:DOPE:DC-Chol (16:8:4 μmole/ml) or PC:Chol:DC-Chol (16:8:4 μmole/ml) revealed that as liposome size and zeta potential values altered very little in the presence of DNA, with the characteristics of DRV(DNA) being relatively similar to empty DRV, the plasmid DNA was localised within the bilayers of these cationic multilamellar DRV liposomes presumably bound to the cationic charges of the inner bilayers when produced by the dehydration-rehydration procedure. Furthermore, DNA retention studies show that very little DNA was bound to the surface of these liposomes, as upon gel electrophoresis a minimal amount of DNA was displaced by competing anionic components from these DRV(DNA) liposomal systems, confirming the presence of entrapped DNA. This may imply that the DNA is likely to remain with the liposomal vesicles when administered *in vivo*, where it will come in contact with interstitial fluids that contain various negatively charged components, which could displace the introduced DNA. In contrast, addition of DNA to empty SUV or 'empty'

DRV liposomes resulted in a significant increase in the z-average diameter compared to empty SUV and DRV, respectively, indicating the formation of SUV-DNA or DRV-DNA complexes or aggregates. These complexes possessed a slightly less positive surface charge, as the majority of the plasmid DNA was localised on the vesicle surface as confirmed by gel electrophoresis in the presence of anionic competitors, in which the complexed DNA was displaced. However, despite this, within all liposomal systems, the associated DNA was protected from degradation by nucleases, as the enzymes were unable to gain access to the highly condensed DNA within the complexes or the entrapped DNA within DRV(DNA). When applied *in vitro*, SUV-DNA complexes transfected COS-7 cells with higher efficiency than liposome-entrapped DNA (DRV(DNA)). It was established that this could be attributed to the inability of the entrapped DNA escaping the vesicles in the time duration of the experimental protocol.

8.2.2 Influence of lipid composition

The high values of DNA entrapment for DRV(DNA) formulated with a cationic lipid is attributed to the electrostatic interaction between the anionic DNA and cationic bilayers of the liposomes. The DNA efficiently binds to the vesicles cationic surface and consequently increasing DNA entrapment, as in the absence of a cationic lipid, the entrapment values were reduced to 51% for neutral liposomes. Liposome aggregation was measured for neutral liposomes due to the lack electrostatic repulsions between vesicles, whereas cationic liposomes were significantly smaller in size, due to the existence of such repulsions.

Carrying out these *in vitro* experiments has enabled us to generate an underpinning understanding of the mechanisms of liposome-mediated DNA transfection. The *in vitro* results presented within this thesis demonstrate that transfection efficiency in COS-7 cells is enhanced when the cationic lipid DC-Chol is added to the formulation, in comparison to other cationic lipids i.e. DOTMA, DOTAP, DDA and DPTAP. The addition of a 'helper' lipid significantly influences the efficiency of gene transfection, however efficiency is highly dependent on the type of 'helper' lipid included within the formulation. When comparing the efficiency of phosphatidylcholines and phosphatidylethanolamines, results demonstrated that both the co-lipid head group and the alkyl-chain length influence transfection efficiency *in vitro*. Decreasing transfection activity was in the order of DOPE > PE > DMPE > DOPC > PC > DMPC, despite the vesicle size and surface charge of all liposome formulations tested

being relatively similar. The effectiveness of DOPE is in line with other previous reports which suggest that the architectures of dioleoyl alkyl lipids can facilitate fusion and disruption of the lipoplexes with cellular and endosomal membranes (Farhood *et al.*, 1995) through their ability to invert into hexagonal lipid arrangements. However it is apparent this can only occur in combination with the PE head-group, which may facilitate the dissociation of the lipoplexes after internalisation. This has been reported (Pedroso *et al.*, 2001) to be due to a weakening of the otherwise strong cationic lipid-DNA electrostatic binding as a result of the PE amine group interacting with the DNA phosphate groups. Previous reports have shown that the inclusion of Chol as a helper lipid is also less efficient than DOPE (Templeton *et al.*, 1997; Sternberg *et al.*, 1998; Hirsch-Lerner *et al.*, 2005), however, *in vitro* Chol as a helper lipid has not been investigated in any great detail, therefore by examining the effect of Chol on transfection efficiency of liposome-DNA complexes, and looking at its effect on liposome bilayers and complex construction, in comparison to the common helper lipid, DOPE, results presented within this thesis reveal that efficiency in the presence of such helper lipids is highly dependent on the cationic liposome to anionic DNA charge ratio and the resultant interactions between them. Results suggest that the transfection efficiency *in vitro* of these formulations is to some extent influenced by complex size, with the optimum measured sizes between 700 and 1000 nm being most effective. Liposome vesicles containing PC reduce transfection efficiency, with efficiency decreasing at a linear rate as the PC content within liposomal formulation is gradually increased.

When a select few liposome formulations and preparations were administered *in vivo*, SUV-DNA complexes composed of PC:Chol:DC-Chol at a molar ratio of 16:8:4 μmole/ml, mixed with 100 μg plasmid DNA, was the most effective at inducing splenocyte proliferation upon exposure to antigen in comparison to control spleens. Once again, showing the enhanced ability of these complexes at transporting the DNA and targeting the appropriate immune cells. PC:Chol:DC-Chol SUV-DNA complexes formulated at the lower and at equal lipid molar ratios were found to be ineffective. However, all formulations and preparations tested were unable to induce an effective humoral immune response. Within *in vivo*, there are a lot more variables to consider than *in vitro*, such as the vesicles ability to not only protect their DNA content from nuclease attack but also deliver it to APC infiltrating the site of injection or in the lymphatics. The liposome system has to reach the target cell before transfection can

occur, during which the liposomes have to be able to withstand the biological milieu of the host.

8.3 Liposome-mediated protein antigen delivery

When developing vaccines for protein antigens, it seems that association with liposomal delivery systems significantly enhances the protective immune response of the subunit antigens, which alone possess poor immunogenicity. Immediately this emphasises the effective role of liposomes as a successful and efficient adjuvant and/or delivery system for subunit antigens and its ability to induce protective immunity against infection with tuberculosis.

8.3.1 Liposomal characteristics: effect of surface charge and liposome morphology

Comparison of the physiochemical characteristics, *in vitro* release and immunological characterisation *in vivo* of the various different formulations tested, it is evident that the surface charge, morphology and the localisation of protein antigen all play a significant role in protein release and inducing protective immunity. Positively charged liposomes composed of DMPC:SA:TDB were significantly larger in size resulting in higher values of protein entrapment than the negatively charged liposomes composed of DMPC:PG:TDB. The immune responses when subcutaneously injected reveal that although the smaller negatively charged liposomes initially produce the most amount of IFN-γ release, this initial response one week after the second immunisation is comparable to the IFN-γ release one week after the third immunisation, indicating that the response does not strengthen overtime. In contrast, the cationic liposomes initially generate a lower amount of IFN-γ, but the response is enhanced after the third immunisation with the release of IFN-γ increasing by approximately 10x. This could be attributed to the rate at which the liposomal protein content is released from these systems. Within the *in vitro* release studies these cationic liposomes retained their protein load for longer showing sustained release overtime. These cationic liposomal systems may act as a depot system slowly releasing the entrapped material thereby maintaining therapeutic levels of protein antigen within the body to enable a sustained and prolonged immunogenic response, in a similar way to the licensed adjuvant alum-based adjuvant systems, whereas, the negatively charged liposomes exhibited a sudden burst release of its protein content into the surrounding medium.

The spatial location of the protein antigen was also shown to be of significant importance. Liposomes prepared by the hand shaking method and subsequently mixed with the fusion protein, to produce MLV-protein liposomes, generated smaller liposomes with reduced protein incorporation values compared to DRV(DNA) of the same formulation. As with the negative PG liposomes, these MLV-protein liposomes initially lead to the production of more IFN-γ than the DRV(DNA) equivalent, yet the over time the depot effect of DRV(DNA) prove to be more effective at eliciting the release of IFN-γ and thus inducing better at inducing protective immunity against infection.

8.3.2 Liposome formulation: effect of lipid composition

When formulating liposomes for protein antigen delivery, the adjuvanticity of the liposomal system has an immense effect on the overall immune response generated against the associated antigen. With the addition of the immunomodulator, TDB, the protective immune response, as indicated by the amount of IFN-γ production, increases considerably with regard to cationic liposomes composed of DSPC:Chol:DDA. Although, the inclusion of TDB within neutral liposomes consisting of DSPC:Chol also enhances IFN-γ production compared to neutral liposomes omitting TDB, the adjuvanticity of this neutral formulation is enhanced to a lesser extent. However, the overall protective immune responses of such neutral liposomes consisting of TDB was effectively maintained over time, when in contrast there was an observed reduction in IFN-γ release and overall protection against infection with tuberculosis for the cationic equivalent. To that end, although the addition of DSPC and Chol reduced the protective immunity of DDA:TDB liposomes, relatively high protection was observed for the neutral counterparts, DSPC:Chol and DSPC:Chol:TDB, which may offer an effective neutral alternative to the DDA cationic system, which, as shown within the DNA transfection studies presented within this thesis, may prove to be too toxic.

8.3.3 Immunological characterisation

The cationic DRV(DNA) formulation, DMPC:SA:TDB and the neutral DRV(DNA) alternative DSPC:Chol:TDB, were taken forward for further analysis. It seems that, although these formulations induced effective production of IFN-γ, higher IgG1 antibody titres were obtained rather than IgG2a, which indicates that the immune response is largely directed towards a Th2-type response, rather than the Th1-type immune response required for effective protection against tuberculosis. Therefore, these liposomal systems can be

effectively utilised as efficient adjuvant systems against other infectious agents, which require a Th2 response, such as malaria.

8.4 Future work

Further research can be conducted in continuation of this work. Differential scanning calorimetry (DSC) studies could be conducted to confirm that both the addition of DOPE depresses the phase transition temperature of phosphotidylcholine based liposomes, as used within *in vitro* shown in chapter 4, and also that the addition of sucrose within the modified DRV procedure (chapter 5) lowers the gel to liquid transition temperature.

Further *in vitro* transfection studies could be conducted. The transfection efficiency of the liposome-mediated DNA formulations administered *in vivo*, could be examined within a macrophage cell line (e.g. RAW 264.7 gamma NO(-)). Therefore, this will assist in our understanding of whether the formulations were unable to effectively deliver the plasmid DNA *in vivo* or whether they are just poor transfecting agents.

Further research could be conducted in order to enhance the promising cell mediated immunity induced by SUV-DNA liposomes composed of PC:Chol:DC-Chol (16:8:4 μmole/ml) and to initiate a sufficient level of humoral immunity. Firstly, the preparation and characterisation of mannosylated liposomes to effectively target the APC could be conducted. Mannan acts as a ligand for mannose receptors expressed on macrophages and dendritic cells. Therefore, mannan coating of the liposomes will effectively enable the liposomes to specifically target APC, to produce both humoral and cell mediated immunity.

In addition, results within this thesis demonstrated that a prime injection of naked DNA considerably enhanced the immune responses to further injections of the hepatitis B surface antigen administered by Freund's adjuvant. The measured cytokine levels for SUV-DNA liposomes composed of PC:Chol:DC-Chol (16:8:4 μmole/ml) were comparable to the positive control group that received the hepatitis B surface antigen by Freund's adjuvant. Therefore, could the immune response against the plasmid DNA, pRc/CMV HBS, within this liposome formulation possibly be enhanced by priming the immune system with an initial dose of free DNA injected intramuscularly?

Alternatively, instead of DNA delivery, a select few formulations that were tested *in vivo* within chapter 6, could be utilised as a delivery system for the hepatitis B surface antigen. Possibly investigating and comparing the effect of DC-Chol and DDA:TDB, which has previously been reported as a highly effective adjuvant for protein antigens.

Immunisation with the fusion protein Ag85B-ESAT-6 against tuberculosis, revealed that liposomes composed of either DMPC:SA:TDB or DSPC:Chol:TDB induced a bias towards a Th2-type immune response. With regard to tuberculosis, a dominant Th1 response is essential, however protection against other infectious diseases, such as malaria caused by *Plasmodium falciparum*, are primarily mediated by induction of antibodies (Cohen et al., 1961), therefore will require a Th2-type response. Therefore, further research could be carried out, by utilising these liposomal formulations for characterisation and immunisation with malaria antigens, such as Merozoite surface protein 1 (MSP1) and the glutamate rich protein (GLURP).

11. Appendices

11.1 Chemical structures of lipids used

Structures and diagrams were obtained from www.avantilipids.com

Cholesterol (Chol)

11.1.1 Phosphatidylcholines

Dioleoyl Phosphocholine (**DOPC;** 18:1 PC)

Dimyristoyl phosphatidylcholine (DMPC; 14:0 PC)

Disteoryl phosphatidylcholine (DSPC; 18:0 PC)

11.1.2 Phosphatidylcholines

Dioleoyl Phosphoethanolamine (DOPE; 18:1 PE)

Dimyristoyl Phosphatidylethanolamine (DMPE; 14:0 PE)

11.1.3 Cationic lipids

Dimethyldioctadecylammonium bromide (DDA; 18:0 DDAB)

3β-[N-(N′,N′-Dimethylaminoethane)-carbamoyl] Cholesterol

Dioleoyl Trimethylammonium-Propane (**DOTAP;** 18:1 TAP)

Dipalmitoyl Trimethylammonium-Propane (DPTAP; 16:0 TAP)

11.1.4 Immunomodulators

Trehalose dibehenate (TDB)

PicoGreen® dsDNA quantification assay

Using the PicoGreen® dsDNA quantitation assay, you can selectively detect as little as 25 pg/ml of dsDNA in the presence of ssDNA, RNA, and free nucleotides (1). The assay is linear over three orders of magnitude and has little sequence dependence, allowing you to accurately measure DNA from many sources, including genomic DNA, viral DNA, miniprep DNA, or PCR amplification products. All graphs and figures shown below were obtained from www.invitrogen.com.

Figure 1 - Quantitation using the PicoGreen® Reagent

Figure 2 - dsDNA specificity

Figure 3 - Spectra — PicoGreen dsDNA quantitation reagent

Fluorescence excitation and emission spectra of PicoGreen dsDNA quantitation reagent bound to DNA.

10. References

Own Publications

McNeil, S., Rosenkrands, I., Agger, E. M., Andersen, P. and Perrie, Y. (2006) – Liposomal adjuvant systems for protein subunit vaccines: effect of liposome formulation and lipid composition. *In preparation.*

McNeil, S., Hanson, P. and Perrie, Y. (2006) – Parameters influencing the transfection efficiency of liposome-DNA complexes in vitro: the effect of bilayer mechanics. *Submitted for publication in Biophysical Chemistry.*

McNeil, S., Hanson, P. and Perrie, Y. – The role of helper lipid in transfection efficiency of liposome-DNA complexes. *12th United Kingdom-Ireland Controlled Release Society Symposium*, AstraZeneca R&D Charnwood, Loughborough, UK, January 2006.

McNeil, S., Hanson, P. and Perrie, Y. (2005) – Optimising transfection efficiency of liposome-DNA complexes: effect of co-lipid, lipid:DNA ratio and complex size. *Journal of Pharmacy and Pharmacology*, 57 (September supplement); S21.

McNeil, S., Hanson, P. and Perrie, Y. (2005) – The importance of co-lipids and phospholipids headgroup type within liposomes for the successful delivery of DNA. *Journal of Pharmacy and Pharmacology*, 57 (September supplement); S22.

McNeil, S., Hanson, P. and Perrie, Y. (2005) – Liposome bilayer mechanics and its effect on transfection efficiency of plasmid DNA *in vitro*. *32nd Annual Meeting & Exposition of the Controlled Release Society Meeting*, Miami Beach, FL, 18-22 June 2005.

McNeil, S. and Perrie, Y. (2005) – The addition of a disaccharide as a cyroprotectant within lipid bilayers and its effect on liposomal characteristics. *11th United Kingdom-Ireland Controlled Release Society Symposium*, Aston University, Birmingham, UK, January 2005.

Perrie, Y., Barralet, J. E., McNeil, S. and Vangala, A. (2004) – Surfactant vesicle-mediated delivery of DNA vaccines via the subcutaneous route. *International Journal of Pharmaceutics*, 284; 31-41.

McNeil, S. and Perrie, Y. (2004) – A modified dehydration-rehydration procedure for the production of small liposome-entrapped DNA. *Journal of Pharmacy and Pharmacology*, 56 (September supplement); S15.

McNeil, S., Hanson, P. and Perrie, Y. (2004) – The incorporation, spatial location and protection of plasmid DNA within various liposomal systems. *10th United Kingdom-Ireland Controlled Release Society Symposium*, GSK House, Brentford, UK, January 2004.

Perrie, Y., McNeil, S. and Vangala, A. (2003) – Liposome-mediated DNA immunisation via the subcutaneous route. *Journal of Drug Targeting*, 11(8-10); 555-563.

McNeil, S., Hanson, P. and Perrie, Y. – Effect of Formulation and Lipid Composition on Liposomal Characteristics and Properties. *6th International conference on Liposome Advances*, London, UK, December 2003.

McNeil, S., Gregoriadis, G. and Perrie, Y. (2003) – The effect of liposome preparation on the incorporation of DNA within liposomes. *Journal of Pharmacy and Pharmacology*, 55 (September supplement); S42.

References

Afrin, F., Anam, K., and Ali, N. (2000) Induction of partial protection against *Leishmania donovani* by promastigote antigens in negatively charged liposomes. *Journal of Parasitology* **86:** 730-735.

Ahearn, A. and Malone, R. (1999) Models of cationic liposome mediated transfection. *Gene Therapy and Molecular Biology* **4:** 159-170.

Ahn, S. J., Costa.J., and Emanuel.J.R. (1996) PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. *Nucleic Acids Research* **24:** 2623-2625.

Akao, T., Osaki, T., Mitoma, J., Ito, A., and Kunitake, T. (1991) Correlation between physiochemical characteristics of synthetic cationic amphiphiles and their DNA transfection ability. *Bulletin of Chemical Society, Japan* **64:** 3677.

Allison, A. G. and Gregoriadis, G. (1974) Liposomes as immunological adjuvants. *Nature* **252:** 252.

Allison, S. D., Molina, M. D., and Anchordoquy, T. J. (2000) Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. *Biochimica et Biophysica Acta* **1468:** 127-138.

Almofti, M. R., arashima, H., hinohara, Y., lmofti, A., ada, Y., and iwada, H. (2003) Cationic liposome-mediated gene delivery: Biophysical study and mechanism of internalization. *Archives of Biochemistry and Biophysics* **410:** 246-253.

Andersen, P. (1994) Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. *Infection and Immunity* **62:** 2536-2544.

Andersen, P., Andersen, A. B., Sorensen, A. L., and Nagai, S. (1995) Recall of long-lived immunity to Mycobacterium tuberculosis infection in mice. *Journal of Immunology* **154:** 3359-3372.

Aramaki, Y., Fujii, Y., Yachi, K., Kikuchi, H., and Tsuchiya, S. (1994) Activation of systemic and mucosal immune response following nasal administration of liposomes. *Vaccine* **12:** 1241-1245.

Audibert, F. M. and Lise, L. D. (1993) Adjuvants: current status, clinical perspectives and future prospects. *Immunology Today* **14:** 281-284.

Avanti Polar Lipids. (2005) Issues to consider when selecting lipids. www.avantilipids.com

Bailey, A. L. and Cullis, P. R. (1997) Membrane fusion with cationic liposomes: effects of target membrane lipid composition. *Biochemistry* **36:** 1628-1634.

Bakouche, Q., David, F., and Gerlier, D. (1987) Impairment of immunogenicity by antigen presentation in liposomes made from dimyristoylphosphatidylethaolamine linked to the

secretion of prostaglandins by macrophages. *European Journal of Immunology* **17:** 1839-1842.

Bally, M. B., Harvie, P., Wong, F. M. P., Kong, S., Wasan, E. K., and Reimer, D. L. (1999) Biological barriers to cellular delivery of lipid-based DNA carriers. *Advanced Drug Delivery Reviews* **38:** 291-315.

Bangham, A. D., Standish, M. M., and Miller, N. (1965) Cation permeability of phospholipid model membranes: effect of narcotics. *Vaccine* **208:** 1295-1297.

Bangham, A. D. (1983) Liposomes: A historical perspective. In *Liposomes*. Ostro, M. J. (ed.) New York: Marcel Dekker, Inc..

Batenjany, M. M., Boni, L. T., Guo, Y., Neville, M. E., Bansal, S., Rubb, R. J. and Popescu, M. C. (2001) The effect of cholesterol in a liposomal Muc1 vaccine. *Biochimica et Biophysica Acta* **1514:** 280-290.

Battersby, B., Grimm, R., Huber, S., and Cevc, G. (2002) Evidence for three-dimensional interlayer correlations in cationic lipid-DNA complexes as observed by cryo-electron microscopy. *Biochimica et Biophysica Acta* **1372:** 379-383.

Baylor, N. W., Egan, W., and Richman, P. (2002) Aluminum salts in vaccines - US perspective. *Vaccine* **20:** S18-S23.

Bhattacharya, S. and Halder, S. (1996) The effects of cholesterol inclusion on the vesicular membranes of cationic lipids. *Biochimica et Biophysica Acta* **1283:** 21-30.

Birchall, J. C., Kellaway, I. W., and Mills, S. N. (1999) Physio-chemical characterization and transfection efficiency of lipid-based gene delivery complexes. *International Journal of Pharmaceutics* **183:** 195-207.

Birnboim, H. C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7:** 1513-1523.

Bramwell, V. and Perrie, Y. (2005) Particulate Delivery Systems for Vaccines. *Critical Reviews in Therapeutic Drug Carrier Systems* **22**.

Bramwell, V. W., Eyles, J. E., and Alpar, H. O. (2005) Particulate delivery systems for biodefense subunit vaccines. *Advanced Drug Delivery Reviews* **57:** 1247-1265.

Brandt, L., Elhay, M., Rosenkrnads, I., Lindblad, E. B., and Andersen, P. (2000) ESAT-6 Subunit Vaccination against *Mycobacterium tuberculosis*. *Infection and Immunity* **68(2):** 791-795.

Brandt, L., Cunha, J. F., Olsen, A. W., Chilima, B., Hirsch, P., Appelberg, R. and Andersen, P. (2002) Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and indcution of protective immunity to tuberculosis. *Infection and Immunity* **70:** 672-678.

Brewer, J. M. and Alexander, J. (1992) The adjuvant activity of non-ionic surfactant vesicles (niosomes) on the BALB/c humoral response to bovine serum albumin. *Immunology* **75:** 570-575.

British National Formulary (2005) [online]. [Accessed 12th September 2005]. Available from World Wide Web: <http://www.bnf.org>

Brown, M. D., Schätzlein, A. G., and Uchegbu, I. F. (2001) Gene delivery with synthetic (non viral) carriers. *International Journal of Pharmaceutics* **229:** 1-21.

Brunel, F., Darbouret, A., and Ronco, J. (1999) Cationic lipid DC-Chol induces an improved and balanced immunity able to overcome the unresponsiveness to the hepatitis B vaccine. *Vaccine* **17:** 2192-2203.

Buckley, R. H. (2002) Gene therapy for SCID-a complication after remarkable progress. *Lancet* **360:** 1185-1186.

Cai, H., Hu, X. D., Yu, D. H., Li, S. X., Tian, X., and Zhu, Y. X. (2005) Combined DNA vaccine encapsulated in microspheres enhanced protection efficacy against *Mycobacterium tuberculosis* infection of mice. *Vaccine* **23:** 4167-4174.

Cambell, P. I. (1983) Toxicity of some charged lipids used in liposome preparations. *Cytobios* **37:** 21-26.

Campbell, R. B., Balasubramanian, S. V., and Straubinger, R. M. (2001) Phospholipid-cationic lipid interactions: influences on membrane and vesicle properties. *Biochimica et Biophysica Acta* **1512:** 27-39.

Cao, Y. and Li, H. (2002) Interfacial activity of a novel family of polymeric surfactants. *European Polymer Journal* **38:** 1457-1463.

Chattergon, M. A., Robinson, T. M., Boyer, J. D., and Weiner, D. B. (1998) Specific immune induction following DNA-based mmunization through in vivo transfection and activation of macrophages/antigen presenting cells. *Journal of Immunology* **160:** 5707-5718.

Chattergoon, M., Boyer, J., and Weiner, D. B. (1997) Genetic immunization: a new era in vaccines and immune therapeutics. *The FASEB Journal* **11:** 754-763.

Childers, N. K., Miller, K. L., Tong, G., Llarena, J. C., Greenway, J., Ulrich, J. T. Michalek, S. M. (2000) Adjuvant Activity of Monophosphoryl Lipid A for Nasal and Oral Immunization with Soluble or Liposome-Associated Antigen. *Infection and Immunity* **68:** 5509-5516.

Chiron Announces Promising Data from Clinical Study of Adjuvanted Avian Influenza Vaccine; Results Confirm Previous Clinical Studies: Chiron's MF59 Adjuvant Significantly Enhances Immune Response (2005) [online]. [Accessed 30th October 2005]. Available from World Wide Web: <http://www.chiron.com/pipeline/vaccinespipe/avian.html>

Chu, J. W. and Sharom, F. J. (1990) Interleukin-2 binds to gangliosides in micelles and lipid bilayers. *Biochimica et Biophysica Acta* **1028:** 205-214.

Ciccarone, V., Hawley-Nelson, P., and Jessee, J. (1993) Cationic liposome-mediate transfection: effect of serum on expression and efficiency. *Focus* **15:** 80-83.

Cohen, J. (2005) High hopes and dilemmas for a cervical cancer vaccine. *Science* **308:** 618-621.

Cole, S. T. (1996) Why sequence the genome of Mycobacterium tuberculosis? *Tuberculosis Lung Disease* **77:** 486-490.

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry III, C. E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Conner, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McClean, J., Moule, S., Murphy, L., Oliver, K., Osbourne, J., Quail, M. A., Rajandream, M.-A., Rogers, J., Rutter, S., Seeger, K., Skelton, R., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S. and Barrel, B. G. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:** 537-544.

Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K., and Falo, L. D. Jr. (1996) DNA-based immunization by *in vivo* transfection of dendritic cells. *Nature Medicine* **2:** 1122-1128.

Congiu, A., Pozzi, D., Esposito, C., Castellano, C., and Mossa, G. (2004) Correlation between structure and transfection efficiency: a study of DC-Chol-DOPE/DNA complexes. *Colloids and Surfaces B: Biointerfaces* **36:** 43-48.

Constant, S. L. and Bottomly, K. (1997) Induction of Th1 and Th2 CD4+ T cell responses: the alternative approahes. *Annual Review of Immunology* **15:** 297-322.

Costa.J. (1996) PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. *Nucleic Acids Research* **24(13):** 2623-2625.

Cox, J. C. and Coulter, A. R. (1997) Adjuvants - a classification and review of their modes of action. *Vaccine* **15:** 248-256.

Crowe, J. H. and Clegg, J. S. (1973) *Anhydrobiosis*. Stroudsburg: Dowden, Hutchinson &Ross.

Crowe, L. M., Crowe, J. H., Rudolph, A. S., Womersley, C., and Appel, L. (1985) Preservation of freeze-dried liposomes by trehalose. *Archives of Biochemistry and Biophysics* **242:** 240-247.

Crowe, L. M., Crowe, J. H., Carpenter, J. F., Rudolph, A. S., Wistrom, C. A., Spargo, B. J. and Anchordoguy, T. J. (1988) Interaction of sugars with membranes. *Biochimica et Biophysica Acta* **947:** 367-384.

Crowe, L. M. and Crowe, J. H. (1995) Freeze-dried liposomes. In *Liposomes, new systems and new trends in their applications*. Peuisieux, F., Couvreur, P., Delattre, J. and Devissaguet, J-P (ed.) pp. 237-272.

Davidsen, J., Rosenkrands, I., Christensen, D., Vangala, A., Kirby, D., Perrie, Y., Agger, E-M. and Andersen, P. (2005) Characterization of cationic liposomes based on dimethyl dioctadecyl ammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6'-dibehenate) - a novel adjuvant inducing both strong CMI and antibody responses. *Biochimica et Biophysica Acta (BBA) – Biomembranes* **In Press**

Davis, D. and Gregoriadis, G. (1987) Liposomes as adjuvants with immunopurified tetanus toxoid: influence of liposomal characteristics. *Immunology* **61:** 229-234.

Davis, H. L., Whalen, R. G., and Demeneix, B. A. (1993) Direct gene transfer in skeletal muscle *in vivo*: factors influencing efficiency of transfer and stability of expression. *Human Gene Therapy* **4:** 151-156.

Davis, H. L. (1996) DNA-based vaccination against hepatitis B virus. *Advanced Drug Delivery Reviews* **21:** 33-47.

De Donato, S., Granoff, D., Minutello, M., Lecchi, G., Faccini, M., Agnello, M., Senotore, F., Verweij, P., Fritzell, B. and Podda, A. (1999) Safety and immunogenicity of MF59-adjuvanted influenza vaccine in the elderly. *Vaccine* **17:** 3094-3101.

de Haan, A., Geerligs, H. J., Huchshorn, J. P., van Scharrenburg, G. J. M., Palache, A. M., and Wilschut, J. (1995) Mucosal immunoadjuvant activity of liposomes: induction of systemic IgG and secretory IgA responses in mice by intranasal immunisation with an influenza subunit vaccine and coadministered liposomes. *Vaccine* **13:** 155-162.

Deamer, D. W. and Barchfield, G. L. (1982) Encapsulation of macromolecules by lipid vesicles under simulated prebiotic conditions. *Journal of Molecular Evolution* **18:** 203-206.

Deck, R. R., DeWitt, C. M., Donnelly, J. J., Liu, M. A., and Ulmer, J. B. (1997) Characterisation of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* **15:** 71-78.

Demel, R. A., Geurts van Kessel, W. S. M., and Van Deenen, L. L. M. (1972) The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **266:** 26-40.

Devaraj, G. N., Parakh, S. R., Devraj, R., Apte, S. S., Ramesg Roa, B., and Rambhau, D. (2002) Release studies on niosomes containing fatty alcohols as bilayer stabilisers instead of cholesterol. *Journal of Colloid and Interface Science* **251:** 265-360.

Development of New Vaccines for Tuberculosis Recommendations of the Advisory Council for the Elimination of Tuberculosis (ACET). (1998) *Morbidity and mortality weekly report* **47**.

Development of Polio Vaccines (2005) [online]. [Accessed 2nd September 2005]. Available from World Wide Web: <http://www. accessexcellence.org/AE/AEC/CC/polio.html>

Dias, R. S., Lindman, B., and Miguel, M. G. (2002) DNA Interaction with Catanionic Vesicles. *Journal of Physical Chemistry B* **106:** 12600-12607.

Duclos, P. and Hofmann, C. A. (2001) Immunisation safety: a priority of the World Health Organization's Department of Vaccines and Biologicals. *Drug Safety* **24:** 1105-1112.

Duclos, P. (2004) A global perspective on vaccine safety. *Vaccine* **22:** 2059-2063.

Eastman, S. J., Siegel, C., Tousignant, J., Smith, A. E., Cheng, S. H., and Scheule, R. K. (1997) Biophysical characterization of cationic lipid:DNA complexes. *Biochimica et Biophysica Acta* **1325:** 41-62.

Eckstein, M., Barenholz, Y., Bar, L. K., and Segal, E. (1997) Liposomes containing *Candida albicans* ribosomes as a prophylactic vaccine against disseminated candidiasis in mice. *Vaccine* **15:** 220-224.

Elouahabi, A. and Ruysschaert, J.-M. (2004) Formation and intracellular trafficking of lipoplexes and polyplexes. *Molecular Therapy* **11:** 336-347.

Escriou, V., Ciolina, C., Lacroix, F., Byk, G., Scherman, D., and Wils, P. (1998) Cationic lipid-mediated gene transfer: effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes. *Biochimica et Biophysica Acta* **1368:** 276-288.

Faneca, H., Simões, S., and Pedroso de Lima, M. C. (2002) Evaluation of lipid-based reagents to mediate intracellular gene delivery. *Biochimica et Biophysica Acta* **1567:** 23-33.

Fang, N., Wang, J., Moa, H. Q., Leong, K. W., and Chan, V. (2003) BCHEM-Chol/DOPE liposomes induced perturbation of phospholipid bilayer. *Colloids and Surfaces B: Biointerfaces* **29:** 233-245.

Farhood, H., Serbina, N., and Huang, L. (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochimica et Biophysica Acta* **1235:** 289-295.

Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M. and Felgner, P. L. (1994) Enhanced Gene Delivery and Mechanism Studies with a Novel Series of Cationic Lipid Formulations. *The Journal of Biological Chemistry* **269(4):** 2550-2561.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987) Lipofectin: a highly efficient, lipid-mediated DNA transfection procedure. *Proceedings of National Academic Sciences, USA* **84:** 7413-7417.

Felgner, P. L. and Rhodes, G. (1991) Gene therapeutics. *Nature* **349:** 351-352.

Felgner, P. L. (1996) Improvements in cationic liposomes for in vivo gene transfer. *Human Gene Therapy* **7(15):** 1791-1793.

Felnerova, D., Viret, J.-F., Gluck, R., and Moser, C. (2004) Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. *Current Opinion in Biotechnology* **15:** 518-529.

Fenske, D. B., MacLachlan, I., and Cullis, P. R. (2002) Stabilized plasmid-lipid particles: A systematic gene therapy vector. *Methods in Enzymology* **346(3):** 36-71.

Ferber, D. (2001) Gene Therapy: Safer and Virus-Free. *Science* **294:** 1638-1642.

Fine, P. E. M. (1995) Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* **346:** 1339-1345.

Floch, V., Loisel, S., Guenin, E., Herve, A. C., Clement, J. C., Yaouanc, J. J., des Abbayes, H. and Ferec, C. (2000) Cation Substitution in Cationic Phosphonolipids: A New Concept To Improve Transfection Activity and Decrease Cellular Toxicity. *Journal of Medicinal Chemistry* **43(24):** 4617-4628.

Florence, A. T. and Attwood, D. (1988) *Physiochemical Principles of Pharmacy*. London: Macmillan.

Florence, A. T., Hillery, A. M., Hussain, N., and Jani, P. U. (1995) Factors affecting the oral uptake and translocation of polystyrene nanoparticles: histological and analytical evidence. *Journal of Drug Targeting* **3:** 65-70.

Fortin, A., Shahum, E., Krzystyniak, K., and Therien, H.-M. (1996) Differential Activation of Cell-Mediated Immune Functions by Encapsulated and Surface-Linked Liposomal Antigens. *Cellular Immunology* **169:** 208-217.

Freise, J. (1984) The preparation of sterile drug-containing liposomes. In *Liposome Technology*. Gregoriadis, G. (ed.) Boca Raton, FL: CRC Press, pp. 131-137.

Freund, J. (1951) The effect of paraffin oil and mycobacteria on antibody formation and sensitization: A review. *American Journal of Clinical Pathology* **21:** 645-656.

Frrkjaer, S., Hjorth, E. L., and Wrrts, O. (1982) Stability and storage of liposomes.

Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C., and Robinson, H. L. (1993) DNA vaccines: protective immunizations by parental, mucosal and gene gun inoculations. *Proceedings of National Academic Sciences, USA* **90:** 11478-11482.

Gao, X. and Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochemical and Biophysical Research Communications* **179:** 280-285.

Gershon, H., Ghirlando, R., Guttman, S. B., and Minsky, A. (1993) Mode of formation and structural feature of DNA-cationic liposome complexes used for transfection. *Biochemistry* **32:** 7143-7151.

Ghosh, Y. K., Visweswariah, S., and Bhattacharya, S. (2000) Nature of linkage between the cationic headgroup and cholesteryl skeleton controls gene transfection efficiency. *FEBS Lett* **473:** 341-344.

Gregoriadis, G. (1990) Immunological adjuvants: a role for liposomes. *Immunology Today* **11(3):** 89-97.

Gregoriadis, G. (1993) *Liposome Technology*. Boca Raton, FL: CRC Press.

Gregoriadis, G., Garcon, N., da S.H., and Sternberg, B. (1993) Coupling of ligands to liposomes independently of sloute entrapment: observations on the formed vesicles. *Biochimica et Biophysica Acta* **1147:** 185-193.

Gregoriadis, G. (1998) Genetic vaccines: strategies for optimisation. *Pharmaceutical Research* **15:** 661-670.

Gregoriadis, G., McCormack, B., Perrie, Y., and Saffie, R. (1998) DNA vaccination: a role for liposomes. In *Medical Applications of Liposomes*. Lasic and Papahadjopoulos (ed.) pp. 61-73.

Gregoriadis, G., McCormack, B., Morrison, Y., Saffie, R., and Zadi, B. (1998) Liposomes in drug targeting. In *Cell Biology: A Laboratory Handbook*. Academic Press, pp. 131-140.

Gregoriadis, G. (2003) Liposomes in drug and vaccine delivery. *Drug Delivery Systems & Science* **2**.

Gregoriadis, G. and Ryman, B. E. (1971) Liposomes as carriers of enzymes or drugs: a new approach to the treatment of storage diseases. *Biochemical Journal* **124:** 58.

Gregoriadis, G. and Davis, C. (1979) Stability of Liposomes in vivo and in vitro is Promoted by their Cholesterol Content in the Presence of Blood Cells. *Biochimica Biophysica Research Community* **90:** 1287-1293.

Gregoriadis, G., Davis, D., and Davis, A. (1987) Liposomes as immunological adjuvants: antigen incorporation studies. *Vaccine* **5:** 143-149.

Gregoriadis, G. (1994) Liposomes as immunoadjuvants and vaccine carriers: Antigen entrapment. *Immunomethods* **4:** 210-216.

Gregoriadis, G., Saffie, R., and Hart, S. L. (1996) High yield incorporation of plasmid DNA within liposomes: Effect of DNA integrity and transfection efficiency. *Journal of Drug Targeting* **3:** 469-475.

Gregoriadis, G., Saffie, R., and de Souza, B. (1997) Liposome-mediated DNA vaccination. *FEBS Lett* **402:** 107-110.

Gregoriadis, G., McCormack, B., Obrenovic, M., Perrie, Y., and Yang, J.-C. (2000) Genetic Vaccines: A Role for Liposomes. Gregoriadis, G. and McCormack, B. (ed.) IOS Press, pp. 92-101.

Gregoriadis, G., Bacon, A., Caparros-Wanderley, W., and McCormack, B. (2002) A role for liposomes in genetic vaccination. *Vaccine* **3462:** 1-9.

Guo, W. and Lee, R. J. (2000) Efficient gene delivery using anionic liposome-complexed polyplexes (LPDII). *Bioscience Reports* **20(5):** 419-432.

Gupta, R. K. and Siber, G. R. (1995) Adjuvants for human vaccines - current status, problems and future prospects. *Vaccine* **13(14):** 1263-1276.

Gupta, R. K. (1998) Aluminum compounds as vaccine adjuvants. *Advanced Drug Delivery Reviews* **32:** 155-172.

Gürsel, M. and Gregoriadis, G. (1997) Interleukin-15 acts as an immunological co-adjuvant for liposomal antigen in vivo. *Immunology Letters* **55:** 161-165.

Harboe, M., Malin, A. S., Dockrell, H. S., Wiker, H. G., Ulvund, G., Holm, A., Jorgensen, M. C. and Andersen, P. (1998) B-Cell Epitopes and Quantification of the ESAT-6 Protein of *Mycobacterium tuberculosis* . *Infection and Immunity* **66(2):** 717-723.

Harvie, P., Wong, F. M. P., and Bally, M. B. (1998) Characterisation of lipid DNA interactions I: destabilisation of bound lipids and DNA dissociation. *Biophysical Journal* **75:** 1040-1051.

Hilgers, L. A. T. and Snippe.H. (1992) DDA as an immunological adjuvant. *Research Immunology* **143:** 494-503.

Hirsch-Lerner, D., Zhang, M., Eliyahu, H., Ferrari, M. E., Wheeler, C. J., and Barenholz, Y. (2005) Effect of "helper lipid" on lipoplex electrostatics. *Biochimica et Biophysica Acta* **Uncorrected Proof**.

Holten-Andersen, L., Doherty, T. M., Korsholm, K. S., and Andersen, P. (2004) Combination of the Cationic Surfactant Dimethyl Dioctadecyl Ammonium Bromide and Synthetic Myco bacterial Cord Factor as an Efficient Adjuvant for Tuberculosis Subunit Vaccine. *Infection and Immunity* **72(3):** 1608-1617.

Huang, D. B., Wu, J. J., and Tyring, S. K. (2004) A review of licensed viral vaccines, some of their safety concerns, and the advances in the development of investigational viral vaccines. *Journal of Infection* **49:** 179-209.

Huebner, S., Battersby, B. J., Grimm, R., and Cevc, G. (1999) Lipid-DNA Complex Formation: Reorganization and Rupture of Lipid Vesicles in the Presence of DNA as Observed by Cryoelectron Microscopy. *Biophysical Journal* **76:** 3158-3166.

Hug, P., Sleight, R. G. (1991) Liposomes for the transformation of eukaryotic cells. *Biochimica et Biophysica Acta* **1097(1):** 1-17.

Hyvonen, Z., Plotniece, A., Reine, I., Chekavichus, B., Duburs, G., and Urtti, A. (2000) Novel cationic amphiphilic 1,4-dihydropyridine derivatives for DNA delivery. *Biochimica et Biophysica Acta* **1509(1-2):** 451-466.

Iezzi, G., Kajalainen, K., and Lanzacecchia, A. (1998) The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* **8:** 89-95.

Johnson, P. A., Conway, M. A., Daly, J., Nicolson, C., Robertson, J., and Mills, K. H. G. (2000) Plasmid DNA encoding influenza virus haemagglutinin induces Th1 cells and protection against respiratory infection despite its limited ability to generate antibody responses. *Journal of Gene Virology* **81:** 1745.

Jonas, A. and Maine, G. T. (1979) Kinetics and mechanism of phosphatidylcholine and cholesterol exchange between single bilayer vesicles and bovine serum high-density lipoprotein. *Biochemistry* **18:** 1722-1728.

Jouanguy, E., Altare, F., Lamhamdi, S., Revy, P., Emile, J. F., Newport, M., Levin, M., Blanche, S., Seboun, E., Fischer, A. and Casanova, J. L. (1996) Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection. *N Engl J Med* **335:** 1956-1961.

Juliano, R. L. and Stamp, D. (1975) The effect of particle size and charge on the clearance rates of liposomes and liposome encapsulated drugs. *Biochemical and Biophysical Research Communication* **63:** 651-658.

Kahl, L. P., Scott, C. A., Lelchuk, R., Gregoriadis, G., and Liew, F. Y. (1989) Vaccination against murine cutaneous leishmaniasis by using *Leishmania major* antigen/liposomes. Optimization and assessment of the requirement for intravenous immunization. *Journal of Immunology* **142:** 4441-4449.

Kaiser, J. (2003) Gene therapy-seeking the cause of induced leukemias in X-SCID trial. *Science* **299:** 495.

Kamiya, H., Tsuchiya, H., Yamazaki, J., and Harashima, H. (2001) Intracellular trafficking and transgene expression of viral and non-viral gene vectors. *Drug Delivery Reviews* **52:** 153-164.

Kaufmann, S. H. E. (2005) Recent findings in immunology give tuberculosis vaccines a new boost. *Trends in Immunology* **26:** 660-667.

Kirby, C. and Gregoriadis, G. (1984) Dehydration-rehydration vesicles (DRV): A new method for high yield drug entrapment in liposomes. *Biotechnology* **2:** 979-984.

Kirby, C. and Gregoriadis, G. (1984) Preparation of liposomes containing factor VIII for oral treatment of haemophilia. *Journal of Microencapsulation* **1:** 33-45.

Koltover, I., Sasldit, T., Radler, J. O., and Safinya, C. R. (1998) An Inverted Hexagonal Phase of Cationic Liposome-DNA Complexes Related to DNA Release and Delivery. *Science* **281:** 78-81.

Kwaura, C., oguchi, A., uruno, T., and akanishi, M. (1998) Atomic force microscopy for studying gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. *FEBS Lett* **421:** 69-72.

Labat-Moleur, F., Steffan, A. M., Brisson, C., Perron, H., Feugeas, O., Furstenberger, P., Oberling, F., Brambilla, E. and Behr, J. P. (1996) An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Therapy* **3:** 1010-1017.

Langer, R. (1997) Tissue engineering: a new field and its challenges. *Pharmaceutical Research* **14:** 840-841.

Langner, M. (2000) Effect of liposome molecular composition on its ability to carry drugs. *Polish Journal of Pharmacology* **52:** 3-14.

Lasic, D. D., Strey, H., Stuart, M. C., Podgornik, R., and Frederik, P. M. (1997) The structure of DNA-liposomes complexes. *Journal of American Chemistry Society* **119:** 832-833.

Legendre, J. and Szoka, F. C. Jr. (1993) Cyclic Amphipathic Peptide-DNA Complexes Mediate High-Efficiency Transfection of Adherent Mammalian Cells. *Proceedings of the National Academy of Sciences* **90:** 893-897.

Lemaire, G., Tenu, J. P., Petit, J. F., and Lederer, E. (1986) Natural and synthetic trehalose diesters as immunomodulators. *Medical Research Reviews* **6:** 243-274.

Lemoine, N. R. (1999) *Understanding Gene Therapy*. Oxford: BIOS Scientific Publishers Ltd.

Leserman, L. (2004) Liposomes as Protein Carriers in Immunology. *Journal of Liposome Research* **14:** 175-189.

Lever, A. M. L. (1996) Gene therapy in the fight against AIDS. *Expert Opinion on Therapeutic Patents* **6:** 161-167.

Levine, H. and Slade, L. (1988) Water as a plasticizer: physio-chemical aspects of low-moisture polymeric systems. In *Water science reviews*. Franks, F. e. (ed.) Cambridge, UK: Cambridge Press, pp. 79-185.

Levine, H. and Slade, L. (1992) Another view of trehalose for drying and stabilizing biological materials. *BioPharm* **5:** 36-40.

Lewis, P. J. and Babiuk, L. A. (1999) DNA vaccines: A review. *Advanced Virus Research* **54:** 129-132.

Li, S., Gao, X., Son, K., Sorgi, F., Hofland, H., and Huang, L. (1996) DC-Chol lipid system in gene transfer. *Journal of Controlled Release* **39:** 373-381.

Lima, K. M., Aparecida dos Santos, S., Rodrigues, Jr. J. M., and Silva, C. L. (2004) Vaccine adjuvant: it makes the difference. *Vaccine* **22:** 2374-2379.

Lindblad, E. B., Elhay, M. J., Silva, R., Appelberg, R., and Andersen, P. (1997) Adjuvant modulation of immune responses to tuberculosis sub-unit vaccines. *Infection and Immunity* **65:** 623-629.

Litzinger, D. and Huang, L. (1992) Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. *Biochimica et Biophysica Acta* **1113:** 201-227.

Liu, F. and Huang, L. (2002) Development of non-viral vectors for systmic gene delivery. *Journal of Controlled Release* **78:** 259-266.

Liu, Y., Mounkes, L. C., Liggit, H. D., Brown, C. S., Solodin, I., Heath, T. D. and Debs, R. J. (1997) Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nature Biotechnology* **15:** 167-173.

Luo, D. and Saltzman, W. M. (2000) Synthetic DNA delivery systems. *Nature Biotechnology* **18:** 33-37.

Madigan, M. T., Martinko, J. M., and Parker, J. (2000) *Biology of Microorganisms*. New Jersey: Prentice-Hall.

Mancini-Bourgine, M., Fontaine, H., Brechot, C., Pol, S., and Michel, M.-L. (2005) Immunogenicity of a hepatitis B DNA vaccine administered to chronic HBV carriers. *Vaccine* **In Press**.

Manickan, E., Karem, K. L., and Rouse, B. T. (1997) DNA vaccines - a modern gimmick or a boon to vaccinology. *Critical Reviews in Immunology* **17:** 139-154.

Mannino, R. J., Allebach, E. S., and Strohl, W. A. (1979) Encapsulation of high molecular weight DNA in large unilamellar phospholipid vesicles. Dependence on the size of the DNA. *FEBS Lett* **101:** 229-232.

Martin, R. M. and Lew, A. M. (1998) Is IgG2a a good Th1 marker in mice? *Immunology Today* **19:** 49.

Matsusue, Y., Hanafusa, S., Yamamuro, T., Shikinami, Y., and Ikada, Y. (1995) Tissue reaction of bioabsorbable ultra high strength poly(L-lactide) rod. A long-term study in rabbits. *Clinical Orthopedics* 246-253.

Matulis, D., Rouzina, I., and Bloomfield, V. A. (2001) Thermodynamics of Cationic Lipid Binding to DNA and DNA Condensation: Roles of Electrostatics and Hydrophobicity. *Journal of American Chemistry Society* **124:** 7331-7342.

May, S. and Ben-Shaul, A. (1997) DNA-lipid complexes: stability of honey-comb-like and spagetti-like structures. *Biophysical Journal* **73:** 2427-2440.

May, S., Harries, D., and Ben-Shaul, A. (2000) The Phase Behaviour of Cationic Lipid-DNA Complexes. *Biophysical Journal* **78:** 1681-1697.

Mayhew, E., Ito, M., and Lazo, R. (1987) Toxicity of non-drug-containing liposomes for cultured human cells. *Experimental Cell Research* **171:** 195-202.

McAllister, D. V., Allen, M. G., and Prausnitz, M. R. (2000) Microfabricated microneedles for gene and drug delivery. *Annual Review of Biomedical Engineering* **2:** 289-313.

Miguel, M. G., Pais, A. A. C. C., Dias, R. S., Leal, C., Rosa, M., and Lindman, B. (2003) DNA-cationic amphiphile interactions. *Colloids and Surfaces A: Physiochemical Engineering Aspects* **228:** 43-55.

Mohammed, A. R., Weston, N., Coombes, A. G. A., Fitzgerald, M., and Perrie, Y. (2004) Liposome fomrulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. *International Journal of Pharmaceutics* **285:** 23-34.

Mok, K. W. C. and Cullis, P. R. (1997) Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA. *Biophysical Journal* **73:** 2534-2545.

Morein, B., Villacres-Ericksson, M., Sjolander, A., and Bengtsson, K. L. (1996) Novel adjuvants and vaccine delivery systems. *Veterinary Immunology and Immunopathology* **54:** 373-384.

Mui, B., Ahkong, Q. F., Chow, L., and Hope, M. J. (2000) Membrane perturbation and the mechanism of lipid-mediated transfer of DNA into cells. *Biochimica et Biophysica Acta* **1467:** 281-292.

Murata, J., Kitamoto, T., Ohya, Y., and Ouchi, T. (1997) Effect of dimerization of the D-glucose analogue of muramyl dipeptide on stimulation of macrophage-like cells. *Carbohydrate Research* **297:** 127-133.

Murdan, S., Gregoriadis, G., and Florence, A. T. (1999) Sorbitan monostearate/polysorbate 20 organogels containing niosomes: a delivery vehicle for antigens? *European Journal of Pharmaceutical Sciences* **8:** 177-185.

Murdan, S., Gregoriadis, G., and Florence, A. T. (1999) Interaction of a nonionic surfactant-based organogel with aqueous media. *International Journal of Pharmaceutics* **180:** 211-214.

Nakanishi, T., Kunisawa, J., Hayashi, A., Tsutsumi, Y., Kubo, K., Nakagawa, S., Fujiwara, H., Hamaoka, T. and Mayumi,T. (1997) Positively charged liposome functions as an efficient immunoadjuvant in inducing immun responses to soluble proteins. *Biochemical and Biophysical Research Communications* **240:** 793-797.

Niven, R., Pearlman, R., Wedeking, T., Mackeigan, J., Noker, P., Simpson-Herren, L. and Smith, J.G. (1998) Biodistribution of radiolabeled lipid-DNA complexes and DNA in mice. *Journal of Pharmaceutical Scienes* **87:** 1292-1299.

O'Hagan, D. T., MacKichan, M. L., and Singh, M. (2001) Recent developments in adjuvants for vaccines against infectious diseases. *Biomolecular Engineering* **18:** 69-85.

O'Hagan, D. T., McGee, J. P., Holmgren, J., Mowat, A. M., Donache, A. M., Mills, K. H. G., Gaisford, W., Rahman, D. and Challacombe, S. J. (2004) Biodegradable microparticles for oral immunization. *Vaccine* **11:** 149-154.

Okayama, R., Noji, M., and Nakanishi, M. (1997) Cationic cholesterol with a hydroxyethylamino head group promotes significant liposome-mediated gene transfection. *FEBS Lett* **408:** 232-234.

Olds, G. R., Chedid, L., Lederer, E., and Mahmoud, A. A. F. (1980) Induction of Resistance to Schistosoma-Mansoni by Natural Cord Factor and Synthetic Lower Homologs. *Journal of Infection and Disease* **141:** 473-478.

Oliver, A. E., Crowe, L. M., and Crowe, J. H. (1998) Methods for dehydration-tolerance: Depression of the phase transition temperature in dry membranes and carbohydrate vitrification. *Seed Science Research* **8:** 211-221.

Olsen, A. W., Van Pinxteren, L. A. H., Okkels, L. M., Rasmussen, P. B., and Andersen, P. (2001) Protection of Mice with a Tuberculosis Subunit Vaccine Based on a Fusion Proetin of Anitgen 85B and ESAT-6. *Infection and Immunity* **69(5):** 2773-2778.

Olsen, A. W., Williams, A., Okkels, L. M., Hatch, G., and Andersen, P. (2004) Protective Effect of a Tuberculosis Subunit Vaccine Based on a Fusion of Antigen 85B and ESAT-6 in the Aerosol Guinea Pig Model. *Infection and Immunity* **72:** 6148-6150.

Orme, I. M., McMurry, D. N., and Belisle, J. T. (2001) Tuberculosis vaccine development: recent progress. *Trends in Microbiology* **9(3):** 115-118.

Ott, G., Barchfeld, G. L., Chernoff, D., Radhakrishman, R., van Hoogevest, P., and Van Nest, G. (1995) MF59: design and evaluation of a safe and potent adjuvant for human vaccines. In *Vaccine Design: The Subunit and Adjuvant Approach.* Powell, M. F. and Newman, M. J. (ed.) New York: Plenum Press, pp. 277-296.

Papahadjopoulos, D., Jacobson, K., Nir, S., and Isac, T. (1973) Phase transitions in phospholipid vesicles. *Biochimica et Biophysica Acta* **311:** 330-348.

Pashine, A., Valiante, N. M., and Ulmer, J. B. (2005) Targeting the innate immune response with improved vaccine adjuvants. *Nature Medicine* **11:** S63-S68.

Patil, S. D., Rhodes, D. G., and Burgess, D. J. (2004) Anionic liposomal delivery system for DNA transfection. *The AAPS Journal* **6:** E29.

Patil, S. D., Rhodes, D. G., and Burgess, D. J. (2005) DNA-based Therapeutics and DNA Delivery Systems: A Comprehensive Review. *The AAPS Journal* **7(1):** E61-E77.

Paukku, T., Lauraeus, S., Huhtaniemi, I., and Kinnunen, P K. (1997) Novel cationic liposomes for DNA-transfection with high efficiency and low toxicity. *Chemistry And Physics Of Lipids* **87(1):** 23-29

Pedroso de Lima, M. C., Simões, S., Pires, P., Faneca, H., and Düzgünes, N. (2001) Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Advanced Drug Delivery Reviews* **47:** 277-294.

Perrie, Y. and Gregoriadis, G. (2000) Liposome-entrapped plasmid DNA: characterisation studies. *Biochimica et Biophysica Acta* **1475:** 125-132.

Perrie, Y., Frederik, P. M., and Gregoriadis, G. (2001) Liposomes-mediated DNA vaccine: the effect of vesicle composition. *Vaccine* **19:** 3301-3310.

Perrie, Y., McNeil, S., and Vangala, A. (2003) Liposome-mediated DNA immunisation via the subcutaneous route. *Journal of Drug Targeting* **11:** 563.

Perrie, Y., Barralet, J. E., McNeil, S., and Vangala, A. (2004) Surfactant vesicle-mediated delivery of DNA vaccines via the subcutaneous route. *International Journal of Pharmaceutics* **284:** 31-41.

Peyre, M., Audran, R., Estevez, F., Corradin, G., Gander, B., Sesardic, D. *et al.* (2004) Childhood and malaria vaccines combined in biodegradable microspheres produce immunity with synergistic interactions. *Journal of Controlled Release* **99:** 345-355.

Phillips, N. C., Moras, M. L., Chedid, L., Lefrancier, P., and Bernard, J. M. (1985) Activation of alveolar macrophage tumoricidal activity and eradication of experimental metastases by freeze-dried liposomes containing a new lipophilic muramyl dipeptide derivative. *Cancer Research* **45:** 128-134.

Phillips, N. C., Gagne, L., Ivanoff, N., and Riveau, G. (1996) Influence of phospholipid composition on antibody responses to liposome encapsulated protein and peptide antigens. *Vaccine* **14:** 898-904.

Pires, P., Simoes, S., Nir, S., Gaspar, R., Duzgunes, N., and Pedroso de Lima, M. C. (1999) Interaction of cationic liposomes and their DNA complexes with monocytic leukemia cells. *Biochimica et Biophysica Acta* **1418:** 71-84.

Pouton, C. W. and Seymour, L. W. (2001) Key issues in non-viral gene delivery. *Advanced Drug Delivery Reviews* **46:** 187-203.

Radler, J. O., Koltover, I., Salditt, T., and Safinya, C. R. (1997) Structure of DNA-cationic liposome complexes: DNA intercalation in multi-lameller membranes in distinct interhelical packing regimes. *Science* **275:** 810-814.

Ramon, G. (1926) Procedres pour acroitre la production des antitoxines. *Annual Institut Pasteur* **40:** 1-10.

Rao, M., Matyas, G. R., Grieder, F., Anderson, K., Jahrling, P. B., and Alving, C. R. (1999) Cytotoxic T lymphocytes to ebola zaire virus are induced in mice by immunization with liposomes containing lipid A. *Vaccine* **17:** 2991-2998.

Rao, M. and Alving, C. R. (2000) Delivery of lipids and liposomal proteins to the cytoplasm and Golgi of antigen-presenting cells. *Advanced Drug Delivery Reviews* **41:** 171-188.

Rao, M., Bray, M., Alving, C. R., Jahrling, P. B., and Matyas, G. R. (2002) Induction of immune responses in mice and monkeys to ebola virus after immunization with liposome-encapsulated irradiated ebola virus: protection in mice requires CD4+ T cells. *Journal of Virology* **76:** 9176-9185.

Ratz, H., Freise, J., Magerstedt, P., Schaper, A., Preugschat, W., and Keyser, D. (1989) Sterilization of contrast media (Isovist) containing liposomes by ethylene oxide. *Journal of Microencapsulation* **6(4):** 485-492.

Reddin, K. M., Easterbrook, T. J., Eley, S. M., Russell, P., Mobsby, V. A., Jones, D. H., Farrar, G. H., Williamson, E. D. and Robinson, A. (1998) Comparison of the immunological and protective responses elicited by microencapsulated formulations of the F1 antigen from *Yersinia pestis*. *Vaccine* **16:** 761-767.

Reed, S. G., Alderson, M. R., Dalemans, W., Lobet, Y., and Skeiky, Y. A. W. (2003) Prospects for a better vaccine against tuberculosis. *Tuberculosis* **83(1-3):** 213-219.

Regelin, A. E., Fankhaenel, S., Gurtesch, L., Prinz, C., von Kiedrowski, G., and Massing, U. (2000) Biophysical and lipofection studies of DOTAP analogs. *Biochimica et Biophysica Acta* **1464:** 151-164.

Regnier, V., Tahiri, A., Andre, N., Lemaitre, M., Le Doan, T., and Preat, V. (2000) Electroporation-mediated delivery of 3'-protected phosphodiester oligodeoxynucleotides to the skin. *Journal of Controlled Release* **67:** 337-346.

Renegar, K. B. and Small, P. A. (1991) Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. *Journal of Virology* **65:** 2146-2148.

Rogerson, A., Cummings, J., Willmott, N., and Florence, A. T. (1988) The distribution of doxorubicin in mice following administration in niosomes. *Journal of Pharmacy and Pharmacology* **40:** 337-342.

Romoren, K., Fjeld, X. T. L., Poleo, A. B. S., Smistad, G., Thu, B. J., and Evensen, O. (2005) Transfection efficiency and cytotoxicity of cationic liposomes in primary cultures of rainbow trout (*Oncorhynchus mykiss*) gill cells. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **Article in Press, Corrected Proof**.

Ropert, C. (1999) Liposomes as a gene delivery system. *Brazillian Journal of Medical and Biological Research* **32:** 163-169.

Rudbach, J. A., Cantrell, J. L., and Ulrich, J. T. (1988) Molecularly engineered microbial immunostimulators. In *Technolological Advances in Vaccine Development*. Alan, R. (ed.) New York: Liss, Inc., pp. 443-454.

Saarinen-Savolainen, P., Jarvinen, T., Taipale, H., and Urtti, A. (1997) Method for evaluating drug release from liposomes in sink conditions. *International Journal of Pharmaceutics* **159:** 27-33.

Sabhani, L., Manocha, M., Sridevi, K., Shashikiran, D., Rayanade, R., and Rao, D. N. (2003) Developing subunit immunogens using B and T cell epitopes and their constructs derived from the F1 antigen of *Yersinia pestis* using novel delivery vehicles. *FEMS Immunology and Medical Microbiology* **38:** 215-229.

Safinya, C. R. (2001) Structures of lipid-DNA complexes: supramolecular assembly and gene delivery. *Current Opinion in Structural Biology* **11:** 440-448.

Salditt, T., Koltover, I., Radler, J. O., and Safinya, C. R. (1997) Two dimensional smectic ordering of linear DNA chains in self-assembled DNA-cationic liposome mixtures. *Physical Review Letters* **79:** 2582-2585.

Schroit, A. J. and Fidler, I. J. (1982) Effects of liposome structure and lipid composisitonon the activation of the tumoricidal properties of macrophages by liposomes containing muramyl dipeptide. *Cancer Research* **42:** 161-167.

Sedegah, M., Hedstrom, R., Hobart, P., and Hoffman, S. L. (1994) Protection against Malaria by Immunization with Plasmid DNA Encoding Circumsporozoite Protein. *Proceedings of National Academic Sciences, USA* **91:** 9866-9874.

Segal, A. W., Willis, E. J., Richmond, J. E., Slavin, G., Black, C. D. V., and Gregoriadis, G. (1974) Morphological observations on the cellular and subcellular destination of intravenously administered liposomes. *British Journal Of Experimental Pathology* **55:** 320-327.

Sehra, S., Chugh, L., and Gangal, S. V. (1998) Polarized Th1 responses by liposome-entrapped allergen and its potential in immunotherapy of allergic disorders. *Clin Ep Allergy* **28:** 1530-1537.

Semple, S. C., Chonn, A., and Cullis, P. R. (1996) Influence of Cholesterol on the Association of Plasma Proteins with Liposomes. *Biochemistry* **35:** 2521-2525.

Senior, J. and Gregoriadis, G. (1982) Is the half-life of circulating liposomes determined by changes in their permeability? *FEBS Lett* **145:** 109-114.

Shahum, E. and Therien, H.-M. (1988) Immunopotentiation of the humoral response by liposomes: encapsulation versus linkage. *Immunology* **65:** 315-317.

Shahum, E. and Therien, H.-M. (1994) Correlation between *in vitro* and *in vivo* behaviour of liposomal antigens . *Vaccine* **12:** 1125-1131.

Simon, S. A. and McIntosh, T. J. (1986) Depth of water penetration into lipid bilayers. *Methods in Enzymology* **127:** 511.

Singer, V. L., Jones, L. J., Yue, S. T., and Haugland, R. P. (1997) Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. *Analytical Biochemistry* **249:** 228-238.

Small, D. M. (1986) *Handbook of Lipid Research:The Physical Chemistry of Lipids, From Alkanes to Phospholipids*. New York: Plenum Press.

Smith, K. R. (2003) Gene Terapy: Theoretical and Bioethical Concepts. *Archieves of Medical Research* **34:** 247-268.

Song, Y. K., Liu, F., Chu, S., and Liu, D. (1997) Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. *Human Gene Therapy* **8:** 1585-1594.

Spack, E. G. and Sorgi, F. L. (2001) Developing non-viral DNA delivery systems for cancer and infectious disease. *Drug Discovery Today* **6:** 186-197.

Sternberg, B., Sorgi, F., and Huang, L. (1994) New structures in complex formation between DNA and cationic liposomes visulised by freeze-fracture electron microscopy. *FEBS Lett* **356:** 361-366.

Sternberg, B., Hong, K., Zheng, W., and Papahadjopoulos, D. (1998) Ultrastructural characterization of cationic liposome-DNA complexes showing enhanced stability in serum and high transfection in vivo. *Biochimica et Biophysica Acta* **1375:** 23-35.

Storm, G. and Crommelin, D. J. A. (1998) Liposomes: quo vadis? *Pharmaceutical Science and Technology Today* **1(1):** 19-31.

Sui, G., Micic, M., Huo, Q., and Leblanc, R. M. (2000) Studies of a novel polymerizable amphiphilic dendrimer. *Colloids and Surfaces A* **171:** 185-197.

Szoka, F. C. J., Yuhong, X., and Zelphati, O. (1996) How are nucleic acids released in cells from cationic lipid-mucleic acid complexes. *Journal of Liposome Research* **6:** 567-587.

Tam, P., Monck, M., Lee, D., Ludkovski, O., Leng, E. C., Clow, K., Stark, H., Scherrer, P., Graham, R. W. and Cullis, P. R. (2000) Stabilised plasmid-lipid particles for systemic gene delivery. *Gene Therapy* **7:** 1867-1874.

Tan, L. and Gregoriadis, G. (1981) A novel positively-charged lipid 1,2-bis(hexadecylcycloxy)-3-trimethyl aminopropane (BisHOP) enhances the adjuvant effect of liposomes on encapsulated tetanus toxoid. *Asian Pac Journal of Allergy and Immunology* **9:** 21-24.

Templeton, N. S., Lasic, D. D., Frederik, P. M., Strey, H. H., and Roberts, D. D. and Pavlakis,G.N. (1997) Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nature Biotechnology* **15:** 647-652.

Therien, H.-M. and Shahum, E. (1996) Differential biodistribution of encapsulated and surface-linked liposomal antigens. *Biochimica et Biophysica Acta* **1280:** 91-97.

Thoelen, S., Van Damme, P., Mathei, C., Leroux-Roels, G., Desombere, L., Safary, A., Vandepapeliere, P., Slaoui, M. and ehues, A. (1998) Safety and immunogenicity of a hepatitis B vaccine formulated with a novel adjuvant system. *Vaccine* **16:** 708-714.

Tomlinson, E. and Rolland, A. P. (1996) Controllable gene therapy: Pharmaceutics of non-viral gene delivery systems. *Journal of Controlled Release* **39:** 357-372.

Tros de Ilarduya, C. and Duzgunes, N. (2000) Efficient gene transfer by transferrin lipoplexes in the presence of serum. *Biochimica et Biophysica Acta* **1463:** 333-342.

Tseng, W. C. and Huang, L. (1998) Liposome-based gene therapy. *Pharmaceutical Science and Technology Today* **1:** 206-213.

Turner, A., Kirby, C., Senior, J., and Gregoriadis, G. (1983) Fate of cholesterol-rich unilamellar liposomes containing 111-I-labelled bleomycin after subcutaneous injection into rats. *Biochimica et Biophysica Acta* **760:** 119-125.

Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, D. G., Felgner, P. L., Darki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedman, A., Hawe, L. A., Leander, K. R., Martinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. L. and Liu, M. A. (1993) Heterologous protection against inlfuenza by injection of DNA encoding a viral protein. *Science*.

Ulmer, J. B. (1990) WHO international working group on the standardisation and control of nucleic acid vaccines. *Report*.

Ulrich, J. T. and Myers, K. R. (1995) Monophosphoryl lipid A as an adjuvant. In *Vaccine design: the subunit and adjuvant approach.* Powell, M. F. and Newman, M. J. (ed.) New York: Plenum Press, pp. 495-524.

Velinova, M., Read, N., Kirby, C., and Gregoriadis, G. (1996) Morphological observations on the fate of liposomes in the regional lymph nodes after footpad injection into rats. *Biochimica et Biophysica Acta* **1299:** 207-215.

Verma, I. M. and Somia, N. (1997) Gene therapy promises, problems and prospects. *Nature* **389:** 239-242.

Wang, R. e. al. (1998) Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* **282:** 476-480.

Wedlock, D. N., Keen, D. L., McCarthy, A. R., Andersen P., and Buddle B.M. (2002) Effect of different adjuvants on the immune responses of cattle vaccinated with *Mycobacterium tuberculosis* culture filtrate proteins. *Veterinary Immunology and Immunopathology* **87:** 79-88.

Weiss, R., Leitner, W. W., Scheilblhofer, S., Chen, D., Bernhaupt, A., Mostbock, S., Thalhamer, J. and Lyon, J. A. (2000) Genetic vaccination against malaria infection by intradermal and epidermal injection of a plasmid containing the gene encoding Plasmodium berghei circumsporozoite protein. *Infection and Immunity* **68:** 5914-5919.

Wimley, W. C. and Thompson, T. E. (1991) Phosphatidylethanolamine enhances the concentration-dependent exchange of phospholipids between bilayers. *Biochemistry* **30:** 4200-4214.

Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner, P. L. (1990) Direct gene transfer into mouse muscle *in vivo*. *Science* **247:** 1465-1468.

Wolkers, W. F., Oldenhof, H., Tablin, F., and Crowe, J. H. (2004) Preservation of dried liposomes in the presence of sugar and phosphate. *Biochimica et Biophysica Acta* **1661:** 125-134.

Wong, F. M. P., Harvie, P., Zhang, Y.-P., Ramsay, E. C., and Bally, M. B. (2003) Phosphatidylethanolamine mediated destabilization of lipid-based pDNA delivery systems. *International Journal of Pharmaceutics* **255:** 117-127.

Wong, J. P., Zabielski, M. A., Schmaltz, F. L., Brownlee, G. G., Bussey, L. A., Marshall, K., Borralho, T. and Nagata, L. P. (2001) DNA vaccination against respiratory influenza virus infection. *Vaccine* **19:** 2461-2467.

Xu, Y. and Szoka, F. C. Jr. (1996) Mechanism of DNA release in cationic liposome mediated gene transfection. *Biochemistry* **35:** 5616-5623.

Yamagami, H., Matsumoto, T., Fujiwara, N., Arakawa, T., Kaneda, K., Yano, I. and Kobayashi, K. (2001) Trehalose 6,6'-Dimycolate (cord factor) of *Mycobacterium tuberculosis* induces foreign-body- and hypersensitivity-type granulomas in mice. *Infection and Immunity* **69:** 810-815.

Yoshioka, T., Sternberg, B., and Florence, A. T. (1994) Preparation and properties of vesicles (niosomes) of sobitan monoesters (Span 20, 40, 60, and 80) and a sorbitan triester (Span 85). *International Journal of Pharmaceutics* **105:** 1-6.

Zabner, J. (1997) Cationic lipids used in gene transfer. *Advanced Drug Delivery Reviews* **27:** 17-28.

Zadi, B. and Gregoriadis, G. (2000) A novel method for the high-yield entrapment of solutes into small liposomes. *Journal of Liposome Research* **10(1):** 73-80.

Zallen, D. T. (2000) US gene therapy in crisis. *Trends in Genetics* **16:** 272-275.

Zelphati, O. and Szoka, F. C. J. (1998) Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. *Pharmaceutical Research* **13:** 1367-1372.

Zemliñska, B., Sochanik, A., Missol-Kolka, E., and Szala, S. (2002) Various cationic carriers for *in vitro* transfection of tumor and endothelial cell lines. *Acta Biochimica Polonica* **49:** 285-290.

Zhang, Y. and Anchordoquy, T. J. (2004) The role of lipid charge density in the serum stability of cationic lipid/DNA complexes. *Biochimica et Biophysica Acta* **1663:** 143-157.

Zhdanov, R. I., Podobed, O. V., and Vlassov, V. V. (2002) Cationic lipid-DNA complexes-lipoplexes-for gene transfer and therapy. *Bioelectrochemistry* **58:** 53-64.

Zhoa, W., Kobayashi, M., Hosokawa, M., and Seth, P. (2002) Adenoviral vectors for cancer gene therapy. *Current Genomics* **3:** 163-180.

Zhou, X. and Huang, L. (1994) DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochimica et Biophysica Acta* **1189:** 195-203.

Zhu, N., Liggit, D., Liu, Y., and Debs, R. J. (1993) Systemic gene expression after intravenous DNA delivery into adult mice. *Science* **261:** 209-211.

Zinkernagel, R. M. (2000) Localization dose and time of antigens determine immune reactivity. *Seminars in Immunology* **12:** 163-171.

Zuidam, N. J., Lee, S. S., and Crommelin, D. J. A. (1993) Sterilization of Liposomes by Heat Treatment. *Pharmaceutical Research* **10:** 1591-1596.

Zuidam, N. J. and Barenholz, Y. (1997) Electrostatic parameters of cationic liposomes commonly used for gene delivery s determined by 4-heptadecyl-7-hydroxycoumarin. *Biochimica et Biophysica Acta* **1329:** 211-222.

Zuidam, N. J. and Barenholz, Y. (1998) Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery. *Biochimica et Biophysica Acta* **1368:** 115-128.

Zuidam, N. J., Hirsh-Lerner, D., Margulies, S., and Barenholz, Y. (1999) Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency. *Biochimica et Biophysica Acta* **1419:** 207-220.

Zuidam, N. J. and Barenholz, Y. (1999) Characterisation of DNA-lipid complexes commonly used for gene delivery. *International Journal of Pharmaceutics* **183:** 185-193.