DOCTOR OF PHILOSOPHY

Design and development of cationic liposomes as DNA vaccine adjuvants

Behfar Moghaddam

2013

Aston University

DESIGN AND DEVELOPMENT OF CATIONIC LIPOSOMES AS DNA VACCINE ADJUVANTS

BEHFAR MOGHADDAM

Doctor of Philosophy

Aston University

December 2012

Aston University

Design and Development of Cationic Liposomes as DNA Vaccine Adjuvants

Behfar Moghaddam

Doctor of Philosophy

2012

Thesis Summary

Cationic liposomes have been extensively explored for their efficacy in delivering nucleic acids, by offering the ability to protect plasmid DNA against degradation, promote gene expression and, in the case of DNA vaccines, induce both humoural and cellular immune responses. DNA vaccines may also offer advantages in terms of safety, but they are less effective and need an adjuvant to enhance their immunogenicity. Therefore, cationic liposomes can be utilised as delivery systems and/or adjuvants for DNA vaccines to stimulate stronger immune responses.

To explore the role of liposomal systems within plasmid DNA delivery, parameters such as the effect of lipid composition, method of liposome preparation and presence of electrolytes in the formulation were investigated in characterisation studies, *in vitro* transfection studies and *in vivo* biodistribution and immunisation studies. Liposomes composed of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) in combination with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or 1,2-stearoyl-3-trimethylammonium-propane (DSTAP) were prepared by the lipid hydration method and hydrated in aqueous media with or without presence of electrolytes. Whilst the *in vitro* transfection efficiency of all liposomes resulted to be higher than Lipofectin, DSTAP-based liposomes showed significantly higher transfection efficiency than DOTAP-based formulations. Furthermore, upon intramuscular injection of liposomal DNA vaccines, DSTAP-based liposomes showed a significantly stronger depot effect at the injection site. This could explain the result of heterologous immunisation studies, which revealed DSTAP-based liposomal vaccines induce stronger immune responses compared to DOTAP-based formulations. Previous studies have shown that having more liposomally associated antigen at the injection site would lead to more drainage of them into the local lymph nodes. Consequently, this would lead to more antigens being presented to antigen presenting cells, which are circulating in lymph nodes, and this would initiate a stronger immune response. Finally, in a comparative study, liposomes composed of dimethyldioctadecylammonium bromide (DDA) in combination with DOPE or immunostimulatory molecule of trehalose 6,6-dibehenate (TDB) were prepared and investigated *in vitro* and *in vivo*. Results showed that although DDA:TDB is not able to transfect the cells efficiently *in vitro*, this formulation induces stronger immunity compared to DDA:DOPE due to the immunostimulatory effects of TDB.

This study demonstrated, while the presence of electrolytes did not improve immune responses, small unilamellar vesicle (SUV) liposomes induced stronger humoural immune responses compared to dehydration rehydration vesicle (DRV) liposomes. Moreover, lipid composition was shown to play a key role in *in vitro* and *in vivo* behaviour of the formulations, as saturated cationic lipids provided stronger immune responses compared to unsaturated lipids. Finally, heterologous prime/boost immunisation promoted significantly stronger immune responses compared to homologous vaccination of DNA vaccines, however, a single immunisation of subunit vaccine provoked comparable levels of immune response to the heterologous regimen, suggesting more immune efficiency for subunit vaccines compared to DNA vaccines.

Keywords: lipoplex, gene delivery, transfection, depot effect, heterologous regimen

Acknowledgment

Writing this thesis has certainly been a long journey, but it would not have been possible were it not for the help and support of a rather special group of people.

First and foremost I would like to express my gratitude and appreciation to my supervisor, Prof Yvonne Perrie for providing me with the opportunity to study in her laboratory and her expert guidance and support throughout the length of this study. My thanks also extended to my associate supervisors Dr Qingou Zheng and Dr Afzal Mohammed.

I would also thank all my friends and fellow colleagues in the drug delivery group at Aston University for their support and Brian and Wayne from biomedical facility for their help during this project. I would like to appreciate Dr Sarah McNeil's guides for *in vitro* studies. I would like to thank Aston University for providing international student bursary.

Last but no means least, I would like to thank my family and friends who have been always there for me and have supported me specially Dr Shirin Ghaderi. My special thanks to my parents who know how much I am grateful to them for their endless love, encouragement and support and I hope that by completing my PhD they will know that all of those supports have paid off well. They have always been supportive of my decisions and pushed me to pursue my dreams and ambitions in life. Thank you Farizeh and Behnam.

"In truth, knowledge is a veritable treasure for man, and a source of glory, of bounty, of joy, of exaltation, of cheer and gladness unto him." – Baha'u'llah

List of publications

Publication from this and related work:

Ali MH, Moghaddam B, Kirby DJ, Mohammed AR, Perrie Y. "*The Role of Lipid Geometry in Designing Liposomes for the Solubilisation of Poorly Water Soluble Drugs*". 2012, Int J Pharm., *In Press*.

Moghaddam B, McNeil S, Zheng Q, Mohammed AR, Perrie Y. "*Exploring the Correlation Between Lipid Packaging in Lipoplexes and Their Transfection Efficacy*". 2011, Pharmaceutics, 3(4). 848-864.

Moghaddam B, Ali MH, Wilkhu J, Kirby DJ, Mohammed AR, Zheng Q, Perrie Y. "*The Application of Monolayer Studies in the Understanding of Liposomal Formulations*". 2011, Int J Pharm., 417(1-2):235-44.

Kaur R, Chen J, Dawoodji A, Cerundolo V, Garcia-Diaz Y, Wojno J, Cox L, Gurdyal, Moghaddam B, Perrie Y. "*Preparation, Characterisation and Entrapment of a Non-glycosidic Threitol Ceramide into Liposomes for Presentation to Invariant Natural Killer T cells*". 2011, J Pharm Sci, 100 (7), 2724-33.

Related abstracts for poster and podium presentations

Moghaddam, B., Zheng, Q., Perrie, Y., (2012), "*Exploring the Effect of Liposomal Characteristics on DNA Vaccine Biodistribution.*" AAPS Meeting, Chicago, Illinois, USA.

Moghaddam, B., Zheng, Q., Perrie, Y., (2012), "*The Role of Liposomal Formulation in DNA Vaccine Biodistribution.*" Academy of Pharmaceutical Sciences UK-PharmSci Conference, Nottingham, UK.

Moghaddam, B., Zheng, Q., Perrie, Y., (2012), "*Investigating the Depot-formation of Cationic Liposome-based DNA Vaccines.*" 39th CRS Meeting, Quebec City, Canada.

Moghaddam, B., Zheng, Q., Mohammed, AR., Perrie, Y., (2012), "*A Novel Prime-Boost Immunisation Strategy Against HBV, Using Cationic Liposomes as Delivery System for pDNA and Protein*" Modern Vaccines Adjuvants & Delivery Systems (MVADS), Copenhagen, Denmark.

Moghaddam, B., Zheng, Q., Perrie, Y., (2012), "*Studying the In vitro Transfection Efficiency of Different DNA Lipoplexes*" UKICRS Symposium, Birmingham, Aston University.

Moghaddam, B., Zheng, Q., Perrie, Y., (2012), "*Investigating the Effect of Characteristic Parameters on the Biodistribution of DNA Lipoplexes*" European Symposium on Controlled Drug Delivery, Egmond aan Zee, Netherlands.

Moghaddam, B., Zheng, Q., Perrie, Y., (2011), "*Probing the Effect of Characteristic Parameters on Lipoplexes In vitro Transfection Efficiency.*" 5th International Liposome Society (ILS) Meeting, London, UK.

Moghaddam, B., Zheng, Q., Perrie, Y., (2011), "*Monolayer Studies - a Way to Translate Molecular Properties of Lipid Monolayers into Liposomal Systems*". APS UK-PharmSci, Nottingham, UK.

Moghaddam, B., Zheng, Q., Perrie, Y., (2011), "*Langmuir Study on Liposome Building Blocks.*" 38th CRS meeting, National Harbor, Maryland, USA.

Moghaddam, B., Zheng, Q., Perrie, Y., (2011), "*Langmuir Study on Monolayer Lipids of Cationic Liposomes.*" 17th UKI-CRS Symposium, Queen's University, Belfast, UK.

Moghaddam, B., Kirby, D.J., Zheng, Q. and Perrie, Y., (2010), "*DNA Lipoplex Formulation: Effect of Salt on Characteristic Properties*", 8thGPEN meeting, University of North Carolina, Chapel Hill, NC, USA.

Moghaddam, B., Zheng, Q. and Perrie, Y., (2010), "*DNA Lipoplex Formulation: Effect of Salt on Characteristic Properties.*" J Pharm Pharmacol 62:(10) p.1263, APS UK-PharmSci-Nottingham, UK.

Moghaddam, B., Zheng, Q. and Perrie, Y., (2010), "*Formulation of Lipoplexes: Interaction of Lipid Geometry*." 37th Annual Meeting and Exposition of the Controlled Release Society, Portland, Oregan , USA.

Moghaddam,B., Zheng, Q. and Perrie, Y., (2010), "*Lipoplex Formulation: Effect of Lipid Transition Temperature.*" 16th UKICRS Symposium, Hertfordshire, UK.

Moghaddam, B., Zheng, Q. and Perrie, Y., (2009), "*The Effect of Salt on the Physico-Chemical Properties of Lipoplexes."* 4TH International Liposome Society (ILS) Meeting, Lonon, UK.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	analysis of variance
APC	antigen presenting cell
BALB	Bagg albino
BCA	bicinchoninic acid
BCG	bacillus calmette-guerin
CAF01	cationic adjuvant formulation 01
CCR7	C-C chemokine receptor 7
CD	cluster of differentiation
CO_2	Carbon dioxide
Con A	concanavalin A
CPM	count per minute
CPP	critical packaging parameter
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DC-Chol	3-[N-(N',N'-(dimethylaminoethane) carbamoyl]-cholesterol
DDA	dimethyldioctadecylammonium
dH_2O	distilled water
DMEM	delbecco's modified eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DNase I	deoxyribonuclease I
DODA	didodecyldimethylammoinuim bromide
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOPG	1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)

DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	1,2-di-O-octadecenyl-3-trimethylammonium propane
DRV	dehydration-rehydration vesicles
DSC	differential scanning calorimetry
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine
DSPG	1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
DSTAP	1,2-stearoyl-3-trimethylammonium-propane
DT	diphtheria toxoid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
FDA	food and drug administration
GFP	green fluorescent protein
HBV	hepatitis B virus
Hib	*Haemophilus influenza* type b
HIV	human immunodeficiency virus
HPV	human papillomavirus
HSCIC	the health and social care information centre
HSV	herpes simplex virus
i.m.	intramuscular
i.n.	intranasal
i.p.	intrapretoneal
i.v.	intravenous
IFN-γ	interferon gamma
Ig	immunoglobulin

IL	interleukin
ISPP	immunisation safety priority project
LSC	liquid scintillation counter
LUV	large unilamellar vesicles
MALT	mucosa-associated lymphoid tissues
MHC	major histocompatibility complex
MLV	multilamellar vesicles
MMR	measles, mumps, and rubella combination vaccine
MVV	multivesicular vesicles
MyD	myeloid differentiation marker
OLV	oligolamellar vesicles
p.i.	post injection
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffer saline
pDNA	plasmid DNA
pg	picogram
PI	polydispersity index
PLGA	poly(lactide-co-glycolide)
PLN	popliteal lymph node
PRR	pattern recognition receptor
PSG	penicillin/streptomyocin/L-Glutamine
RBF	round bottom flask
s.c.	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
siRNA	small (short) interfering ribonucleic acid

SOI	site of injection
SUV	small unilamellar vesicles
TB	tuberculosis
TBE	tris borate EDTA
T_c	transition temperature
TDB	trehalose dibehenate
TDM	trehalose dimycolate
Th	T helper cell
TLR	toll-like receptor
TNF-α	tumour necrosis factor-alpha
UV	ultra violet
WHO	world health organisation

Chapter 1:
General Introduction

1.1. Vaccines

The World Health Organisation have noted that the two public health interventions that have the greatest impact on the World's health are clean water and vaccines (Andre et al., 2008). The aim of a vaccine is to exploit the natural defence mechanisms of a body's immune system to promote long term immunological protection against establishment of an infection. Vaccines can be prepared in a range of formats, including live attenuated forms of a virus (e.g. rubella or measles vaccine) or live attenuated bacteria (e.g. BCG vaccine). Alternatively, inactivated preparations of the virus or bacteria can be used (e.g. influenza vaccine). A third class of vaccines includes those prepared from extracts of pathogens or detoxified exotoxins (e.g. Tenanus toxoid vaccine).

1.1.1. Historic evolution of vaccines

The impact of vaccination is most clearly demonstrated in the eradication of Smallpox, thanks to the development of a vaccine based on the early work of Edward Jenner (Jenner, 1798). The earliest evidence of smallpox has been found on the mummified body of Pharaoh Ramses V of Egypt, who died over 3000 years ago (Hopkins, 1980; Li et al., 2007). Medical writings from ancient India (1500 B.C.) and China (1122 B.C.) (Li et al., 2007) show the presence of smallpox in that region. Rhazes, a Persian physician, was the first to publish a written account attempting to distinguish the measles from smallpox in "fi al-Judari wa al-Hasbah" ("A Treatise on the Smallpox and Measles") in 9th century A.D. (Cohen, 2008; Otri et al., 2008; Rhazes, 910). Smallpox was described as an acute infectious disease with a mortality rate of at least 30 % (Stewart and Devlin, 2006). Recovery was seen to be accompanied with long-life immunity against the disease (Li et al., 2007). It has been reported that a procedure called inoculation (also known as variolation) was possibly first performed by Asians to prevent smallpox. This technique involved nasal administration of dried powders of smallpox scabs or

scratching the recovered materials from smallpox lesions into the skin of a non-infected patient (Hilleman, 2000; Maurer et al., 2003). This method decreased the rate of smallpox infection, but still was a threat, as the virus could become active despite being in these dried formats and cause smallpox infection (Stewart and Devlin, 2006).

Inoculation was introduced to UK in 1721, and its successful treatment led to an extensive acceptance across the Europe and USA (Riedel, 2005; Stewart and Devlin, 2006). This method was introduced to Edward Jenner who was born in Gloucestershire, England in 1749, as he was inoculated in his childhood (Stewart and Devlin, 2006). As a practitioner, he used the same method for his patients. He was also aware of the fact that milkmaids who caught cowpox from their cows were protected against smallpox. Therefore, in 1796, he inoculated his gardener's son James Phipps with cowpox from a local milkmaid and afterwards exposed James to smallpox. Successfully, James showed immunity against smallpox (Hilleman, 2000; Jenner, 1798; Riedel, 2005) and this was start of vaccinology and immunology. The term vaccine was derived from use of the term "cowpox" (Latin "variolæ vaccinæ", adapted from the Latin "vaccïne-us, from "vacca" cow).

It took 80 years for another vaccine to be discovered. In 1885 Louis Pasteur introduced rabies vaccine, which was the first live attenuated vaccine (Nicolle, 1961; Pearce, 2002). Moreover, the nineteenth century had other fundamental events regarding immunology, such as proving of the germ theory of disease, commencing of the sciences of microbiology and immunology, discovery of large numbers of bacteria due to the great work of Koch and his colleagues, and by the end of the century viruses were discovered as a new class of microbes (Artenstein, 2009; Hilleman, 1998, 1999, 2000; Plotkin, 2005). The understanding and knowledge of such systems continued to the 20th century,

with the discovery of more vaccines such as toxoid, cholera, typhoid, tuberculosis, plague, yellow fever, influenza, polio and many more bacterial and viral vaccines (Hilleman, 2000; Norrby, 2007). Besides discovery of the vaccines, understanding of the role of antibodies in immune responses, concepts of humoural and cellular immune systems and different vaccinations have increased (Hilleman, 2000; Plotkin, 2005).

However, despite huge improvements in vaccinology in the last century, there remains the urgent need for new vaccines to be developed for both existing uncontrolled diseases (e.g. HIV, TB and malaria) and new diseases like tumour associated diseases. To achieve this goal, large amounts of research is in progress across the globe and World Health Organisation (WHO) have encouraged governments to invest more in vaccine technology (Taylor et al., 2009).

1.1.2. Overview of immunological concepts

The immune system is responsible for protecting the host from pathogens, including external microbes and viruses or autoimmune syndrome and mutation. To do so, the immune system, which is composed of biological structures and processes, should be activated at the right time and respond effectively (Schijns, 2000). To achieve this, the immune system is divided into innate and adaptive (acquired) systems, although there are many crossover reactions between the two systems.

1.1.2.1. Innate immune system

The innate immune system is described as the first line of immune defence against the pathogen. The response of the innate immune system is non-specific and rapid, and its duration of action is short, therefore the host will not be protected (immunised) against future infection with the same pathogen (Medzhitov, 2007; Storni et al., 2005). In

addition, failure of this system can lead to the development of autoimmune diseases (Rifkin et al., 2005). This response includes mechanisms such as fever, mucosal secretions, chemical mediators and phagocytic cells. The innate immune system senses the pathogen through receptors called pattern recognition receptors (PRRs). The targets of PRRs are named pathogen-associated molecular patterns (PAMPs) (Hashimoto et al., 1988), particularly when there are bacterial or fungal PAMPS. For viral pathogens instead, the main target of PRRs are viral nucleic acids, because all viral components are developed within host cells. In the presence of pathogens, the macrophages and dendritic cells (DCs) are responsible for eliminate of the intruders by phagocytosis. This is followed by antigen presentation, which triggers the adaptive immune system. The pathogen or antigen uptake has three main pathways: initially, antigen presenting cells (APCs) submerge the microorganisms, then phagocytes recognise the pathogen surface for the PRRs and finally phagocytic cells take up the soluble substances by macropinocytosis (Sallusto et al., 1995).

There are different classes of PRRs and the best characterised class is called Toll-like receptors (TLRs). They are known to evoke inflammatory and antimicrobial responses (Medzhitov, 2007) and their stimulation results the production of important mediators of innate immunity such as IL-6, IL-12, IL-18 and IFN-α and IFN-γ. It is also reported that TLRs signal through two main intracellular pathways, including MyD88-dependant (TLRs 2,4,5 and 9) and MyD88-independent (TLRs 3 and 4). It is shown that signalling via both pathways has a synergistic impact on their ability to produce pro-inflammatory cytokines in mice (Kawai et al., 1999; Milicic et al., 2012). Based on their cellular localisation, TLRs can be divided into plasma membrane (TLRs 1, 2, 4, 5 and 6) and intracellular counterparts (TLRs 3, 7, 8 and 9) (Milicic et al., 2012; O'Hagan and De Gregorio, 2009).

1.1.2.2. Adaptive immune system

In contrast to the innate immune system, the adaptive (acquired) immune system is specific with long-term effect, however, it does not respond quickly. This system is mediated by two types of antigen receptors, called T-cell and B-cell receptors. Two types of lymphocytes that express antigen receptors are conventional and innate-like lymphocytes. Conventional lymphocytes include conventional T cells and B cells. Innate like lymphocytes on the other hand consist of B1 cells, marginal zone B cells, natural-killer T cells and subsets of γδ T cells. Being taken up by APCs, antigens are delivered to the lymph nodes or spleen, so they will be recognised by conventional lymphocytes, which circulate through the lymph nodes (Bendelac et al., 2001; Medzhitov, 2007).

Conventional T cells have two types: T-helper (Th) cells and cytotoxic T cells. The former cells are marked by the co-receptor $CD4^+$ on the cell surface and the latter ones express $CD8^+$. In fact, recognition of the antigen in association with major histocompatibility complex (MHC) II molecules can lead to activation of $CD4^+$ T cells and differentiation to Th1 and Th2 (Pierre et al., 1997; Seder and Hill, 2000). $CD8^+$ cells, in contrast are activated by MHC I molecules. A group of TLRs, which is located in intracellular counterparts (TLRs 3, 7, 8 and 9) can enhance antigen presentation rate by DCs and through the MHC I pathway. This process results in the increase of $CD8^+$ T cell responses (Edwards et al., 2002; Schubert et al., 2000; Seder and Hill, 2000).

Conventional B cells can recognise any antigen by binding to the epitopes (a specific three-dimensional molecular determinant). However, the innate-like B cells (B1 cells) emerge in peripheral cavities and produce IgM antibodies against bacterial pathogens. More differentiation of B cells to naïve B cells leads to expression of IgD. Naïve B cells

circulate in lymph nodes, spleen and mucosa-associated lymphoid tissues (MALT) to encounter possible antigens (Bendelac et al., 2001; Medzhitov, 2007; Storni et al., 2005). When a microbial antigen such as lipids, glycolipids and formylpeptides are presented by non-classical MHC molecules, innate-like T cells are responsible for their recognition and this would be enough to signal the presence of infection (Janeway et al., 1988).

1.1.2.3. Innate control of adaptive immune responses

It was discussed that some PRRs such as TLRs are responsible for the induction of adaptive immune responses by specialised signals for conventional lymphocytes. Therefore, an association between the antigens recognised by lymphocytes and the PAMPs recognised by TLRs is the basic principle of innate control of adaptive immunity (Janeway, 1989).

This process for T cells is initiated by DCs recognising the pathogen. The DCs then take up the pathogen by phagocytosis and antigenic peptides of pathogens are presented by MHC I or II molecules at the DC cell surface (Blander and Medzhitov, 2006). DCs also become activated by TLRs, so they produce cytokines and express cell-surface signals. DCs migrate to lymph nodes and present the antigen and cytokines to the T cells, which leads to the activation of T cells and differentiation of them to Th1 and Th2 by interference of $CD4^+$ (Banchereau and Steinman, 1998; Seder and Hill, 2000). For B cells, co-engagement of B cell receptor and a TLR can establish a direct association between the antigen and a PAMP (Medzhitov, 2007).

T cells can differentiate into different subsets, including Th1, Th2 and Th17 cells (Reinhardt et al., 2006). Th1 cells produce IFN-γ and also induce B cells to produce antibodies of the IgG2 subclass (Medzhitov, 2007; Seder and Hill, 2000). Th1 cells

represent a class of immune responses called cell mediated or cellular immune responses. Th2 responses can produce IL-4, IL-5 and IL-13. IL-4 is also involved in production of antibodies of the IgE subclass from B cells (Nelms et al., 1999; Stetson et al., 2004). Th2 cells represent humoural immune responses. Th17 cells produce IL-17, which induces haematopoietic cell types and is involved in protection against bacteria and fungi (LeibundGut-Landmann et al., 2007; Weaver et al., 2007). Differentiation of T cells is mostly due to the production of cytokines by antigen presenting cells in response to the TLR activation, so the whole process is controlled and initiated by the innate immune system. It has been reported that TLR activity induces IL-12 production; this causes differentiation of Th1 cells from Th cells. In addition, IL-6 can induce a TLR, as part of a differentiation process of Th cells to Th17. A similar route is predictable for Th2 cell generation (Medzhitov, 2007; Seder and Hill, 2000; Stetson et al., 2004). Since MHC class II molecules are responsible for $CD4^+$ T cells activity, $CD8^+$ T cells are induced by MHC class I molecules. $CD8^+$ T cells are responsible for production of cytokines such as IFN-γ and tumour necrosis factor (TNF-α) (Seder and Hill, 2000). Interestingly, it has been shown that innate immunity induces the activity of adaptive immunity and the latter has impact on performance of the former (Banchereau and Steinman, 1998; Medzhitov, 2007). TLRs from the innate immune system activate the adaptive immunity and induce production of T cells, thus T cells produce appropriate cytokine which activates a particular route of the innate immune system. For example, Th1 and Th2 cells activate macrophages and eosinophils, respectively (Reinhardt et al., 2006).

1.1.2.4. Immunisation

The main purpose of vaccination is to protect the patient from the recurrence of the infection in future exposures of the host cells and the pathogens. This has been called

immunisation and is provided through immunological memory, which is the specific consequence of the adaptive immune system. Immunological memory is the ability of the immune system to respond more effectively to pathogens that have been exposed before, and using memory B cells and T cells, it induces a strong response against the pathogen (Storni et al., 2005). When the immune system successfully removes the antigens from the system, it enters the phase of memory development (Zinkernagel, 2002).

Differentiation of naïve B lymphocytes into the memory B lymphocytes is triggered by $CD4^+$ Th cells through CD40 ligation. This process starts at the final phase of primary immune response and takes place in germinal centres, where the naïve B cells experience clonal expansion, somatic hypermutation, affinity maturation and possibly isotype switch during the adaptive immune response. B cells that survive and did not differentiate to plasma cells, change to memory B cells (Arpin et al., 1997; Gray et al., 1994a; Gray et al., 1994b).

The mechanism of generation of memory T cells has not been completely understood, although, it has been described that upon exposure of the antigen to naïve T cells in lymph node and spleen, T cells become activated and divided. T cell expansion proceeds over the course of a week and leads to a remarkable increase in the number of antigen reactive T cells and, after reaching to the optimum level, this falls back to a level which is notably higher than initial levels (100 to 1000 fold higher). T cells are now called memory T cells and have two subsets: central memory T cells and effector memory T cells (Sallusto et al., 1999). Central memory T cells express CCR7 for recirculation through secondary lymphoid tissues such as splenic T zone, lymph nodes and peyer's patch (Moser, 2003). These central memory T cells are long-lived in the

absence of antigen and are able to face systemic pathogenic infections. In contrast, effector memory T cells are dependant of antigen presentation and they are short-lived (Opferman et al., 1999). This process is stimulated by production of cytokines and interaction of accessory molecules on the APC (Garside et al., 1998; McCullough and Summerfield, 2005; Sallusto et al., 1999).

In summary, upon encounter of antigen to the host cell, the innate immune system reacts to the antigen and triggers the adaptive immune system. This activates different signalling cascades leading to activation of effector T cells and finally removal of the antigens. In the last phase of defensive reactions against antigen, memory B and T cells will be generated and circulate in the lymphatic system to protect the body from secondary infection. At this stage, the body is immunised from the infection by microorganism and this type of immunity is called active immunity. Vaccination technology can provide this immunity using killed or attenuated live microorganisms, or its subunits thereof, to trigger the immune system and provide long time immunity. A key consideration on all this is that upon secondary and subsequent responses of immune system, responses are only mediated by memory cells and not by naïve cells, so the ability of the vaccine to promote memory responses should be at the centre of attention in designing a vaccine.

1.1.3. Types of vaccines

As mentioned traditional vaccines are categorised into three main groups: live attenuated vaccines, inactivated vaccines and subunit vaccines. However, there are more types of vaccines such as peptide vaccines and DNA vaccines.

Attenuated vaccines are produced via the induction of physical or chemical changes to the microorganism so that its pathogenic effect weakens and, as a result, will be safe for the vaccine recipient (Perrie, 2006). These microorganisms are able to infect target cells but the infection is mild and the replication of the microorganisms is limited. These vaccine are able to stimulate both humoural and cell mediated immune responses; however, because of the risk of reversion to a more pathogenic state, this type of vaccines cannot be considered for use in immunosuppressed patients (Chambers et al., 2004; Perrie, 2006). Some important vaccines of this group are polio, Bacillus Calmette-Guerin (BCG), measles, mumps, rubella combination vaccine (MMR) and influenza virus vaccine (Arvin and Greenberg, 2006; Harper et al., 2003; Nichol et al., 1999; Wareing and Tannock, 2001).

Inactivated or killed vaccines contain microorganisms, which have been inactivated by heat or chemicals and are unable to replicate or produce toxins, so while these vaccines are not infectious they can retain their immunogenicity (Perrie, 2006). The main advantage of this group of vaccines is their safety. However, these vaccines are less effective than live attenuated vaccines and they fail to produce cellular immune responses (Black et al., 2010; Mackett and Williamson, 1995). Examples of vaccines from this group are cholera, hepatitis A and polio vaccines (Fiore et al., 2006; Perrie, 2006).

Subunit vaccines consist of small parts of the organism such as cellular extracts, parts of cells, a surface antigens that are highly purified and reproducible (Perrie, 2006). They induce strong immune responses and are not able to revert to the infectious format. Important developed vaccines of this group are influenza and Hepatitis B virus vaccines (Mischler and Metcalfe, 2002; Perrie et al., 2008). DNA recombinant technology

facilitated the production of subunit vaccines. For instance, hepatitis B vaccine is now manufactured using biotechnology as it cloned in yeast; however, in the past it used to be purified from the blood of hepatitis B carrier patients (Edlich et al., 2003; Perrie, 2006). Although subunit vaccines have less adverse effects compared to live attenuated and inactivated vaccines, they are less immunogenic and require adjuvants to promote the immune response to the antigen. In addition, since the duration of immune response initiated by subunit vaccines is short, several boosts are needed to achieve the protection (Bramwell and Perrie, 2005b; Perrie, 2006; Perrie et al., 2008).

There are some microorganisms that produce toxins to cause the disease. Inactivated forms of toxins are called toxoids, which can also be used in vaccines. The most common toxoids are tetanus and diphtheria vaccines. Whilst used as a vaccine in their own right, toxoids can also be used to increase immunogenicity of the other vaccines; for example *Haemophilus influenza* type b (Hib) vaccine contains polysaccharide unit from the bacterium conjugated to diphtheria or tetanus toxoid (Perrie, 2006).

1.1.4. Challenges in using traditional vaccines

Since their invention, vaccines have been among the most attractive and successful ways of prevention of infectious disease and cancers. They have eradicated many pathogens all over the world such as smallpox, tetanus, diphtheria and measles (Gregoriadis, 1998; Perrie et al., 2007). However, there are still some untreated viruses such as human immune deficiency virus (HIV), herpes simplex virus (HSV), and influenza virus or recurred diseases such as tuberculosis (TB), which traditional vaccines have yet to eradicate. (Andersen, 2007; Fairman et al., 2009; Hong et al., 2010; Tirabassi et al., 2011; Toda et al., 1997). Furthermore, there is a large amount of research currently in progress on discovering vaccines to have a therapeutic or prophylactic effect on cancers

or autoimmune diseases, which need a new generation of vaccines. So far, only two prophylactic cancer vaccines have been approved by FDA which are hepapitis B (HB) vaccine and Gardsil™ that prevent the infection with HBV and human papillomavirus (HPV) respectively (Giarelli, 2007). These viruses are believed to be the leading causes of liver cancer and cervical cancer respectively (Hamdy et al., 2011). Cancer vaccine-based immunotherapy is shown as a novel therapeutic strategy for cancer treatment. A recent study (Wang et al., 2012a) demonstrated that use of TLR 3 agonists as an adjuvant in combination with cationic liposomes such as DOTAP could enhance vaccine-induced tumour-specific cytotoxic T lymphocyte (CTL) response and IFN-γ production. Particulate vaccine delivery systems have been shown to be effective for therapeutic cancer vaccination, as they are capable of stimulating $CD8^+$ T cell immunity, which enhances the activation of cytotoxic lymphocytes that can kill cancer cells (Foged et al., 2012).

Concerns about safety of vaccines and their adverse effects give some challenges for traditional vaccines. This becomes a more problematic issue in a developed environment where disease incidence is much less than a developing or undeveloped society. For instance, concerns over the safety of the MMR vaccine has led to a 10% reduction in vaccine uptake across the UK and 20% just in London (Fitzpatrick, 2004; Perrie, 2006). Whilst uptake is now improving, as the HSCIC's NHS Immunisation Statistics England, 2011-12 has reported, MMR vaccine uptake is still below the required 95% as it has reached to 91.2% in 2011-12 (HSCIC, 2012). Most of the licensed vaccines, whether live attenuated or inactivated, have shown rare but serious adverse effects. These adverse responses include headache, nausea, encephalitis, neurological reactions and even death (Bramwell and Perrie, 2005a; Huang et al., 2004). Based on this, and as a part of Immunisation Safety Priority Project (ISPP) launched in 1999 by

WHO (Duclos and Hofmann, 2001), the main focus of vaccine research is in the development of a new generation of vaccines using highly purified proteins or synthetic peptides. However, to have a highly potent and immunogenic vaccine, these antigens should be delivered along with adjuvants (Chen et al., 2002; Kenney and Edelman, 2003; Moser et al., 2003).

Given the fact that licenced adjuvants such as Alum and squalene-oil-water emulsion (MF59) are not suitable for the new generation of vaccines as they only generate humoural immune responses (Holten-Andersen et al., 2004), suggests there remains a need to develop new adjuvant systems or delivery vehicles which are focused on subunit and DNA vaccines (Gregoriadis, 1998; Henriksen-Lacey et al., 2011c; Perrie et al., 2007; Perrie et al., 2008).

1.1.5. DNA vaccines

The concept of DNA vaccination is based on the fundamental experiment by Wolff et al; (1990), which showed that intramuscular inoculation of plasmid DNA (pDNA) resulted in expression of its encoded protein. They showed upon direct injection of antigen-encoding plasmid DNA and its uptake by cells, the DNA can enter into the nucleus and transfect the cell. The expressed antigens trigger the immune system of the host, similar to the way that foreign antigens do, so DNA injection can induce protective humoural and cell mediated immunity (Gregoriadis, 1998; Tang et al., 1992; Ulmer et al., 1993).

DNA vaccines have several advantages over conventional immunisation. 'Naked' DNA vaccines are inexpensive and easy to produce, due to one step cloning of target coding sequence into plasmid vectors; they are also more temperature stable than live vaccines

(Chen and Huang, 2005; Gregoriadis et al., 2002; Li et al., 2012). In addition, expression of the antigens inside the live cell gives the advantage of presenting the antigens in the same condition as in a viral infection, but without being infectious; so antigenic structure includes all the important post-translational modifications that are the same as the native protein. Furthermore, DNA vaccines are able to stimulate Th1 and Th2 $CD4^+$ and $CD8^+$ T cells as well as B cells and induce both humoural and cellular immune responses, and were reported to achieve this without use of conventional adjuvants (Li et al., 2012; Liu, 2011; Wahren, 1996). Indeed, several studies in 1990s showed the promising immunogenic characteristics of naked DNA tested on a variety of different animal models of infectious disease, including rabies (Lodmell et al., 1998), influenza virus (Ulmer et al., 1993), malaria, (Becker et al., 1998; Sedegah et al., 1994), HIV (Donnelly et al., 1997), tuberculosis (Tascon et al., 1996) and herpes simplex virus (Manickan et al., 1995).

Following the encouraging results of DNA vaccines, these formulations have been tested in several human clinical trials (Levine, 2010; Liu, 2011; Liu and Ulmer, 2005). Overall results showed an acceptable safety but disappointingly low immune response for the DNA only vaccines. However, this technology had a successful profile in animal vaccination, with four licenced DNA vaccines (Liu, 2011). The latest was Oncept™ (Merial, Lyon, France) approved in 2010, which was an immunotherapeutic DNA vaccine as a therapy for malignant melanoma in dogs (Li et al., 2012; Liu, 2011).

Despite poor human clinical trial results, considering the successful development of DNA vaccines for veterinary use and advantages of this type of vaccine over traditional vaccines has led to the development of new strategies for vaccination, such as

prime/boost vaccination and enhancing the delivery method of plasmid DNA using different kinds of vectors such as viral, bacterial and liposomal systems.

1.1.6. Delivery of nucleic acids

Although it has been shown that direct injection of naked DNA allows transgene expression in muscle (Vassaux et al., 2006), in most cases naked DNA molecules are not able to enter cells efficiently due to their large size, negative charge and the nuclease mediated degradation in systemic blood stream (Al-Dosari and Gao, 2009). Therefore, a delivery vehicle (vector) is needed to carry the gene into the target cell to save the gene from the above dangers. There are two classic categories for gene vectors: biological and non-biological systems. As shown in Table 1.1, biological systems or viral vectors are viruses or bacteria, which mediate gene transfer (El-Aneed, 2004; Vassaux et al., 2006). Non-viral delivery systems include physical and chemical methods (Al-Dosari and Gao, 2009; Mae et al., 2009) (Table 1.1).

Viral vectors provide a high efficiency, although, there remains concerns about inducing toxicity and immunogenic reactions. In fact, there has been a fatality reported by adenoviral vector gene therapy (Marshall, 1999). Therefore, non-viral vectors are under more attention to reach the ideal vector (Whitehead et al., 2009), which should have stability, high efficiency, minimal toxicity, and unrestricted size limitation for nucleotide acid and easy preparation with up -scaling capacity and low cost (Lul and Haung, 2003).

Particulate delivery systems are categorised among non-viral gene delivery systems and are limited to those delivery systems that use non-living components for the purpose of carrier systems (Bramwell and Perrie, 2005a). Particulate delivery systems have been

used for delivery of protein and peptide and DNA and mainly for vaccination purposes. Several particulate delivery systems have been introduced for DNA delivery, such as gold nanoparticles (Fynan et al., 1993), polymers such as poly(lactide-co-glycolide) PLGA (Jones et al., 1997) and chitosan (Mumper et al., 1995) and liposomes (Allison and Gregoriadis, 1974).

Table 1.1. Different gene transfer systems (Al-Dosari and Gao, 2009; El-Aneed, 2004; Galanis et al., 2001; Mae et al., 2009; Vassaux et al., 2006).

Biological Gene Transfer Systems	**Bacterial Vectors**	*Salmonella, E.Coli, Shigella, Yersinia, Listeria.*
	Viral Vectors	*Retrovirus, Adenovirus, Adeno-associated Virus, Herpes simplex Virus, Epstein-Barr Virus, Poxivirus, Newcastle Disease Virus,*
Non-biological Gene Transfer Systems	**Physical Methods**	*Needle/Jet Injection, Hydrodynamic Gene Transfer, Gene Gun, Electroporation, Sonoporation.*
	Chemical Methods	*Cationic Lipids, Cationic Peptides, Cationic Polymers, Cell Penetrating Peptides, Inorganic Nanoparticles*

Liposomes were first identified as being effective immunological adjuvants for diphtheria toxoid (DT) by Alison and Gregoriadis (1974) and, since then, liposomes have attracted extensive attention as adjuvant systems for DNA and subunit protein vaccines (Gregoriadis, 1998). Adjuvants are substances which are able to enhance a specific immune response and increase humoural and cell mediated immunity to the antigen compared to the free antigen (O'Hagan et al., 2001). Opposite to other adjuvants, liposomes are found to be safer with no local or systemic toxicity. Liposomes also do not produce allergic reactions or any other side effects (Gregoriadis et al., 1999).

1.2. Liposomes

Liposomes were first described by Bangham et al., 1965 while studying cell membranes. They are vesicular structures consisting of hydrated bilayers, which form when lipids (generally phospholipids) are dispersed in water (Bangham et al., 1965). In 1970s Gregoriadis (1974) proposed a new drug delivery system using liposomes. Subsequently, Felgner et al. (1987) demonstrated the use of cationic liposomes to promote gene expression *in vitro*. Since these early studies, numerous investigations have been performed in this area, with, for example, Karmali and Chaudhuri (2007) showing DNA mediates high levels of transgene expression *in vivo* after incorporation into cationic liposomal systems. A key component of these systems is the cationic lipid and several studies have been performed on the effect of cationic lipids on nucleic acid delivery, and many achievements have been obtained, such as inn the use of DNA vaccines (Perrie et al., 2002), improving in *vivo* lung transfection efficacy (Majeti et al., 2004), hepatocyte-selective gene transfection by using galactosylated cationic liposomes (Fumoto et al., 2004), and development of folate-conjugated cationic lipid-based transfection complexes which cause *in vivo* transgene expression in mice tumours (Hofland et al., 2002).

1.2.1. Liposome morphology

Lipids are amphiphilic and have a polar head and a non-polar fatty acid chain. They form a closed structure in aqueous media, called liposomes. The polar heads, when exposed to the water, form a single layer of lipid, with the non-polar parts form, in the second layer of the bilayer structure shielded have the water, that will form liposomes. Because of their structure, liposomes can carry both hydrophilic and lipophilic drug molecules. Hydrophilic drugs can be localised in the aqueous phase and lipophilic and amphiphilic drugs can be included in the lipid bilayers (Muller-Goymann, 2004).

Liposomes can be classified by their structural properties. The main types are listed and their characteristics are outlined in the Table 1.2 followed by a schematic representation in Figure 1.1.

Table 1.2: Size and number of lipid bilayers of different categories of liposomes. (Rongen et al., 1997)

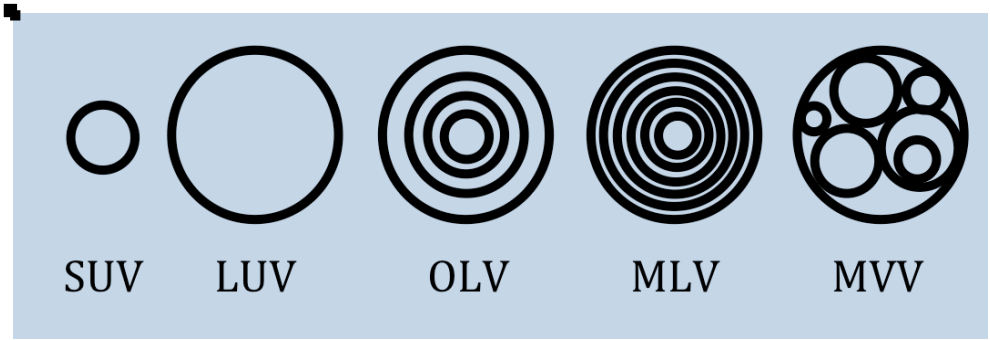

Figure 1.1: Schematic representations of five types of liposomes.

1.2.2. Liposome preparation methods

There are many different methods for the preparation of liposomes, depending on the type of vesicle formation (Kirby and Gregoriadis, 1984; Lasch et al., 2003; Rongen et al., 1997).

1.2.2.1. Hydration method

In this method, a mixture of lipids are dissolved in an organic solvent such as methanol or chloroform. The solvent is then removed by rotary evaporation at reduced pressure. The dried film of lipids, which has been deposited onto the wall of a round-bottom flask, is hydrated and shaken to give a milky suspension of equilibrated MLV (Kirby and Gregoriadis, 1984; Lasch et al., 2003) (Figure1.2).

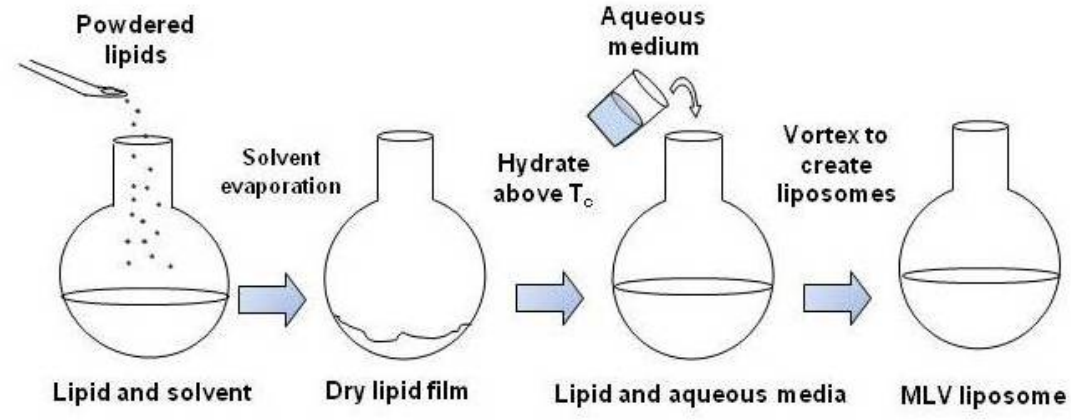

Figure1.2: Schematic representation of hydration method of liposome preparation

1.2.2.2. Ultrasonication method

In the ultrasonication method, the aqueous lipid dispersion, which has been prepared with hydration method, will be sonicated. Sonication can be performed by bath sonication or probe sonication. The resulting liposomes will be SUV. It should be considered that in the case of probe sonication, the vial of the sample must be put in ice or a water bath to avoid localised overheating (Lasch et al., 2003). However, in the case of low T_c lipids, liposome suspensions are more effectively reduced in size when they are sonicated at temperatures above T_c.

1.2.2.3. Dehydration- rehydration method

This method begins with SUV mixed with the drug to be entrapped, after which they are freeze-dried. Then, the vesicles are rehydrated under controlled conditions and larger vesicles are formed, which are DRV and it gives a high encapsulation efficiency of the drug (Kirby and Gregoriadis, 1984) (Figure 1.3).

Figure 1.3: Schematic representation of the dehydration-rehydration method. In brief, MLV prepared according to the lipid hydration method outlined above was sonicated to produce SUV. These SUV were mixed with the antigen, frozen at -70 °C and freeze-dried overnight. Controlled rehydration of the dried powder led to the formation of antigen containing DRV vesicles.

1.2.2.4. Reverse-phase evaporation method

The procedure starts by adding the drug to the mixture of dissolved lipid in an organic solvent. Then an emulsion will be gained by vortexing. Rotary evaporating and removing the organic solvent results in formation of a gel and by shaking of the gel or continuing the rotary evaporator the gel will change to a dispersion of large liposomes which are reverse-phase evaporation vesicles. These liposomes may be LUV or MLV in morphologies, depending on the water and organic phase ratio (Lasch et al., 2003; Rongen et al., 1997).

1.2.2.5. Ether evaporation method

Ether vaporisation or solvent injection method is another method of liposome preparation. In this method, after dissolving lipids in organic solvent, the resulting mixture is injected slowly into an aqueous phase. By this method, large unilamellar vesicles will be produced with a high entrapment efficiency (Rongen et al., 1997).

1.2.2.6. Freeze-thaw extrusion method

This method is based on repeated cycles of quick freezing of liposome dispersion in liquid nitrogen and thawing in warm water. The liposome dispersion is first formed by the film method. The final vesicles are MVV or LUV with a high entrapment ratio (Rongen et al., 1997).

1.2.2.7. Detergent removal method

One of the most important methods for detergent depletion is detergent dialyses method. In this method, an aqueous solution of drug is added to the mixture of lipids in an organic solvent. After forming the film and removal of the organic solvent, a detergent is added to solubilise the lipids and this will form a lipid-detergent micelle. Subsequent removal of the detergent by dialyses will cause formation of liposomes (Rongen et al., 1997). There are other methods of detergent removal instead of dialyses such as dilution, gel filtration and adsorption (Lasch et al., 2003).

1.2.3. Cationic liposomal systems

Cationic lipids were first introduced for gene therapy by Flegner and his colleagues in 1987, they used N-(1-[2,3-dioleyloxy]propyl)-N,N,N-trimethylammonium chloride (DOTMA) to deliver DNA and RNA into animals and human cell lines (Felgner et al., 1987).

1.2.3.1. Cationic lipid structure

Cationic lipids are amphiphilic molecules, which are positively charged and have a cationic polar head group (e.g. an amine) and a hydrophobic domain (generally alkyl chains or cholesterol). A linker connects the polar head group to the non-polar tail. There are several cationic lipids that have been used in gene delivery, such as DOTAP, DC-Cholesterol, and DSTAP (Lonez et al., 2008). Each of the three domains of a cationic lipid plays a role in the quality of gene delivery by affecting the cellular toxicity, transfection efficiency and stability of the lipoplex.

1.2.3.2. DNA liposome complex- lipoplex

A lipoplex is a complex of a cationic liposome and a polynucleotide like DNA. The positive charge of the amine head group of the cationic lipid and negative charge of phosphate in nucleic acid cause an interaction between them, thus forming the lipoplex (Figure 1.4). At this stage, the cationic lipid wrapped around the nucleic acid and the lipoplex will be in its highest stable condition, as all potential interaction sites are blocked and the tendency of adjacent complexes to further undergo extensive lipid mixing has largely ceased (Wasungu and Hoekstra, 2006). The size of the lipoplex is thought to be in a range of 80-400 nm; however, this varies and can depend upon cationic lipid/DNA ratio, type of lipids used and method of preparation.

1.2.3.3. Mechanism of delivery to the cells and transfection

The aim of liposomal mediated gene transfer is to have the DNA or other polynucleotides inside the cell where it should be translated to a protein or peptide.

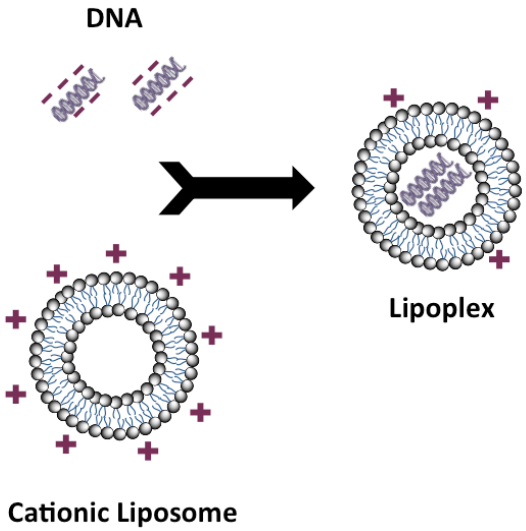

Figure 1.4: Schematic representation of lipoplex formation.

The lipoplex surface has a positive charge and these charges will make an electrostatic interaction with negative charges of cell surfaces. This is the first step of the transfection process after making the lipoplex. Due to their cationic nature, incubation of lipoplexes with cultured cells promotes internalisation of the lipoplexes through the vesicular pathway and then the DNA can be released in the cytoplasm. Some fractions of released DNA will be trafficked to the cell nucleus, followed by transcription and translation of it to the protein (Figure 1.5). Internalisation pathways of lipoplexes are mainly three endocytotic routes: 1) clathrin-mediated endocytosis, 2) caveolae-mediated endocytosis, 3) macropinocytosis. Of these, the most common pathway for lipoplexes is clathrin-mediated endocytosis, as caveolae is mostly for polyplexes and macropinocytosis is still a potential entry, which is poorly characterised (Uyechi-O'Brien and Szoka, 2003; Wasungu and Hoekstra, 2006). A crucial point in transfection is DNA escape from the endosome, because as much as it stays there, the chance of plasmid degradation increases. The ability of lipoplexes to move into hexagonal phase is helpful here. In

contrast, adenovirus, which is a well-known vector for gene delivery, solves this problem by lysis of the endosomal membrane structure. Lipoplexes do not have such an enzyme, but some interactions occur between the inverted hexagonal layer of the lipoplex and the endosomal layer of the cell membrane. In fact, there will be a competition between the DNA and the negatively charged endosomal membrane lipids to interact with the cationic lipids of lipoplexes, thus the DNA will release to the cytosol (Xu and Szoka, 1996). DNA entrance to the nucleus relates to the size of DNA and also the time of transfection. Some studies show if transfection occurs in S or G_2 expression will be 30-500 fold more than G_1 (Lul and Haung, 2003; McNeil and Perrie, 2006).

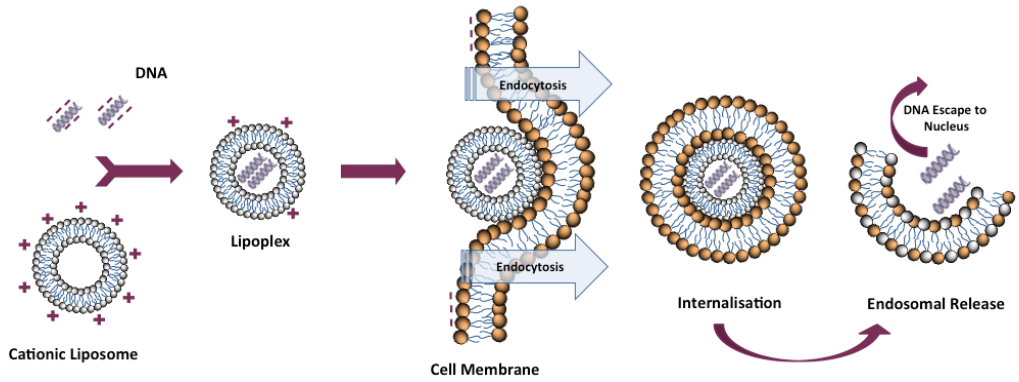

Figure 1.5: Proposed mechanisms of cationic lipoplex condensation and uptake. In brief, cationic liposomes are attracted by electrostatic interactions to the negative charges of DNA forming a lipoplex. Lipoplex binding to the cell surface followed by internalisation and then release of DNA from the lipoplex. DNA enters the nucleus and in the nucleus, RNA will be transcribed.

1.2.3.4. Effect of helper lipid on transfection

The theory of using helper or co-lipid comes from the origin research of Flegner et al, in 1987. They demonstrated that the transfection activity of DOTMA when formulated with dioleoylphosphatidylethanolamine (DOPE) is more than when it is formulated with dioleoylphosphatidylcholine (DOPC). The reason is the effect of DOPE to promote the transition from lamellar phase to an inverted hexagonal phase (Uyechi-

O'Brien and Szoka, 2003). The ability to achieve this is related to the structural attributes of the lipids used in lipoplexes. Cationic lipids are amphiphilic molecules and geometry of amphihile is an important property, which is related to the lipid application as a vector. When cationic lipids suspend in an aqueous phase, they can make different structural phases, such as micellar, lamellar and inverted hexagonal phases (Figure 1.6).

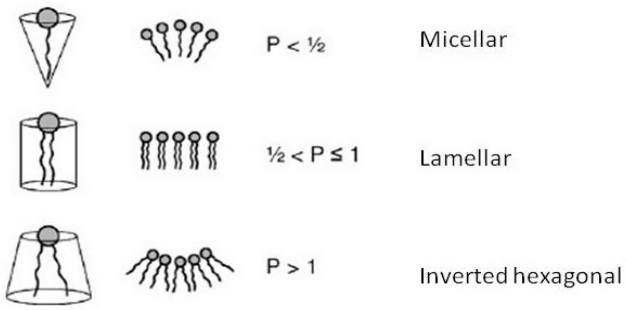

Figure 1.6: Schematic representation of the phase structure of cationic lipids as a function of packing parameter (Adapted from Wasungu & Hoekstra, 2006).

A factor known as the critical packing parameter (P) can determine which structure a particular cationic lipid will make. A critical packing parameter $P= v/al_c$ is defined as the ratio of the hydrocarbon volume (v) and the product of effective head-group area (a) and the critical length of the lipid tail (l_c). In fact, (P) describes the ratio of the area occupied by the hydrophobic region versus that of the hydrophilic region. Therefore, when P>1 the area occupied by the hydrophobic chain is larger than that of hydrophilic head groups and the lipid will adopt the inverted hexagonal (H_{II}) phase (Figure 1.6). This structural phase is a bilayer destabilising structure and it is essential for liposomal gene delivery to the cytosol (Israelachvili and Mitchell, 1975).

Figure 1.7: Cartoons of cationic lipids and helper lipids shown as a micelle, single composition and mixed composition liposomes, and in an inverted hexagonal phase. Proposed lipid mixing model of fusion of cationic liposome with a target, i.e., endosomal, membrane (Uyechi-O'Brien and Szoka, 2003).

Helper lipids such as DOPE and cholesterol also promote conversion of the lamellar lipoplex phase into a non-lamellar structure. They are fusogenic and have strong destabilising effect towards bilayer lipids. These properties are the cause of transfection efficiency improvement by helper lipids, Figure 1.7 (Ciani et al., 2004). However, whilst this helper effect of DOPE has been shown to be effective *in vitro, in vivo* studies shows cholesterol is better than DOPE due to enhanced stability and transfection (Lul and Haung, 2003; McNeil and Perrie, 2006).

1.2.3.5. Transition temperature

The phase transition temperature is described as required temperature to change the lipid physical state from gel phase to the liquid phase. In the gel phase, the hydrocarbon chains are fully extended and closely packed. However, in the liquid phase the hydrocarbon chains are randomly oriented and fluid. Hydrocarbon length, unsaturation, charge and head-group species are main factors that affect the phase transition temperature (Cevc, 1991; Tristram-Nagle and Nagle, 2004).

Van der Waals forces are the most important bonds between the hydrocarbon chains and they are responsible for the in ordered state of hydrocarbon chains. As the hydrocarbon length is increased, van der Waals interactions become stronger, requiring more energy to disorder the ordered packing and, consequently, the phase transition temperature increases (Cevc, 1991). To form stable liposomes, the formulation should be prepared above the transition temperature (T_c) of the lipid.

1.2.4. Langmuir monolayer studies

A common idiom, 'pouring oil on troubled water', is a figurative way of suggesting that attempts are made to calm a contentious or problematic situation. This relates to the fact that a thin layer of oil can calm choppy water, a technique referred to as wave damping, where sailors poured oil onto the sea to prevent waves being formed. This method was first described by Aristotle and Plinius (Fulford, 1968). To achieve this effect, very little oil is required; it need only be a surface coating of 1 molecule thick, that is to say a monolayer. Marangoni effects are the basis of this wave damping effect produced by oil. The Marangoni effect is a phenomenon whereby movement of a liquid occurs due to local differences in the surface tension of the liquid (Kuroda et al., 2000). Sudden local increases in surface area lead to enhanced surface tension, resulting in a surface tension

gradient which, in turn, promotes contraction of that area and thus, further surface area growth is prohibited.

Basically, the Marangoni flow opposes the flow associated with the wave action. Benjamin Franklin, having seen this phenomenon of wave calming behind ships on which the cooks used sea water to rinse the fat off dishes, undertook to scientifically investigate this further (Lyklema, 2000). On a lake near Clapham Common in London, Franklin noted that one teaspoon of oil was enough to calm several hundred square meters of the lake's surface, with the wind having a much reduced effect on treated areas of the water surface compared to the untreated parts (Franklin et al., 1774). Later, John Shields carried out large-scale wave-damping experiments in Scotland and lodged a patent based on this in 1879 (Lyklema, 2000). Lord Rayleigh also followed this research area, and he noted that water surface tension could be lowered by contamination and oil films. Although he had no method of exactly measuring the thickness of the films, he estimated them to be monomolecular, with a thickness of 1–2 nm, and noted that by using such films, information on the size of molecules was obtainable (long before the existence of molecules was generally accepted) (Lyklema, 2000; Rayleigh, 1890, 1899). However, the first surface pressure versus area measurements (as they are now referred to) were reported by Agnes Pockels (Pockels, 1891) a German Scientist, who made a basic surface balance in her kitchen. Using this system, she was able to determine surface contamination as a function of the surface area for different oils, and further observed that by compressing monolayers below a certain area, the surface tension falls rapidly. Pockels methods were further developed by Irvine Langmuir with his film balance system, a method that still bears his name.

One of the advantages the Langmuir trough offered was that a direct measurement of the film pressure could be derived from the deflection of a movable float, separating the film from clean water. Using this trough, Langmuir studied monolayer lipids more systematically and confirmed that the films are monomolecular. He also showed that the molecules in these monolayers are orientated on the aqueous surface, with the hydrophilic portion of the surfactants in contact with the liquid, whilst the hydrophobic region of the surfactants is pointing up towards the air (Langmuir, 1917; Langmuir, 1920).

1.2.4.1. Information gained from monolayer studies

From Figure 1.8, various phases of the monolayer are shown: as the concentration of the molecules at the surface is increased (i.e. the distance between the barriers is contracted) the monolayer changes from a very dilute 'gaseous' monolayer (G), where the molecules are far apart and there is low interfacial pressure, to a 'liquid' state. Often there are two liquid states: a 'liquid expanded' (LE) and a 'liquid condensed' (LC) monolayer, which are determined by the proximity and orientation of the surfactants (Figure 1.8B). Only a small reduction in the area is required to move the monolayer from the LC to the 'solid' (S) state. In this state, all the amphiphilic molecules are closely packed and the hydrophobic tails are aligned in parallel, with the area per molecule corresponding with the S-phase which is equal to the closed-packed molecular cross-sectional area (Lyklema, 2000). Further compression of the monolayer results in the monolayer collapsing (molecules breaking out of the monolayer, by forming micelles, or multilayers in the case of phospholipids, for example) which leads to a sharp break in the isotherm (Figure 1.8B).

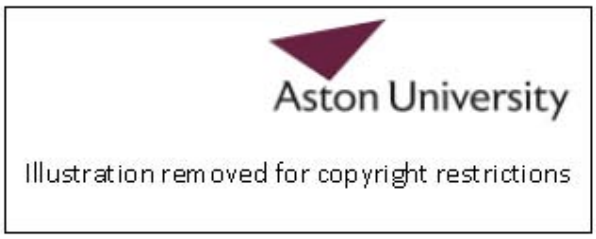

Figure. 1.8: (A) Schematic example of isotherm, Π-A exhibiting a variety of phases which can occur. G: gaseous, LE: liquid expanded, LC: liquid condensed, and S: solid. Curves like this are typical for lipid monolayers. (B) Schematic representation of lipid packaging at the monolayer interface. Figure modified from (Lyklema, 2000).

As is shown in Figure 1.8A, the transition between these phases is not always distinct and often more than one phase may be present. There is also a question as to the orientation of the surfactant tails in the gaseous phase: are they orientated flat on the interface (as in Figure 1.8Bi), or are their tails out into the nonaqueous phase (Figure 1.8Bii and iii). It is thought that situation (ii) maybe entropically more favourable (Lyklema, 2000), but the overall molecular shape of the molecules in question would need to be considered and often option (iii) is used schematically to represent the general concept.

1.2.4.2. The use of monolayer studies in liposome research–example applications

As extensively reported, liposomes are bilayer vesicles, first described by (Bangham et al., 1965) whilst studying cell membranes. They are vesicular structures consisting of hydrated bilayers, which form when phospholipids are dispersed in water (Bangham et al., 1965). Whilst there are very many reported variations on the theme (e.g. niosomes, virosomes, bilosomes, etc.), all have the same basic bilayer construction. Since their first description as a possible drug delivery system (Gregoriadis and Ryman, 1971), these constructs have been extensively investigated and there are now several liposome-based products clinically used. Given their structure, it is not surprising that monolayer studies of phospholipids and other surfactants are undertaken and the findings extrapolated to liposomal bilayers. Indeed, such studies can be highly informative, giving insights into areas such as bilayer lipid packaging configuration (Ali et al., 2010; Dynarowicz-Latka and Hac-Wydro, 2004), drug–lipid interactions (Ali et al., 2010) and liposome stability (Christensen et al., 2008; Demel et al., 1998; Lambruschini et al., 2000). However, since they are effectively half a membrane, monolayer studies are less suited to study certain aspects, such as trans-membrane processes, and consideration to this overall difference in morphology should always be borne in mind.

1.3. Liposomal vaccines

As mentioned earlier, liposomes are being used as vaccine adjuvants due to their immunogenic properties and their capability to induce both cellular and humoural immunity (Gregoriadis et al., 1999). It has been reported that the immunogenicity of liposomes originates from the presentation of the liposomes to antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs) (Killion and Fidler, 1998; Rao and Alving, 2000). In addition, association of liposomes and antigen leads antigen to gain access to both MHC class I and II pathways in APCs (Rao and Alving, 2000; Zhou

and Huang, 1994a), therefore liposomal antigens can stimulate humoural and cellular immune systems.

1.3.1. Role of cationic liposomes in enhancement of immunity

In contrast to neutral or anionic liposomes, cationic liposomes can interact effectively with negatively charged molecules such as DNA, siRNA, proteins and peptides based on electrostatic interactions between the liposome cationic charge and anionic charge of the molecules (Bramwell and Perrie, 2005a; Felgner et al., 1987; Gregoriadis, 1998; Henriksen-Lacey et al., 2010c; Malone et al., 1989; McNeil and Perrie, 2006; Perrie et al., 2001; Perrie et al., 2008).

It has been shown that adjuvant activity of cationic liposomes has resulted from the enhanced protection of the antigen, the ability to form a depot at the site of injection and efficient uptake by cells (Gregoriadis, 1994; Henriksen-Lacey et al., 2010b). Furthermore, the positive surface charge of the cationic lipids enhances the uptake of the liposome and entrapped antigen uptake by APCs and their presentation to the responder cells (Gregoriadis et al., 2002; Korsholm et al., 2007). In addition, cationic liposomes with the help of fusogenic lipids, such as DOPE and cholesterol, can destabilise the endosomes allowing the delivery of antigens into the cytoplasm of the APCs and promoting $CD8^+$ CTL responses (Burger and Verkleij, 1990; McNeil and Perrie, 2006; Zhou and Huang, 1994a). It is also been reported that cationic liposomes have an impact on activation of cell signalling pathways through activation of MAP kinases, leading to the expression of co-stimulatory molecules (CD80 and CD86) and chemokines in dendritic cells (Iwaoka et al., 2006; Ouali et al., 2007; Tanaka et al., 2008; Yan et al., 2007). Therefore, cationic liposomes have been investigated

extensively as an adjuvant for subunit antigens (Davidsen et al., 2005; Vangala et al., 2006) or DNA vaccines (McNeil and Perrie, 2006).

1.3.2. Subunit liposomal vaccines

Subunit vaccines include highly purified peptides or protein antigens and are being investigated extensively due to their safety, reproducibility and low manufacturing cost compared with the conventional live attenuated or killed vaccines (Wilson-Welder et al., 2009). It has been shown that liposomes are able to protect small peptide/protein antigens from enzymatic breakdown by host cells (Gregoriadis, 1994; Gregoriadis et al., 1999) and co-delivery of the subunit antigen and liposomes enhances the immunity (Christensen et al., 2012). It is shown that cationic lipids such as DDA or DC-Chol can be used as adjuvants for subunit protein vaccines (Andersen, 1994). For instance, DC-Chol has been shown to be able to overcome the non-responsiveness to hepatitis B vaccine (Brunel et al., 1999) and induce high levels of antibody and cell factors. Cationic lipids have also been extensively investigated as adjuvant for tuberculosis-unit vaccines (Holten-Andersen et al., 2004), with a combination of DDA and monophosphoryl lipid A promoting elevated immune responses (Brandt et al., 2000; Holten-Andersen et al., 2004).

In addition, incorporation of trehalose 6,6'-dibehenate (TDB), which is a synthetic analogue of trehalose 6,6'-dimycolate (TDM) an immunostimulatory component of the mycobacteria cell wall (Olds et al., 1980; Pimm et al., 1979), with DDA, has shown an effective adjuvant activity, which enhances high level of immunity, compared to the DDA liposome formulation alone (Davidsen et al., 2005). To evaluate the potential of DDA:TDB formulations and fulfil the requirements for the clinical studies, several studies were performed on this formulation to investigate the impacts of vesicle size

(Henriksen-Lacey et al., 2011b), surface charge (Henriksen-Lacey et al., 2010c), depot formation (Henriksen-Lacey et al., 2010b), and pegylation of DDA:TDB (Kaur et al., 2012a, b) on its immune response. Furthermore, DDA immunogenicity was compared with other cationic liposomes such as DOTAP and DC-Chol (Henriksen-Lacey et al., 2011a). Also monolayer properties (Christensen et al., 2008) and morphological characteristics (Davidsen et al., 2005; Vangala et al., 2006) of DDA:TDB were studied. Recently, phase I clinical studies on this system in combination with Ag85B-ESAT-6 antigen called CAF01, have been completed (ClinicalTrials.gov, 2012). The effectiveness of DDA:TDB is not only due to the immunogenic effects of DDA (Gall, 1966) but is also owed to the effect of immunostimulant TDB. The mechanism of action of TDB is not fully understood, yet, can be related to the fact that TDB is a synthetic analogue of TDM that is part of *Mycobacterium tuberculosis* cell wall and has antigenic effect, therefore can activate the innate immune system. Given the high immunogenicity as TB vaccine, DDA:TDB was studied as an adjuvant for other antigens such as HBsAg, an antigen related to hepatitis B (Vangala et al., 2007) and the study showed high immune responses, suggesting further evaluation should be performed on its clinical potential. Moreover, CAF01 has been studyied in other disease models such as malaria, chlamydia and influenza (Agger et al., 2008; Christensen et al., 2010; Vangala et al., 2006). Multi parameter flow cytometry has determined the stage of memory and effector T cell differentiation based on the expression of selected cytokine combinations (Seder et al., 2008). Upon immunisation with DDA:TDB incorporated with Ag85B-ESAT-6, central memory (IFN-γ^+ TNF-α^+ IL-2^+ and TNF-α^+ IL-2^+) and effector memory (IFN-γ^- TNF-α^+) cells, which are essential for the development of long term immunity, became activated (Christensen et al., 2010; Kamath et al., 2009). The liposomal system induced high level of IFN-γ and IgG2 production (Davidsen et al., 2005; Henriksen-Lacey et al., 2010b; Henriksen-Lacey et al., 2010c), showing the effect

of CAF01 on enhancement of the Th-1 immune response. Although DDA does not act via TLRs, TDB activates NF-κB through syk-CARD9/Bcl10/Malt-1 intracellular pathway. This results in up-regulation of co-stimulatory molecules, including CD40 and CD86 on DCs (Kamath et al., 2009; Schoenen et al., 2010; Werninghaus et al., 2009). It is known that incorporation of other immunostimulatory molecules or adjuvants can change the mechanism of action of liposomal subunit vaccines (Henriksen-Lacey et al., 2011c; Nordly et al., 2011).

In summary, there are MHC I and MHC II molecules which are on the surface of APCs and injected antigen can interact with them to induce immunity (Figure 1.9). Upon interaction of the antigen, the MHC molecules will be transported via cytosol to be expressed on the surface of APC, thereafter the complex of MHC-antigen will interact with T cell receptors (Madigan et al., 2000). It is believed that the type of MHC molecules involved is related to the source of protein antigen. In the case of subunit protein antigens, these antigens cannot be presented by MHC I molecules because they cannot reach to the cytosol of the target cell. Therefore, MHC II molecules present the subunit antigens to the Th1 pathway by $CD4^+$ T cells, leading to induction of humoural immune responses (Spack and Sorgi, 2001). This explains why free subunit antigens are not able to induce CTL immune response, which is activated by MHC class I antigen presentation. Hence, liposomal systems can induce the MHC I antigen presentation, resulting in release of Th1 cytokines, which induces a strong cellular immune response for the subunit protein vaccines (Rao and Alving, 2000; Spack and Sorgi, 2001).

1.3.3. DNA liposomal vaccines

Cationic liposomes, which are able to protect the plasmid DNA (Gregoriadis et al., 1996), have been shown to be able to enhance the uptake of DNA vaccines by the APCs

(Gregoriadis, 1990; Gregoriadis et al., 1997). Two types of DNA liposomal vaccines that have shown more interest are DRV and SUV vesicles. For DRV systems, DNA is entrapped within the aqueous compartments between bilayers (Perrie et al., 2001), whereas SUV form lipoplexes.

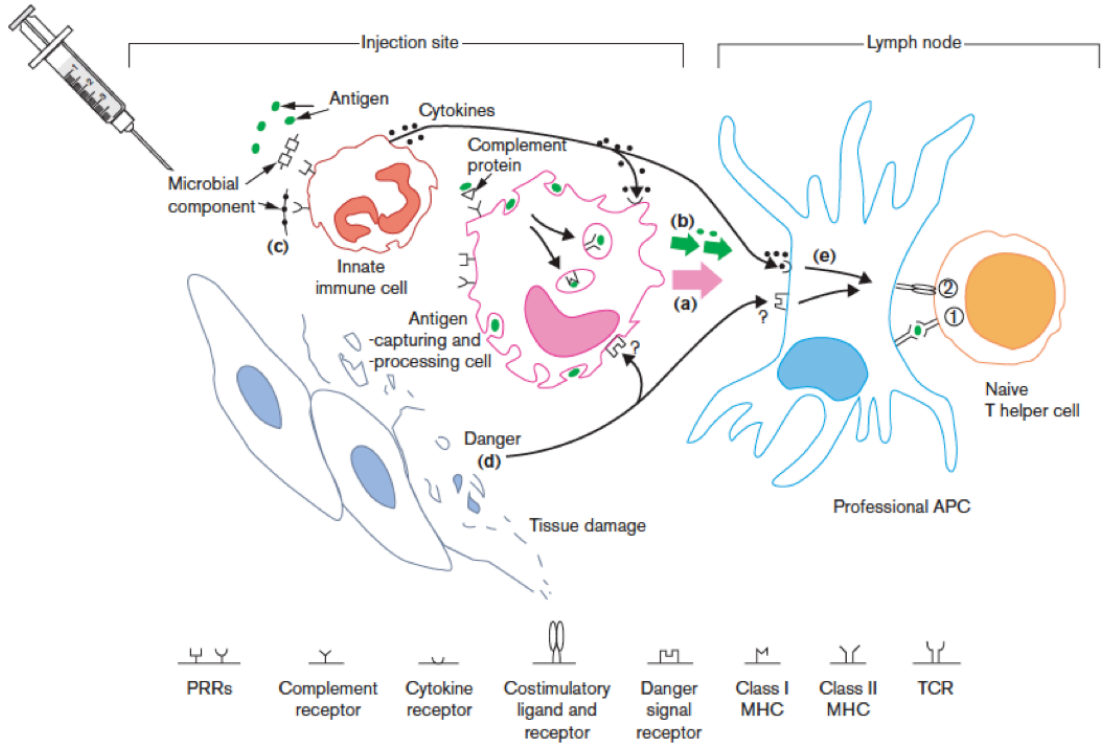

Figure 1.9: Presentation of the essential steps of different concepts of adjuvant activity. **(a)** Facilitation of antigen transport, uptake and presentation by antigen-capturing and processing cells in the lymph node draining the vaccine injection site. **(b)** Repeated or prolonged release of antigen to lymphoid tissues (depot effect). **(c)** Signalling of release of PRRs activates innate immune cells to release cytokines necessary for upregulation of costimulatory molecules. **(d)** Danger signals from stressed or damaged tissues alert the antigen-presenting cells to upregulate costimulatory molecules. **(e)** Signalling by recombinant cytokines or costimulatory molecules mimics classical adjuvant activity. Adapted from (Schijns, 2000)

It has been reported (Gregoriadis, 1990) that local injection of liposomal DNA vaccines leads to APCs infiltrating the injection site or the lymph nodes and uptake of the plasmid DNA. Consecutive studies have been performed using plasmid DNA of (pRc/CMV HBS) which encodes the S region of the hepatitis B surface antigen (HBsAg) (Gregoriadis et al., 1997; Perrie et al., 2001). Results showed a remarkably higher

antibody response when the animal (BALB/C mice) immunised with liposomal DNA vaccines (e.g., DOTAP or DC-Chol) rather than naked DNA. Cytokine production levels for IFN-γ and IL-4 showed the same trend and using dehydration rehydration method to prepare the liposomes led to an increase in the immune responses for DRV liposomes compared to MLV liposomal vaccines. These results revealed the capability of liposomal DNA vaccines to enhance both humoural and cellular immune responses (Gregoriadis et al., 1997). These studies are in agreement with the notion that liposomes facilitate the uptake of liposomal DNA vaccines by APCs in the lymphoid tissues. In addition, expression of transgene-encoded protein (green fluorescent protein (GFP)) was also reported by these studies. Oral administration of liposomal DNA vaccines studied by Perrie et al (Perrie et al., 2002) shows that DSPC DRV liposomes containing DNA induce immune responses via the oral route. Another study showed DOTAP-based formulations produce longer-lived immunity compared to DC-Chol (Perrie et al., 2003). DNA lipoplexes (SUV liposomes/DNA complexes) may use different strategies, such as the use of surface ligand for cell targeting. Some studies used mannosylated cholesterol derivative to target macrophage mannose receptors and showed higher *in vitro* transfection and *in vivo* gene expression compared to DC-Chol (Kawakami et al., 2000). Using RGD peptides (Anwer et al., 2004) and use of biodegradable PLA (Bramwell et al., 2002) in combination with lipoplexes are other approaches of DNA delivery using SUV liposomes, which caused enhanced production of Th1 and Th2 responses. Different mechanisms (Figure 1.10) have been proposed to explain the immunogenicity of DNA vaccines; it was reported earlier that DNA-encoded antigens are presented by somatic cells through their MHC class I pathway to $CD8^+$ T cells (Figure 1.10a) (Davis et al., 1993). However, other studies show that myocytes are not efficient enough to produce such high immune responses via MHC class I pathways (Gregoriadis, 1998; Spier, 1996). These studies have proposed that the CTL responses induced by DNA

vaccines result from the transfer of antigenic material between the muscle cells and professional APC (Figure 1.10c) and also the plasmid is taken up directly by DCs at the local lymph nodes, which present the antigen to the $CD8^+$ T cells (Figure 1.10b).

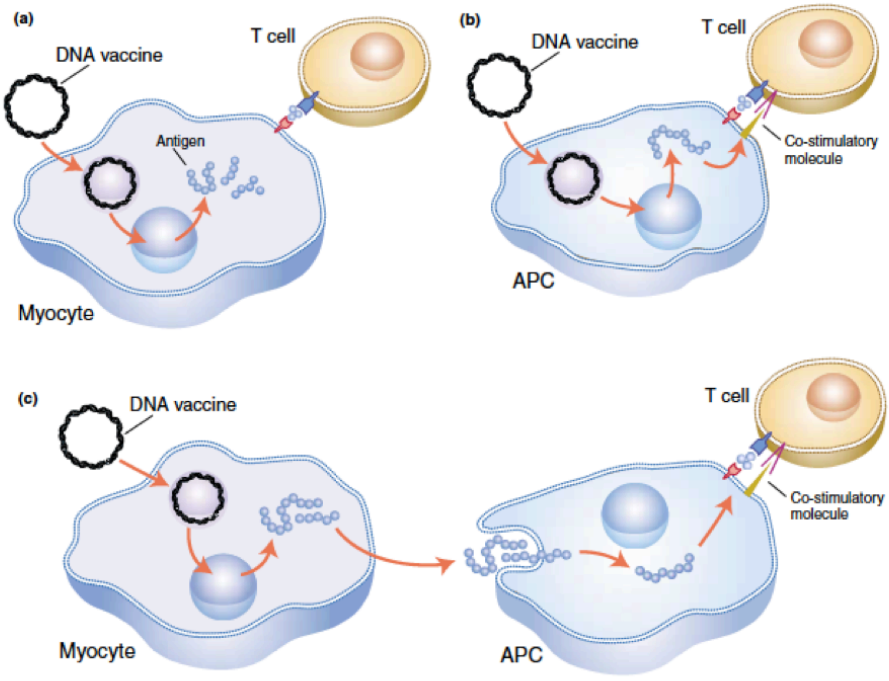

Figure 1.10: Mechanism of antigen presentation for activation of T lymphocytes (CTL) following plasmid DNA immunisation. a) DNA encoded antigens are presented by myocytes through their MHC class I pathway to $CD8^+$ T cells. b) DNA vaccination led to direct transfection of APCs. c) Cross-priming results from transfected myocytes being phagocytosed by APCs, which then present the antigen to T cells. Adopted from (Liu, 2003).

The effect of liposomes on this mechanism is to offer protection of the plasmid DNA from degradation so more DNA can transfect the cell and, as a result, higher CTL responses will be induced (Gregoriadis, 1998; Liu, 2003). Furthermore, cationic liposomes can induce innate immune response upon the immunisation and initiate the humoural immunity. Finally, applying fusogenic lipids in the structure of cationic liposomes would destabilise the cell membrane and enhance the delivery of the plasmid DNA into the cell and eventually to the nucleus (McNeil and Perrie, 2006).

1.3.4. Prime/boost DNA vaccine strategies

Prime/boost immunisation regimen has been defined as a vaccination strategy, which includes prime immunisation of plasmid DNA followed by the immunisation with the same antigen, which was encoded by the prime plasmid DNA. An early study (Schneider et al., 1998), has shown that DNA prime immunisation followed by a single protein boost of the same modified vaccina virus Ankara (MVA) antigen induced high levels of $CD8^+$ T cells, and as a result, induced complete protection in challenges. Later studies (Carstens et al., 2011; Deshmukh et al., 2007; O'Hagan et al., 2004; Tirabassi et al., 2011; Vaine et al., 2010; Wang et al., 2008; Wierzbicki et al., 2002; Yang et al., 2008) have also shown the resulting immune response to the heterologous vaccination can be significantly higher than homologous plasmid DNA or protein vaccines (eg., DNA/DNA or protein/protein). This was also tried in human studies, which have shown high immune responses of prime-boost vaccination compared to homologous DNA vaccine regimens (Lu et al., 2008). Recent studies have also investigated heterologous regimens for influenza and HIV vaccines (Churchyard et al., 2011; De Rosa et al., 2011; Koblin et al., 2011; Ledgerwood et al., 2011), which are showing promising immune responses and are currently undergoing human clinical trials.

The immunologic mechanisms behind the effectiveness of heterologous prime/boost strategies are not well understood, but it can be related to the lower antigen expression of DNA prime immunisation compared to protein vaccines, and this may preferentially prime T-helper cell responses, with the humoural response subsequently being boosted by the high dose protein boost (De Mare et al., 2008; Li et al., 2012). In fact, following the prime immunisation, the rate and efficacy of antigen recognition and immune response are increased, therefore with booster immunisation, these provided high affinity lymphocytes to be further developed in size and in the ability of epitope

recognition. This leads to an increase in the efficiency of the immune system (McCullough and Summerfield, 2005).

1.4 Aims and objectives

As described, liposomes have shown great potential as adjuvants and delivery systems for subunit antigen and plasmid DNA vaccines, through enhancing the immunogenicity of these molecules as well as effective and efficient delivery of them. It was also mentioned that different aspects such as cationic lipid composition, choice of helper lipid, vesicle size and surface charge of the liposome and method of liposome preparation impact on *in vitro* transfection, *in vivo* delivery and immunogenicity of the vaccines. Therefore, the aim of this thesis was to evaluate how differences in physicochemical characteristic properties of the liposome formulations correlate with their *in vitro* transfection efficiency, *in vivo* biodistribution and immunological function. To achieve this aim following objectives have been considered:

- To investigate a range of cationic liposome-DNA formulations with different lipid/DNA +/- charge ratios and study the physicochemical characteristics of them.
- To investigate the role of lipid structure on molecular packaging of the lipids.
- To study the effect of the presence of electrolytes and lipid composition on physicochemical properties and *in vitro* transfection of the DNA liposomal formulations.
- To evaluate the biodistribution of liposomes and their associated plasmid DNA with the aim to correlate these to their physicochemical properties and immunological function.

- To perform prime/boost immunisation experiments with selected cationic liposomal vaccines to evaluate the effect of cationic lipid composition, electrolytes and type of the liposomes on immunogenicity of the liposomal vaccines.

Chapter 2:
Materials and Methods

2.1. Materials

Material	Supplier
1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)	Avanti Polar Lipids, Alabaster, AL, USA
1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG)	Avanti Polar Lipids, Alabaster, AL, USA
1,2-dioleoyl-sn-glycero-3-phsphoethanolamine (DOPE)	Avanti Polar Lipids, Alabaster, AL, USA
1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG)	Avanti Polar Lipids, Alabaster, AL, USA
1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)	Avanti Polar Lipids, Alabaster, AL, USA
1,2-stearoyl-3-trimethylammonium-propane (DSTAP)	Avanti Polar Lipids, Alabaster, AL, USA
^{125}I (NaI in NaOH solution)	Perkin Elmer, Waltham, MA, USA
2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)	Sigma-Aldrich, Poole, Dorset, UK
^{32}P-dATP	Perkin Elmer, Waltham, MA, USA
^{3}H-Cholesterol	Perkin Elmer, Waltham, MA, USA
African green monkey kidney cells (COS-7)	European collection of cell cultures (ECACC) (Salisbury, UK).
Agarose	Sigma-Aldrich, Poole, Dorset, UK
Alhydrogel-2 %	InvivoGen San Diego, CA, USA
BALB/C mice	Charles River, Margrate, UK
Bicinchoninic acid (BCA) protein assay kit	Sigma-Aldrich, Poole, Dorset, UK
Boric acid	Biomedicals, Inc. Ohio, USA
Bromophenol blue	Sigma-Aldrich, Poole, Dorset, UK

C57BL/5Jico	Charles River, Margrate, UK
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	Promega, Madison, WI, USA
Chloroform	Fisher Scientific, Leicestershire, UK
Citric acid	Sigma-Aldrich, Poole, Dorset, UK
Concanavalin A	Sigma-Aldrich, Poole, Dorset, UK
Delbecco's Modified Eagles Medium (DMEM)	Biosera, Leicestershire, UK
Deoxyribonuclease I	Sigma-Aldrich, Poole, Dorset, UK
Dimethyldioctadecylammoniumbromide (DDA)	Avanti Polar Lipids, Alabaster, AL, USA
Distilled Water; DNase/RNase Free	Gibco, Invitrogen, Paisley, UK
DNA ladder (2-10 kb)	Promega, Madison, WI, USA
Ethanol	Fisher Scientific, Leicestershire, UK
Ethidium bromide	Sigma-Aldrich, Poole, Dorset, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Poole, Dorset, UK
Foetal bovine serum (FBS)	Biosera, Leicestershire, UK
Illustra ProbeQuant G-50 Micro Column	GE Healthcare, Amersham, UK:
Goat anti-mouse IgG, IgG1, IgG2b	AbD Serotec, Oxford, UK
gWiz™ Luciferase	Genovac GmbH, Germany
Heparin	Sigma-Aldrich, Poole, Dorset, UK
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer solution	Sigma-Aldrich, Poole, Dorset, UK
Hydrogen Peroxide	Sigma-Aldrich, Poole, Dorset, UK
Iodogen® pre-coated iodination tubes	Pierce Biotechnology, Rockford, IL, USA
L-glutamine/Penicillin-Streptomycin	Biosera, Leicestershire, UK

Lipofectin™ reagent	Invitrogen Life Technologies, UK
Loading buffer	Promega, Madison, WI, USA
luciferase assay system	Promega, Madison, WI, USA
Magnesium chloride	Sigma-Aldrich, Poole, Dorset, UK
Marvel milk	Premier Int. Foods Ltd, Lincs, UK
Methanol	Fisher Scientific, Leicestershire, UK
Mouse DuoSet capture ELISA IL-1ß, IL-2, IL-5, IL-6, IL-10, IFN-γ	R & D Systems, Abingdon, UK
Nick Translation kit	GE Healthcare, Amersham, UK:
Phosphate buffer saline tablets (PBS)	Sigma-Aldrich, Poole, Dorset, UK
PicoGreen® reagent	Invitrogen Life Technologies, UK
Plasmid DNA (pRc/CMV HBS)	Aldevron, Fargo, USA
Pontamine blue (Chicago Sky Blue 6B)	Sigma-Aldrich, Poole, Dorset, UK
Potassium chloride	Sigma-Aldrich, Poole, Dorset, UK
Potassium phosphate	Sigma-Aldrich, Poole, Dorset, UK
Protease inhibitor cocktail	Sigma-Aldrich, Poole, Dorset, UK
Reagent diluent	R & D Systems, Abingdon, UK
RPMI 1640	Biosera, Leicestershire, UK
Sephadex™ G-75	Sigma-Aldrich, Poole, Dorset, UK
Serum free and antibiotic free medium (Opti-MEM®)	Gibco, Invitrogen, Paisley, UK
Sodium bicarbonate	Sigma-Aldrich, Poole, Dorset, UK
Sodium carbonate	Sigma-Aldrich, Poole, Dorset, UK
Sodium chloride	Sigma-Aldrich, Poole, Dorset, UK
Sodium dodecyl sulphate	Sigma-Aldrich, Poole, Dorset, UK
Sodium hydroxide	Sigma-Aldrich, Poole, Dorset, UK

Sodium phosphate dibasic	Sigma-Aldrich, Poole, Dorset, UK
Solvable™	Perkin Elmer, Waltham, MA, USA
Stop solution	R & D Systems, Abingdon, UK
Sucrose	Sigma-Aldrich, Poole, Dorset, UK
Substrate solution	R & D Systems, Abingdon, UK
Synthetic hepatitis B surface antigen (HbsAg) (ayw subtype)	Aldevron, Fargo, USA
Trehalose 6,6′-dibehenate (TDB)	Avanti Polar Lipids, Alabaster, AL, USA
Triton X – 100	Sigma-Aldrich, Poole, Dorset, UK
Trizma-Base	Sigma-Aldrich, Poole, Dorset, UK
Trypan blue	Sigma-Aldrich, Poole, Dorset, UK
Trypsin/EDTA	Gibco-Invitrogen, Carlsbad, CA, USA
Tween-20	Sigma-Aldrich, Poole, Dorset, UK
Ultima Gold Scintillation Fluid	Perkin Elmer, Waltham, MA, USA

2.2. Monolayer studies

To investigate the surface pressure of monolayer lipids, Langmuir-Blodgett technique has been used. It is an automated controlled film balance apparatus (Figure 2.1) (KSV Langmuir Mini-trough, KSV Instruments Ltd., Helsinki, Finland) equipped with a platinum Wilhemy plate and placed on a vibration-free table. This instrument was used to collect the surface pressure-area isotherms. The size of the trough was 24225.0 mm^2 enclosing a total volume of about 220 mL; the subphase was filtered deionised water or PBS solution. The compounds were prepared at fixed total concentration of 1 mg/mL of lipid in chloroform. 20 μL of each solution was spread onto the air/water interface with a Hamilton micro-syringe, precise to ± 0.2 μL (Figure 2.2). The monolayer was left

for about 15 minutes to allow chloroform to evaporate. Then a constant compression with a rate of 10 mm/minute was performed on the molecules until the required surface pressure was attained. The temperature of the subphase was kept constant at 20 °C ± 1 °C by use of an external water bath circulation system.

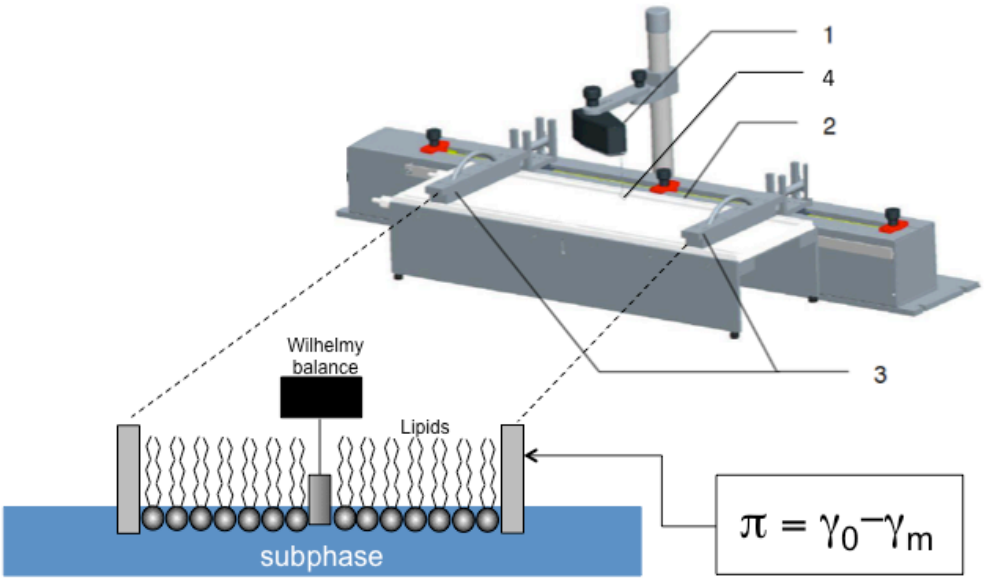

Figure 2.1: Representation of Langmuir mini trough, kindly supplied by KSV.Ltd and modified. Different parts of the instrument: 1) Balance 2) Trough filled with clean subphase e.g. water 3) Movable barriers 4) Wilhelmy Plate (Reproduced with permission from (Moghaddam et al., 2011)).

The following compounds were tested in this study in both deionised water and PBS subphases: DOPE, DOTAP, DSTAP, DSPE, DOPE:DOTAP, DOPE:DSTAP, DSPE:DOTAP, DSPE:DSTAP. DDA, DDA:TDB and DOPE:DDA were also studied in deionised water. Each experiment was compressed once and performed at least three times. To analyse the data KSV software (KSV Instruments Ltd., Helsinki, Finland) was used.

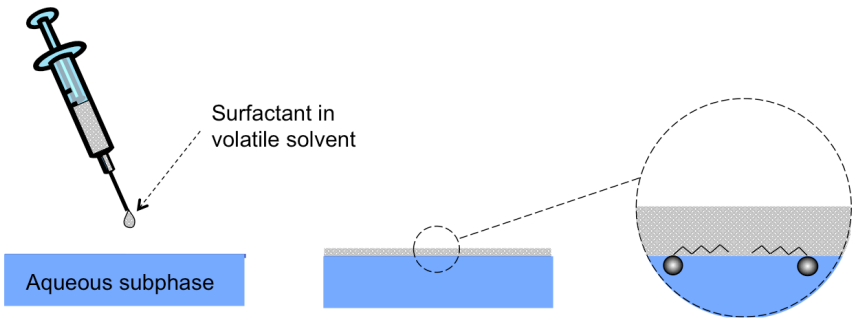

Figure 2.2: Spontaneous spreading of a liquid of surfactant molecules (adapted from (Lyklema, 2000))

2.3. Liposome preparation by lipid hydration method

2.3.1. Production of multilamellar vesicles (MLV)

All cationic liposomes were prepared by the lipid hydration method based on the work of Bangham (Bangham et al., 1965). To prepare the liposomes, the required amount of each lipid were taken and dissolved in an appropriate volume of stock solution of chloroform:methanol (9:1 v:v ratio) in a round bottom flask (RBF). The organic solvent was then evaporated and a thin film coated in RBF by using rotary evaporator. After flushing of the RBF with N_2 to ensure complete remove of solvent, 1 mL of hydration phase (either distilled water, PBS or Tris buffer) was added to the RBF followed by 2 minutes vortex and the contents kept 10 °C above transitional temperature (T_c) for 30 minutes to form multilamellar vesicles (MLV) e.g. the T_c for DOPE, and DOTAP is below room temperature but it is 47 °C for DDA, 62.9 °C for DSTAP and 75 °C for DSPE (Davidsen et al., 2005; Regelin et al., 2000).

2.3.2. Production of small unilamellar vesicles (SUV)

To produce small unilamellar vesicles (SUV), MLV were sonicated (Figure 2.3A) for approximately 2 minutes with a probe sonicator (Soniprep 150) with the power of 5 amplitude microns using a probe tip with a diameter of approximately 4 mm. Since sonication tips tend to release titanium particles into the lipid suspension this was removed by centrifugation prior to use. Details of each liposome formulation are shown in Table 2.1.

Table 2.1: Studied liposome formulations

Liposome formulation	Lipid ratio (µmol)	Hydration medium
DOPE:DOTAP	(8:8)	dH_2O/Sucrose
DOPE:DOTAP	(8:8)	PBS
DOPE:DSTAP	(8:8)	dH_2O/Sucrose
DOPE:DSTAP	(8:8)	PBS
DSPE:DOTAP	(8:8)	dH_2O
DSPE:DOTAP	(8:8)	PBS
DDA:DOPE	(8:8)	dH_2O/Sucrose
DDA:TDB	(8:1)	Tris buffer

For biodistribution and immunisation studies, to keep the isotonicity of the formulation *in vivo,* 10 % solution of sucrose was used as hydration media instead of dH_2O in formulations. Also for DDA:TDB 10 % trehalose was added to the Tris buffer to have an isotonic buffer.

2.3.3. Production of dehydration-rehydration vesicles (DRV)

To produce dehydration-rehydration vesicles (DRV) (Kirby and Gregoriadis, 1984), the empty SUV suspension was kept at -70 °C for one hour and then placed in freeze dryer over night with a shelf temperature of -20 °C. Controlled rehydration procedure was performed after complete freeze drying. Minimal amount of dH_2O added to the lipid cake (100 μL per 16 μmoles lipid) and then vortexed for a short time (less than one minute), this stage repeated until the dry lipid was redispersed. The rehydrated formulation was kept at $>T_c$ for 30 minutes to allow vesicle formation. When the suspension was diluted for further experiments, it was left to stand for 30 minutes before any experiment was carried out (Figure 2.3B).

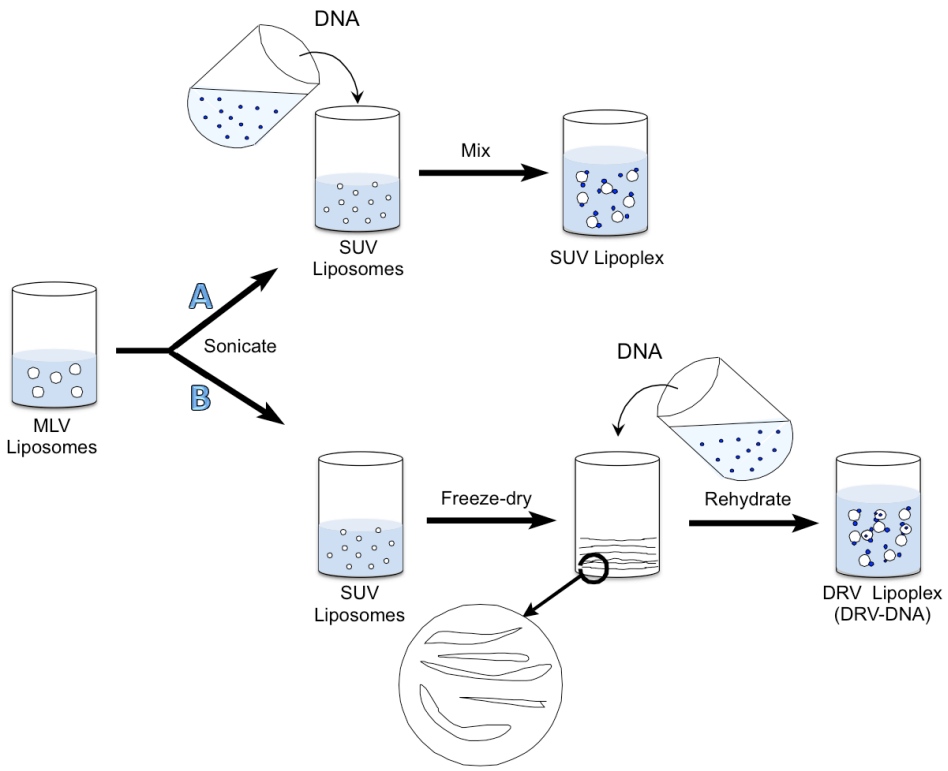

Figure 2.3: Liposomal preparations: **A)** Small unilamellar vesicles complexed with DNA (SUV-DNA), **B)**Preformed empty dehydration-rehydration vesicles complexed with DNA, (DRV-DNA). (insert illustrates flattened liposomal membrane in their dried state after freeze drying).

2.3.4. DNA lipoplex preparation

Cationic liposome-DNA complexes were prepared by incubating either empty SUV or DRV (Figure 2.3) with the required amount of gWiz Luciferase or plasmid DNA (pRc/CMV HBS) for 30 minutes. Lipid films were hydrated in either dH_2O, 10 % sucrose, PBS or for DDA:TDB in Tris buffer (10mM, pH 7.4).

2.3.5. Protein-liposome complex preparation

To produce protein-liposome complexes, empty SUV or DRV were incubated with the appropriate amount of protein for one hour at room temperature (RT).

2.4. Determination of vesicle size

The z-average diameter of lipoplexes was determined by dynamic light scattering using the photon correlation spectroscopy (PCS) technique measured on a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). Using the cuvettes supplied by Malvern, 20 to 50 µL of the sample was diluted by the hydration phase up to 1.5 mL and the vesicle size was measured at 25 °C.

2.5. Measuring Zeta potential

The zeta potential of the complexes was measured on Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK) at 25 °C in distilled water, PBS or Tris buffer as appropriate. To measure the zeta potential 100 µL of liposome suspension was diluted in 2 mL of its aqueous phase whether dH_2O or 0.001 M PBS.

2.6. Evaluating antigen association

The level of associated plasmid DNA within lipoplexes, as well as absorbed protein, associated the liposomes was determined based on following protocols:

2.6.1. Plasmid DNA association

To measure the association of the DNA with liposomes, 25 µL of the liposome was removed and diluted to 1 mL with PBS. The diluted samples were centrifuged by Optima^{TM} Max-xp Ultra Centrifuge, (Beckman Coulter, USA) for one hour with the speed of 125,000 x g at 4 °C. Following to the centrifugation, the supernatant were collected and the pellets were resuspended in 1 mL PBS and the centrifuge repeated. Supernatants, which contain unassociated pDNA, were incubated with similar volume of fluorescent dye of PicoGreen® for 5 minutes at RT in a black 96 well plate (the plate was covered by foil to avoid photodegradation). The absorbance was read by SpectroMax Gemini EM (Molecular Device) plate reader with the excitation maximum at 495 nm and an emission peak at 525 nm. So the amount of free pDNA (washed pDNA) was quantified. To measure the liposome-associated pDNA, the liposome 'pellet' was resuspended in PBS and exposed to 10 µL of 10 % Triton X-100 with final concentration of 0.1 %. The mixture was shaken vigorously prior to addition of Picogreen® and then the absorbent was read to quantify the associated pDNA. Incorporation values of DNA with liposomes were calculated by following equation (including any dilution):

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

2.6.2. Protein adsorption

2.6.2.1. Radiolabelling of the proteins

In order to determine the entrapment of protein in each liposomal vaccine formulation, radiolabelling was applied using ^{125}I. The protein antigen HBsAg, was radiolabelled using the Iodo-gen® pre-coated iodination tubes. The protein antigen was diluted to 50 µL PBS buffer (0.01 M, Ph 7.4) and added to the pre-coated iodination tube following by addition of ^{125}I equal to 3.7 MBq. The mixture was left for 45 to 60 minutes with swirling every 15 minutes to ensure complete exposure of protein and ^{125}I to the iodination tube. The labelled antigen was separated from unlabelled antigen by applying the mixture of protein and ^{125}I onto a 5 mL Sephadex G-75 gel column.

To prepare the gel column, 2 g of the Sephadex G-75 was rehydrated in 30 mL of distilled water and stored in the fridge over the night. Then the swollen gel was packed to give a final gel volume of 5 mL. The gel was washed by 10 mL PBS to ensure PBS hydrated the whole gel column.

Radioactivity of the samples was measured using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA).

2.6.2.2. Detection of the liposome-associated protein.

To determine the presence of protein, bicinchoninic acid (BCA) assay was used; 25 µL of each sample was transferred to the wells of 96 well plate. 200 mL of BCA reagent (ratio of 50:1 for reagent A to B) was added to each well. Then the plate was incubated for half an hour in 37° C. This was followed by reading the absorbance at 562 nm by microplate reader (BioRad, model 680). The radioactivity and absorbance readings for

each 0.25 mL aliquot were plotted to confirm the presence of radiolabelled protein antigen.

2.6.2.3. Quantification of liposome-adsorbed protein

The aliquots containing the radiolabelled antigen were collected and the required amount was added to the liposome suspension. The mixture was left at RT for one hour. The radioactivity of each liposome formulation was measured using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA). To separate the entrapped and unentrapped antigen, the liposome suspension was diluted by PBS to 3.9 mL and centrifuged for one hour at 4° C at 125,000 x *g* using a TL-100 rotor on an Optima™ Max-xp Ultra Centrifuge; Beckman Coulter, USA. The Pellet and supernatant were separated and after redispersing of the pellet in 1 mL of PBS, the radioactivity of them was measured on the gamma counter. The amount of entrapped antigen was calculated by dividing the radioactivity of the pellet by the radioactivity of the unwashed sample multiplied by 100 %:

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

2.7. Agarose gel electrophoresis

Gel electrophoresis was used to determine the retention of plasmid DNA in the lipoplexes under various conditions. Agarose gel was prepared by dissolving the desired amount of Agarose powder in 1x Tris borate EDTA (TBE) buffer solution to reach to 1 % concentration (w/v). 10 µL ethidium bromide (1 mg/mL) was also added to the solution and followed by heating the mixture in the microwave for 5 minutes. A clear and transparent solution was obtained and left to cool down to (50 to 60) °C and then

was poured in the tray. When the gel hardened the comb was taken off the gel, and the tray, which is filled with the gel, was placed in the chamber filled with pre-chilled 1x TBE buffer. Samples of DNA ladder, 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).

2.8. Protection of DNA from degradation

A vial of Deoxyribonuclease I (DNase I) reconstituted with 1 mL of cold sodium chloride (NaCl) (0.15 M) to prepare a standard solution of DNase I with a concentration of 2000 Kunitz units/mL. From this, aliquots of 60 μL were pipetted into vials and frozen at -20 °C for future use. 100 μL of naked DNA (10 μg) was taken and 150 μL of magnesium chloride ($MgCl_2$) (5 mM) was added to it. Samples were then incubated in the presence (i.e. positive test) or absence (i.e. negative test) of 100 units of DNase I (50 μL from the stock solution), at 37 °C for 10 minutes. This protocol was repeated for the DNA lipoplex formulations. Adding 100 μL of EDTA (0.1 M; pH 8.0) after 10 minutes stopped the reaction. 8 μL from each sample was removed and 2.5 % w/v SDS was added (1:1 v/v), in order to rupture 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.

2.9. DNA release studies

DNA association within liposome formulations was determined as detailed in section 2.6.1. 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, 192 hours), DNA association 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 the amount of DNA released was determined as detailed in section 2.6.1.

2.10. *In vitro* studies

2.10.1. Cell culture protocols

2.10.1.1. Reviving the frozen COS-7 cell line

To revive the frozen cell line, the ampoule was removed carefully from liquid nitrogen tank and kept in 37 °C water bath for 2 minutes, until cells defrosted. Under sterile conditions, the cell solution was removed from the ampoule and slowly pipetted into a 75 cm2 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 (PSG), in order to dilute out the toxic effects of Dimethyl Sulfoxide (DMSO). Cells were incubated at 37 °C and 5 % CO_2 under sterile conditions.

2.10.1.2. Subculture of COS-7 cells

In order to passage the cell lines, the previous media (DMEM) was removed and 5 mL of trypsin/EDTA added to the flask ensuring that all cells are covered. The cells were incubated at 37 °C and 5 % CO_2 for 5 minutes. Cells were examined under an inverted microscope to ensure all cells have detached from the flask. 5 mL of fresh DMEM supplemented with 10 % FBS and 1 % PSG was added to the cell suspension to dilute the trypsin/EDTA solution, the cell suspension was then 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 resuspended in 10 mL DMEM/FBS/PSG. 1 mL of cell suspension is added to a 75 cm^2 containing 19 mL DMEM and incubated at 37 °C and 5 % CO_2 under sterile conditions.

2.10.1.3. Cell quantification

As explained in section 2.10.1.2 adherent COS-7 cells were brought into solution. Then 200 μL of this cell suspension was removed and added to a microcentrifuge tube followed by addition of 200 μL of Trypan Blue. 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 observed by inverted microscope. 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. The following equation was used to calculate the cell number:

$$\text{No of cells/mL} = \text{number of cells per square} \times \text{dilution factor} \times 10^4$$

- no. cells per square is the average of 10 squares in the hemocytometer
- Dilution factor is 2 if equal volumes of resuspended cells and tryphan blue are used
- 10^4 is the multiplication factor related to the volume of the hemocytometer grid

2.10.1.4. Cryopreservation

Adherent cells were brought into suspension and quantified as explained in sections 2.10.1.2 and 2.10.1.3. Cells were then centrifuged at 200 x g for 10 minutes at 15 °C and the pellet resuspended in FBS containing 10 % DMSO with a cell concentration of 4 x 10^6 cells/mL. 1 mL aliquots of cell suspension were pipetted into cryopreservation

ampoules, and frozen at -70 °C overnight. The ampoules were then placed and stored in a liquid nitrogen storage container.

2.10.2. Transfection studies

2.10.2.1. Cell preparation and plating for *in vitro* transfection

Adherent COS-7 cells were brought into suspension and quantified as detailed in section 2.10.1.2 and 2.10.1.3. The cell suspension was then centrifuged at 200 x g for 10 minutes at 15°C and the pellet resuspended to a cell concentration of 1 x 10^5 cells/mL with supplemented DMEM. COS-7 cells were plated 24 hours prior to transfection, at a cell concentration of 1 x 10^5 cells/mL in 1 mL of medium in a 12-well plate and incubated overnight at 37 °C and 5 % CO_2 under sterile conditions for 24 hours.

2.10.2.2. DNA lipoplex preparation for *in vitro* transfection

To perform *in vitro* studies, lipoplexes was prepared by diluting 17.5 μL of SUV solution (16 μmoles) to 0.35 mL with Opti-MEM, and then incubated for 40 minutes at room temperature. After incubation, 0.35 mL of Opti-MEM containing 3.5 μg plasmid DNA was added, mixed with liposome solution and incubated again for a further 15 minutes at room temperature. The resultant lipoplex mixture was then diluted to a final volume of 3.5 mL with Opti-MEM. The lipid/DNA charge ratio for *in vitro* study was +1.7/1.

Prior to transfection, cells, which were incubated for 24 hours, were washed with 1 mL of Opti-MEM before lipoplexes were added to the cells. 1 mL of the lipoplex solution (0.0078 μmole total lipid content containing 1 μg plasmid DNA) was added to each well, each in triplicate. In addition, Lipofectin reagent and free DNA were added as positive and negative controls respectively. After 5 hours incubation at 37 °C in 5 %

CO_2, the Opti-MEM medium was replaced with growth medium (DMEM) containing 10 % FBS and the cells were incubated for 48 hours in the same condition.

2.10.2.3. Luciferase assay

Transfection efficiency of each formulation was determined using luciferase assay system. Luciferase was used as a reporter to assess the transcriptional activity in cells that are transfected with the DNA by producing light emission so the luciferase activity of the cell can be determined by detecting the produced light using a illuminometer.

Therefore to determine the transfection efficiency of lipoplexes, transfected cells were exposed to 80 μL/well of lysis buffer and detached using cell scrapper. Detached cells spanned down at 12,000 x g for 15 seconds at room temperature and 10 μL of the supernatant was removed and pipetted onto a 96-well plate. To quantify the luciferase activity prior to addition of luciferase reagent, the plate was read using illuminometer (Spectra Max Gemini XPS, Molecular Probes) with 30 reads/well. Then luciferase assay reagent was added to the cells (100 μL/well) and the plate was read again.

Measuring the amount of detected light for each sample, the transfection efficiency was reported as the percentage of the produced activity of each formulation to the Lipofectin™.

2.10.3. Cytotoxicity study

Cell preparation and plating procedure for cytotoxicity study is same as transfection as described in section 2.10.2.1 and 2.10.2.2 except that 100 μL of cells were pipetted into 96 well plates before incubation for 24 hours. After 5 hours incubation, the medium was replaced by 100 μL of supplemented DMEM and incubated for 24 hours, then 20 μL of

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) reagent, (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) was added to each well. This protocol is based on the fact that cells bioreduce the MTS reagent into a red formazan product. Plates were incubated for 4 hours at 37 °C, in a 5 % humid CO_2 condition. After that the quantity of produced formazan was measured on microplate reader (Thermo Scientific Molecular Spectrum plate reader) at A_{490}. The absorbance reading is directly proportional to the number of living cells in the medium. In this study cell viability is calculated by comparing the results to the positive control (*i.e.*, cells and medium) and expressed as a percentage.

2.11. Biodistribution studies in mice

The biodistribution of several radiolabelled lipoplex formulation was studied in female 6-8 week old (18 – 21) g, BALB/c mice (Charles River, Margrate,UK). Groups of 4 mice were used to study biodistribution of naked DNA and seven liposomal formulations (Table 2.2) for each time point and three time points were selected for termination of the mice which were 1, 4 and 8 days after the injection. So 3 experimental groups (12 mice) were used to study each formulation and in total 96 mice were used in this study. Mice were housed under conventional conditions (22 °C, 55 % humidity, 12 h day/night cycle) in their experimental groups and were given a standard diet ad-lib.

All experimentation undertaken strictly adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to ethical review and were carried out in a designated establishment.

Table.2.2: Lipoplex specifications of present study

Formulation	Lipid ratio (μM)	Lipid/DNA charge ratio	Rehydration medium
SUV DOPE:DOTAP	8:8	+4/1	Sucrose
SUV DOPE:DOTAP	8:8	+4/1	PBS
SUV DOPE:DSTAP	8:8	+4/1	Sucrose
SUV DOPE:DSTAP	8:8	+4/1	PBS
SUV DDA:DOPE	8:8	+4/1	Sucrose
SUV DDA:TDB	8:1	+4/1	Tris
DRV DOPE:DSTAP	8:8	+4/1	Sucrose

2.11.1. Pontamine blue injection

Three days before injection of the formulations, 200 μL of sterile filtered (0.2 μm) Pontamine blue (0.5 % w/v in PBS) was injected subcutaneously (s.c.) into the neck scruff of the mice as a marker for lymph nodes and monocytes (Tilney, 1971b).

2.11.2. Preparation of radiolabelled vaccines and their injection

Three days after injection of Pontamine blue, mice were injected a single intramuscular (i.m.) injection into the right quadriceps muscle with 50 μL of the plasmid:liposomes complex (lipoplex). To be able to investigate the biodistribution of the lipoplexes and detect lipid and pDNA *in vivo*, formulations radiolabelled with dual radiolabelling technique (Figure 2.4). Liposomes were radiolabelled by incorporation of [^{3}H]-Cholesterol to the liposomal bilayer. [^{3}H]-Cholesterol was added to the lipid mixture prior to solvent evaporation and the liposomes were prepared by lipid-film hydration method detailed in section 2.3. The amount of [^{3}H]-Cholesterol added was based on the radioactivity of (^{3}H) and the concentration of cholesterol in the liposomes so that the physicochemical properties of the liposomes were not changed. The radioactivity of

[^{3}H]-Cholesterol was 37 MBq/mL, which was ideal for this study with a high radioactivity and low cholesterol interference in liposomal characteristic parameters. So to gain a 150 kBq/dose radioactivity, a weight ratio of 1:10000 [^{3}H]-Cholesterol:lipid was used. Plasmid DNA was labelled by the incorporation of [α-^{32}P]-dATP by nick translation procedure (Kelly et al., 1970; Rigby et al., 1977). Therefore a Nick Translation Kit N5500 (GE Healthcare, Amersham, UK) was used, according to the instructions of the supplier and 2 μg of pDNA was labelled with 66 pmole of [α-^{32}P]-dATP. After the DNA and [α-^{32}P]-dATP reaction, the free label was removed using Illustra Probe Quant G-50 Micro Columns (GE Healthcare, Amersham, UK). The appropriate amount of labelled and unlabelled pDNA were mixed to achieve a 150 KBq/dose activity and the mixture then added to the radiolabelled liposomes to prepare the dual radiolabelled lipoplexes (Figure 2.4). The injected dose volume was 50 μL containing 150 kBq of [^{32}P] and 150 kBq of [^{3}H]. There was also 50 μg of gWiz luciferase plasmid DNA in each dose and the lipid/DNA ratio was +4/1. All mice were injected intramuscularly to the right quadriceps muscles.

2.11.3. Processing of the tissues

At time points of one day, four days and eight days post injection (p.i.), mice were terminated by cervical dislocation and various tissues collected. The collected tissues include site of injection (SOI), popliteal lymph node (PLN), liver, kidney, spleen and lung. Each tissue was weighted and transferred to scintillation vial and 1.5 mL of Solvable™ was added to each vial and incubated for 24 hours at 50 °C to digest the tissues completely. To avoid the colour quenching effect on counting of radioactivity of the samples, 200 μL of oxygen peroxide was added to each sample and kept at room temperature for 8 to 10 hours. 10 mL of Ultima Gold™ scintillation fluid was added to the fully bleached samples and made ready to be counted for their radioactivity.

2.11.4. Quantification of the proportion of vaccine components in tissues

The activity of [^{32}P] and [^{3}H] of the samples were counted on a Packard Tri-Carb liquid scintillation counter (LSC), GMI Inc. using separate standard detection protocols for [^{32}P] and [^{3}H]. The data are expressed as percentage of the injected dose per tissue (%ID). Also for investigating doses at the PLN, the data was expressed as percentage of the injected dose per milligram of the PLN. To calculate the dose percentage for each formulation, triplicate samples of the original dose were processed the same as tissue processing and were counted at each time point.

It has been reported (Carstens et al., 2011; Zamecnik et al., 1982) that ^{32}P energy spectrum overlap with the ^{3}H spectrum; therefore to determine the radioactive count of each radionuclide a standard curve was plotted. In a range of 20 cpms to 150 kBq, triplicate samples of ^{32}P were prepared and counted on scintillation counter by both ^{32}P and ^{3}H protocols. A plot of the ^{32}P values (x-axis) was made against the cpm values derived from ^{3}H (y-axis) and the line of best fit and equation derived for samples below 50,000 cpm and those above 50,000 cpm. These two equations were used to calculate out the effect of ^{32}P on the ^{3}H values.

2.12. Immunisation studies in mice

2.12.1. Immunisation plan and vaccine formulations

Female C57BL/5Jico mice (18-20 g) purchased from Charles River, UK, were placed in groups of 5, with a total of 16 groups. Vaccination was performed in a heterologous DNA prime-protein boost regimen. This was composed of two immunisations with 50 μg/dose pRc/CMV HBS plasmid DNA (Free pDNA for controls or various pDNA-liposome complexes) and a third injection with 3 μg/dose HBsAg (ayw subtype)

dissolved in PBS, Alhydrogel 2 % or incorporated with liposomes of various formulations. For all of the groups, intramuscular (i.m) injection was applied to the quadriceps muscle of the right leg of the animal and the volume of each dose was 50 μL. Each group of 5 mice (excluding the naïve group) have received this vaccination regimen at 2 week intervals.

Several control groups were designed as follows: a group of five mice that received one single free protein injection on day 28 (Boost control, --P), second group received the prime control which composed of two injections of free pDNA on days 1 and 14 (DD-) and third group received the prime-boost control that includes two free pDNA injections on days 1 and 14 followed by injection of free protein on day 28 (DDP). Same schedule was applied for DOPE:DSTAP liposomes which formed in sucrose or PBS. To compare the effect of aluminium salts on immunisation one group of 5 mice specified for receiving of a single injection of HBsAg in alhydrogel 2 %.

Other liposomal vaccine formulations were injected under prime-boost (DDP) regimen. One day before each injection, one week after third injection and three weeks after third injection, (days 0, 13, 27, 36 and 49) blood samples were collected from the tail vein. Three weeks after the last immunisation mice were terminated and spleen and the site of injection (SOI) were collected to analyse the immune response.

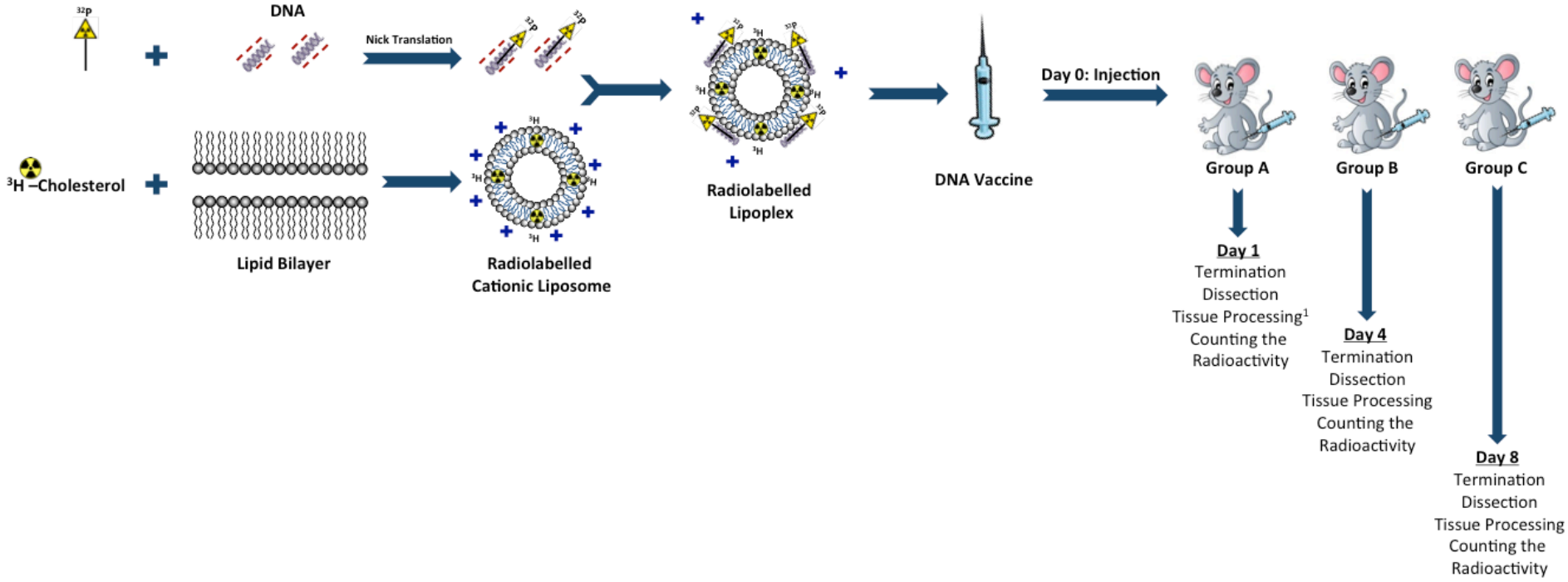

Figure 2.4: Schematic diagram of dual radiolabelling method to prepare radiolabelled lipoplexes and the biodistribution schedule plan.

2.12.2. Antibody analysis

To perform the antibody enzyme-linked immunosorbent assay (ELISA), blood samples were taken by making a small cut in the tail vein and collecting 50 μL of blood sample within a capillary tube, which was coated with 1 % heparin. Heparin was dissolved in PBS. The collected bloods were transferred to microcentrifuge tube containing 450 μL PBS to give a 1/10 dilution. Blood dilutions were centrifuged (Micro Centaur) at 13,000 g for 5 minutes. The supernatant was collected and stored at -20 °C. If the volume ratio of haematocrit is assumed 50 %, consequently the stored serum in each microcentrifuge tube was 20 times diluted in PBS.

As it is shown in Figure 2.5, ELISA plates (flat bottom, high binding) were coated with 60 μL of 2 μg/mL HBsAg 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 to remove any unbound antigen. PBST buffer were made by dissolving 40 g NaCl, 1 g KCl, 1 g KH_2PO_4 and 7.2 g $Na_2HPO_4(2H_2O)$ in 5 litres distilled water and with 0.4 mL Tween 20. To remove any non-specific binding antigen, the plates were coated with 100 μL of 4 % (w/v) Marvel (dried skimmed milk powder) and incubated for 1 hour at 37 °C. Then plates were washed again three times with PBST buffer. 190 μL of PBS was added to each well of first row (Row A) of the 96 well ELISA plate and 100 μL PBS was added to the rest of the wells in rows B to H. 10 μL of the serum sample was taken and added to the specific wells of row A and mixed well. Each sample was investigated in duplicate. Then 50 μL of the mixtures in row A were taken and added to row B and this procedure repeated to row H to make the serial dilution of the serum for each column of the plate. 50 μL was removed from row A and H so that the total volume of the serially diluted serum in all the wells was 100 μL.

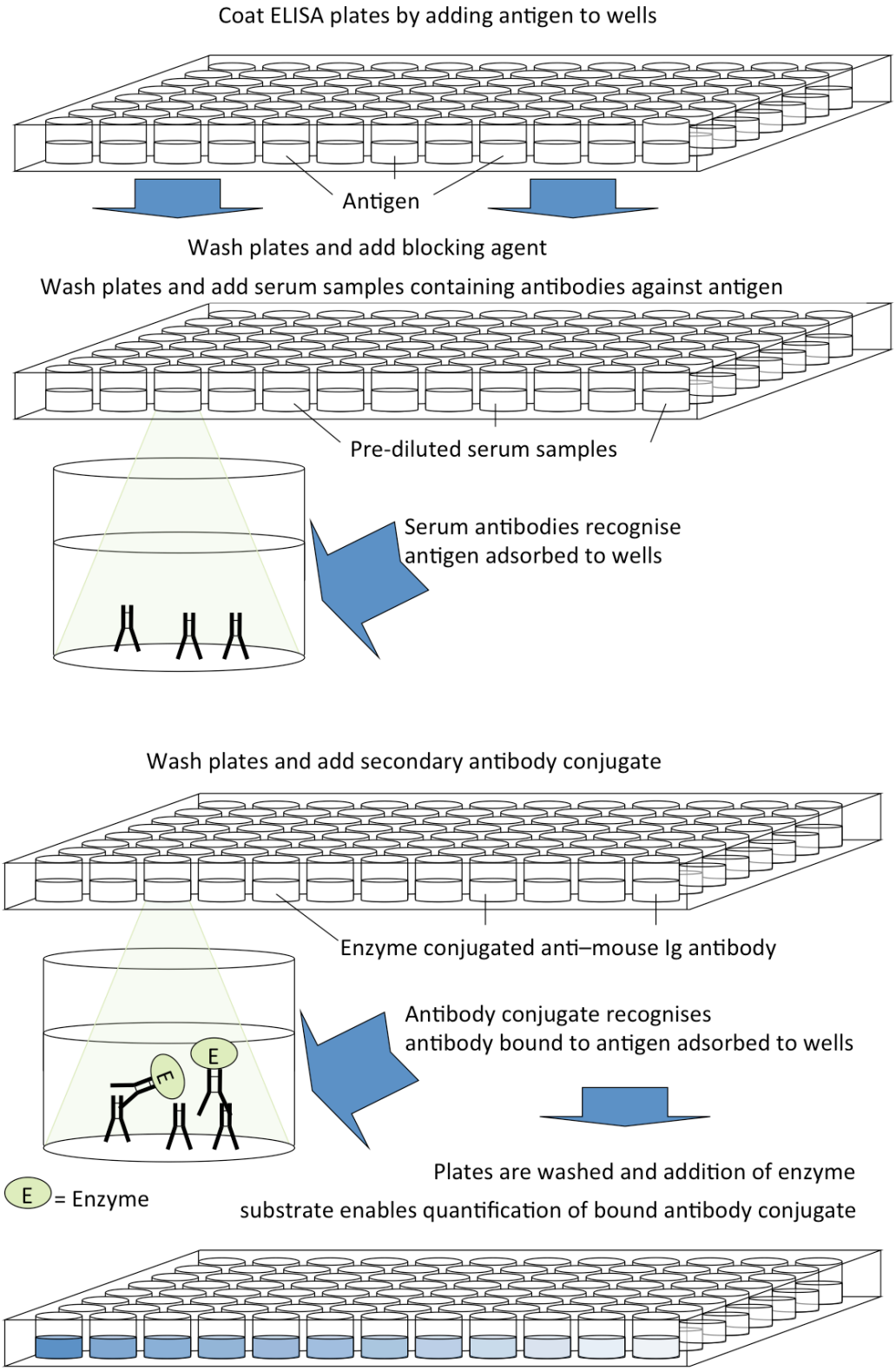

Figure 2.5: Schematic diagram of mouse antibody enzyme-linked immunosorbent assay (ELISA).
Elisa plates were coated with HBsAg prior to numerous wash stages with PBST and blocking with Malvern milk 4 % (w/v). Washing prior to adding serum samples, antibody conjugates and enzyme substrate took placed with incubation at 37 °C and 5 % CO_2. The absorbance was measured using plate reader at 405 nm.

Samples incubated for one hour at 37 °C followed by 5 times wash with PBST. 50 μL of diluted isotype specific immunoglobulin (IgG, IgG1 and IgG2b diluted in PBS as 1:500 for IgG and 1:4000 for IgG1 and IgG2b) were added to the appropriate plates. Then they were incubated for one hour at 37 °C followed by five PBST washes. 50 μL colouring agent (substrate) added per well. The substrate was made by dissolving of 6 x 10 mg tablets of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid in 100 mL of citrate buffer (2.3 g citric acid, 4.89 g Na_2HPO_4 in 250 mL ddH_2O) incorporating 10 μL of hydrogen peroxide (Figure 2.5).

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). Previous known positive sera and naïve mouse sera were employed as positive and negative controls, respectively, on each ELISA plate. The results were expressed as the $\log_{10}$ value of the reciprocal of the end point dilution, which gave an optical density (O.D) of 0.2 or above.

2.12.3. Splenocyte proliferation study

Mice were terminated on day 49 and their spleens were removed and placed into 5 mL ice-cold sterile PBS. Under sterile conditions, each spleen was gently mashed on a fine wire mesh to give a suspension of spleen cells and added to 10 mL RPMI 1640 supplemented with 10 % FBS and 1 % PSG. The cell suspension was left for 5 minutes to allow to the cell debris to settle, and then the supernatant was transferred to the sterile 15 mL falcon tubes and centrifuged 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. The cells were

counted as described in section 2.10.1.3 and the cell number was adjusted to 1 x 10^7cells/mL.

100 μL of serially diluted HBsAg (0.05, 0.5 and 5 μg/mL) in RPMI, was added to the sterile 96 well plate. The negative control wells contained medium only, and positive control was 5 μg/mL dilution of concanavalin A (Con A) in RPMI. 100 μL of splenocytes (1 x 10^7cells/mL) were added to the wells and the plates were incubated for 72 hours in a sterile incubator with 37 °C and 5 % CO_2 and 95 % humidity. After incubation, 40 μL of 18.5 kBq [^{3}H]-thymidine within sterile supplemented RMPI was added to each well of a 96-well plate and incubated for further 24 hours under the same conditions. Cells were harvested onto a quartz filter mats using a cell harvester (Titertek). For harvesting well contents were aspirated onto the quartz filter mat and kept to dry. The discs representing each well were punched from the filter mats into 15 mL plastic scintillation vial followed by addition of 5 mL Ultima Gold™ scintillation fluid. The radioactivity of each sample was counted on a Packard Tri-Carb liquid scintillation counter (LSC), GMI Inc. by ^{3}H scintillation counting protocol.

2.12.4. Cytokine analysis from *in vitro* restimulated splenocytes

Restimulated splenocytes with HBsAg or Con A and cells were prepared as outlined in section 2.12.3. Dilutions of 5 μg/mL HBsAg in RPMI were made and 100 μL was added per well of a sterile 96 well plate. Negative and positive controls were again medium only and 5 μg/mL Con A respectively. To have enough supernatant, 12 wells of each plate were used for each condition. 100 μL of splenocytes (1 x 10^7cells/mL) were added to the wells and the plates were incubated for 48 hours at a sterile incubator with 37 °C and 5 % CO_2 and 95 % humidity. The supernatants were then removed and added to the microcentrifuge tubes and frozen at -70 °C until use.

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 protocol was taken from the manufacturer catalogue (R&D Systems, Abingdon, UK). The ELISA plates were 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 overnight. Plates were then washed three times with PBST buffer and blocked by 300 μL block buffer. Block buffer for IL-2 and IFN-γ was 1 % BSA in PBS with 0.05 % NaN_3 and for IL-5, IL-6 and IL-10 was their reagent diluent which was 1 % BSA (in PBS, pH 7.2-7.4). Plates were then incubated for 1 hour at room temperature, followed by three washes with PBST buffer. 100 μL of samples added to each well. At this stage standards were also diluted in reagent diluent and pipetted into the plate. For IL-2 and IFN-γ reagent diluent was, 0.1 % BSA, 0.05 % Tween 20 in Tris-buffer Saline (20 mM Trizma base, 150 mM NaCl) pH 7.2-7.4. After incubation, plates were washed three times with PBST buffer. The cytokine standards were 2-fold serial dilutions of the supplied cytokine diluted in 0.2 μm sterile filtered reagent diluent. Each standard was diluted 6 times and included a 0 pg/mL control. The samples were added without any dilution. After incubation 100 μL detection antibody for the corresponding cytokine was added to each well and the plates were covered by new microplate slip and 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, and be avoided from the direct light. Plates were washed three times with PBST buffer and 100 μL of substrate solution was added per well. Substrate solution was 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 while

was avoided from the direct light. Then by adding 50 μL of stop solution (2N H_2SO_4) the reaction stopped and the blue light indicating the reaction changes to golden yellow. The optical density of each well was measured immediately using a microplate reader (Bio-Rad, model 680) set to 450 nm.

2.12.5. Cytokine analysis from *in vitro* restimulated cells from the site of injection

The production of IL-1β at the site of injection (SOI) was investigated by the method described by Sharp et al. (2009). Muscle from the SOI was excised 3 weeks after the final injection and flushed freeze by liquid Nitrogen and kept frozen at -70 °C. On the day of experiment, leg was defrosted and the quadriceps muscle was removed and separated from the bone. After weighing the muscle using a manual homogenisation tube (Figure 2.6), each leg's muscle homogenised on ice in 2.5 mL of homogenisation buffer (500 mM NaCl/50 mM Hepes, pH 7.4, containing 0.1 % Triton X-100, Sigma protease inhibition mixture and 0.02 % NaN_3).

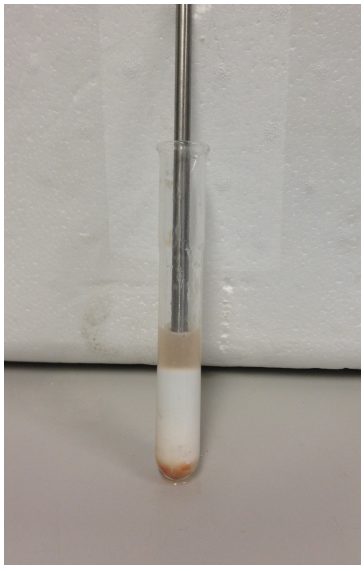

Figure 2.6: Teflon homogenisation tube to homogenise the leg muscle.

Samples were sonicated and centrifuged (6000 x g, 20 minutes, 4 °C) prior to detection of IL-1β in supernatants. After centrifugation, three layers were separated from up to down of the 15 mL Falcon tube which were fat layer, supernatant or cells and at the bottom of the tube there was debris and hair. The supernatant was removed carefully from the tube and pooled into the microcentrifuge tubes and kept in -20 °C until use. The same method, which used for splenocytes in section 2.12.4 was used to figure out the production level of IL-1β at the site of injection. Results were shown as pg of IL-1β per milligram of each muscle.

2.13. Statistics

Means and standard deviation were calculated for all experiments. The one-way analysis of variance (ANOVA) was performed on all data to determine statistical significance. The statistical significance determined to 0.05 confidence intervals ($P<0.05$). To compare the difference of significance of different conditions, Tukey's post hoc test was performed.

Chapter 3:

The Effect of Alkyl Chain and Electrolytes on Characteristics of Transfection Agent Lipoplexes

Papers relating to this chapter:

Moghaddam, B., McNeil, S.E., Zheng, Q., Mohammed, A.R., Perrie, Y., 2011. Exploring the Correlation Between Lipid Packaging in Lipoplexes and Their Transfection Efficacy. Pharmaceutics, 3, 848-864.

3.1. Introduction

Cationic liposomes have been widely investigated as a non-viral delivery system for gene delivery (Bedi et al., 2011; Gjetting et al., 2011; Gregoriadis et al., 2002; Perrie et al., 2003; Wang et al., 2012b; Zabner, 1997) and the electrostatic interaction between positive charge of cationic liposomes and negative charge of DNA make a complex of cationic liposome-DNA generally referred to as lipoplexes (McNeil et al., 2010).

To develop lipoplexes with high transfection and low toxicity, several parameters should be taken into account such as: size, lipid/DNA charge ratio, net positive charge of the lipoplex, chemical structure of cationic lipid and helper lipid, and finally the structure of the complex itself (Congiu et al., 2004). However, given the dynamic nature of these structures, many factors, in addition to those already listed, can contribute to the resultant physico-chemical attributes of the lipoplexes, including the rate of mixing of the various components, the temperatures used and even the presence of electrolytes in the buffers used (Congiu et al., 2004; McNeil and Perrie, 2006, 2007). Indeed, research has shown that the presence of electrolyte within the aqueous media can influence both the physicochemical properties and the *in vivo* efficacy of lipoplexes, with the authors demonstrating that the addition of low concentrations of sodium chloride to cationic liposomes during complex formation lead to an improved vaccine adjuvant action (Yan and Huang, 2009).

Thus, the aim of the work in this chapter was to investigate the molecular interactions of lipids and the resultant lipoplexes properties and to attempt to correlate these with the transfection attributes of the system in a controlled *in vitro* environment. Therefore, lipid monolayers were studied by Langmuir-Blodgett trough, as such monolayers can be considered as building blocks for bilayer vesicles, consequently, studying these

monolayers in an aqueous media (dH_2O or PBS) at air/water interface, in combination with the lipid attributes, may give insights into bilayer lipid packaging configuration and liposomes stability which could influence transfection (Ali et al., 2010; Christensen et al., 2008). Cationic liposomes were prepared in dH_2O or the commonly used phosphate buffered saline (PBS) and the liposome physicochemical characteristics considered, such that the effect of electrolytes could be considered, and correlated with the Langmuir studies. Of the cationic liposome systems tested, the combination of the fusogenic lipid DOPE (Figure 3.1) with the cationic lipid DOTAP, is a frequently used composition due to its high *in vitro* transfection efficiency and optimal immune response (Ciani et al., 2007; Guo and Lee, 2000; Li et al., 2010; Liu, 2003; McNeil et al., 2010; Perrie et al., 2001).

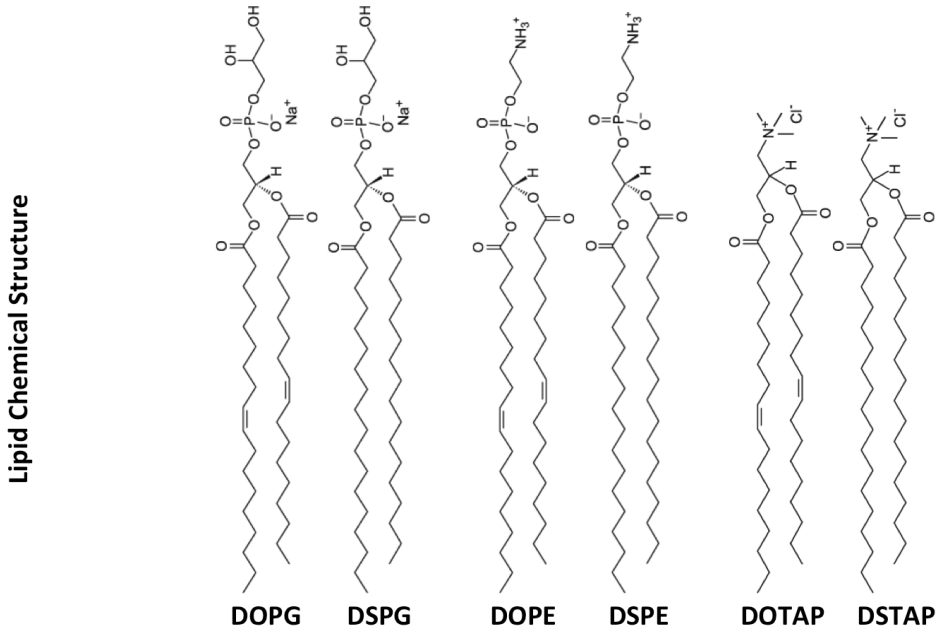

Figure 3.1: Molecular structure of used anionic lipids (DOPG and DSPG), fusogenic lipids (DOPE and DSPE) and cationic lipids (DOTAP and DSTAP).

Therefore, this formulation was chosen for further investigation. To consider the effect of lipid acyl chains on their molecular packaging and lipoplex characteristics, DOPE and DOTAP were also systematically compared with their disteroyl equivalents (DSPE and DSTAP). To investigate if the finding from these studies were cationic lipid specific, or if it was achievable by anionic lipids, two anionic lipids (DOPG and DSPG) were also studied (Figure 3.1).

3.2 Liposome preparation: Initial studies

3.2.1. The effect of buffer concentration on zeta potential

Prior to performance of characterisation studies on liposomes, the effect of different PBS concentrations on zeta potential was measured. First, multilamellar vesicles (MLV) of a model cationic liposome formulation (DOPE:DC-Chol) was prepared with PBS as the hydration phase. Then a serial dilution of PBS at concentrations of [0.0001, 0.001, 0.01, 1, 10 mM] was prepared (Figure 3.2).

For measuring the zeta potential, 100 µL of the liposome suspension was diluted in 3 mL of the above concentrations. Liposomes diluted in dH_2O were also tested. Results show an initial increase in zeta potential (≈ 55 mV) by using PBS even at very low concentrations (0.01 mM) comparing to distilled water (≈ 30 mV), however, as the PBS concentration increases the zeta potential decreases to ≈ 40 mV (Figure 3.2).

Electrolytes are known to influence the electrical double layer which surrounds charged particles and interfaces (Florence and Attwood, 2006); this also leads to changes in the zeta potential. It is reported that increasing electrolyte concentration can condense the electrical double layer and thus reduce the zeta potential on a charged surface and this may explain the reduction in zeta potential at higher PBS concentrations (Florence and

Attwood, 2006). However, it was also shown there is no significant difference in the zeta potential of liposomes suspended in PBS at 1 mM and 10 mM ($P>0.05$) suggesting that 1 mM PBS can be used for characterisation studies (Perrie and Gregoriadis, 2000).

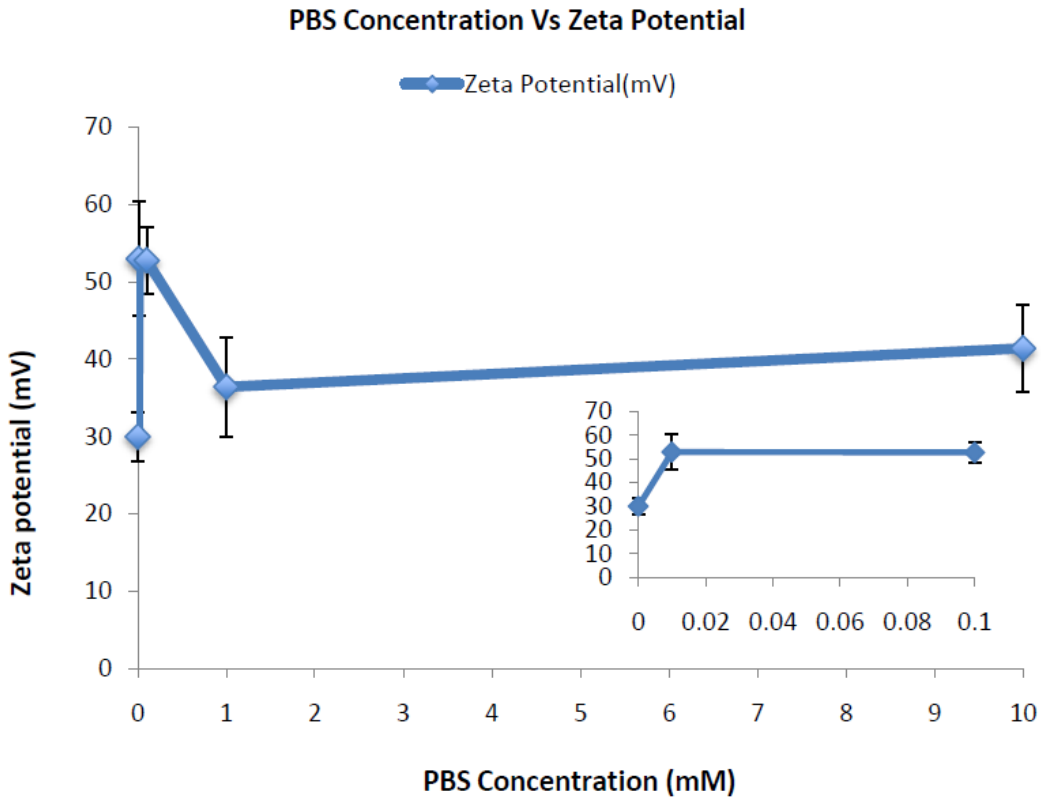

Figure 3.2: The effect of electrolyte concentration on the zeta potential of cationic liposomes. Results represent mean ± SD from 3 independent batches.

3.2.2. Optimisation of sonication time in SUV preparation

Whilst it is common to reduce the size of MLV via probe sonication, it has previously been reported that prolonged probe sonication can cause an increase in size due to lipid damage and particle aggregation; high temperatures produced by sonication were reported to be responsible for this (Ferdous et al., 1998). In addition, titanium particles from the probe can influence the formulation integrity. To reduce these disadvantages of probe sonication, a study was performed to select the shortest and the most efficient

time of sonication for the production of SUV. After measuring the size and zeta potential of MLV liposomes of DOPE:DOTAP, DOPE:DSTAP, DSPE:DOTAP hydrated in distilled water or PBS, the suspension was sonicated for 15 s and size and zeta potential were measured at this point. Then, the sonication and measuring of the size and zeta potential has continued every 15 s for 2 minutes.

Results in Figure 3.3A show the mean particle size of MLV for DOPE:DOTAP (8:8 μmol) hydrated in dH_2O was ≈ 540 nm and the size decreases immediately after first sonication to ≈ 113 nm. By continued sonication, the size reduced to ≈ 99 nm after 60 s sonication, with no further decrease in size or PI (Figure 3.3). Therefore, in further experiments with this formulation and concentration, 60 s sonication will be performed to make SUV. Measuring the zeta potential of these vesicles during this process also demonstrated that their cationic nature remained unaffected by sonication (≈ 39-42 mV; Figure 3.3B), as would be expected unless lipid damage occurred.

Results in Figure 3.3A also demonstrated that sonication of MLV liposomes formed in PBS were ≈ 810 nm in size and decreased to ≈ 170 nm after 15 s, with further sonication reducing the size of the vesicles slightly (≈ 150 nm) and producing the lowest PI, suggesting 60 s sonication as an appropriate time to produce SUV (Figure 3.3A). As with the vesicles dispersed in distilled water, the zeta potential of the vesicles remained positive and constant (≈42-51mV; Figure 3.3B). Comparing between the formulations prepared in the presence/absence of electrolytes show that, whilst MLV prepared in PBS were larger in size than those in dH_2O, appropraite sonication could produce SUV in the same size range in either aqueous media (Figure 3.3A).

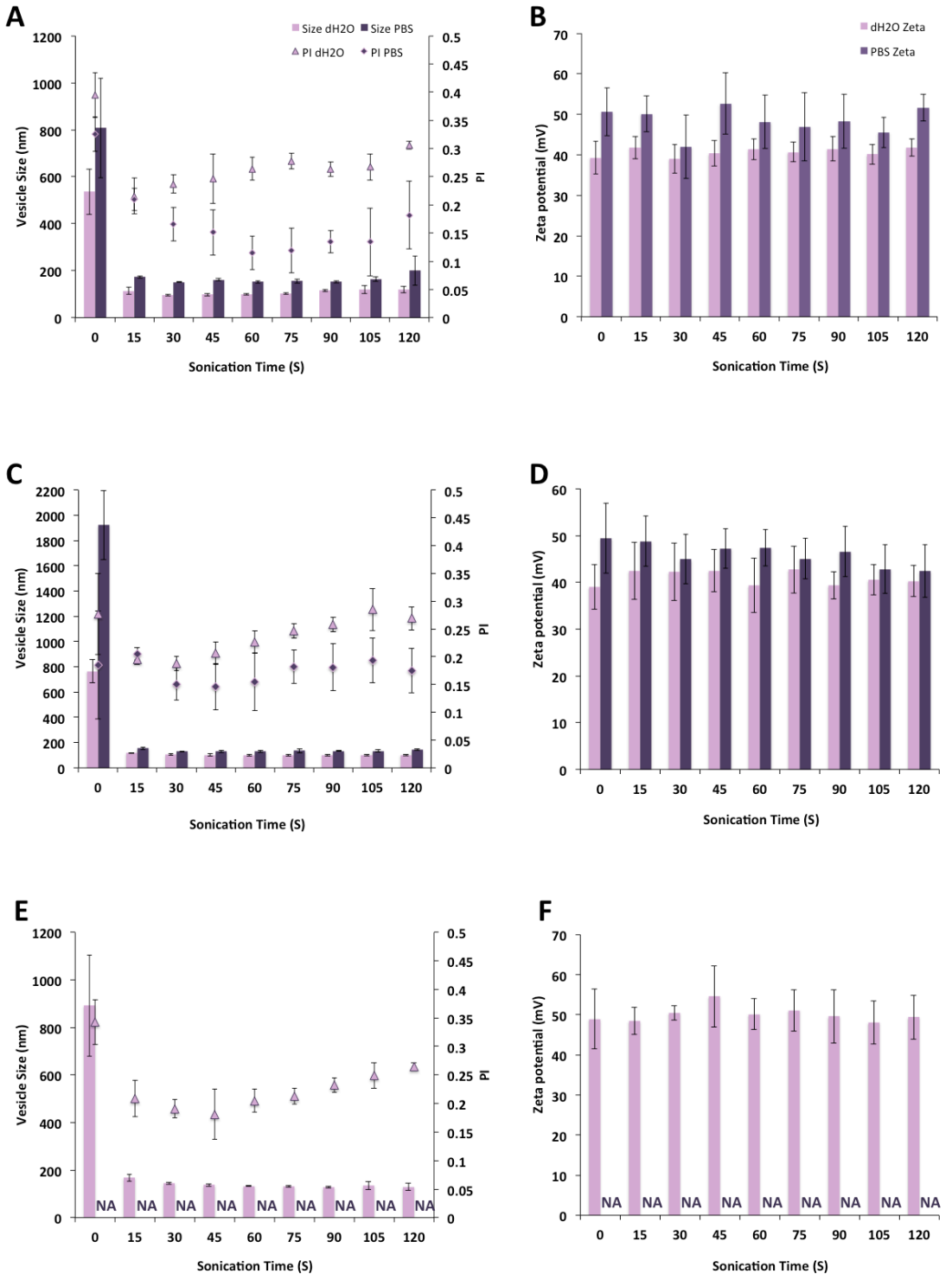

Figure 3.3: Effect of sonication time on liposomes characteristics. **A**,**C** and **E** show vesicle size and polydispersity index for DOPE:DOTAP, DOPE:DSTAP and DSPE:DOTAP respectively. **B**, **D** and **F** demonstrate the zeta potential values for DOPE:DOTAP, DOPE:DSTAP and DSPE:DOTAP respectively. All formulations hydrated in both distilled water or PBS and with the molar ratio of (8:8 µmol). Results represent mean ± SD, from 3 independent batches.

When considering the choice of cationic lipid, replacing the unsaturated DOTAP with its saturated counterpart DSTAP, shows the mean vesicle size of MLV for DOPE:DSTAP hydrated in distilled water was approximately 800 nm, and after sonication decreases to ≈ 120 nm after brief sonication, and after 60 s a minimum vesicle size of ≈ 97 nm was produced (Figure 3.3C).

Changing the hydration media to PBS increased the MLV size to ≈ 2 μm, although SUV with the size of around 130 nm could be produced with sonication (Figure 3.3C), which is comparable to previous studies looking at these formulations hydrated in NaCl (0.85 % W/V) where the vesicle size is about 120 nm (Filion and Phillips, 1997), and is slightly smaller than other research reporting DOPE:DSTAP vesicle sizes of ≈ 160 nm (Regelin et al., 2000). Therefore, whilst the DSTAP MLV were significantly larger in size ($P<0.05$) than DOTAP MLV, comparably sized SUV could be produced with either cationic lipid. Zeta potential measurements for the DOPE:DOTAP formulation (Figure 3.3D) show zeta potential for the liposomes which are hydrated in distilled water are in a constant range of ≈ 39 to 43 mV, and ≈ 42 to 49 mV for liposomes hydrated in PBS (Figure 3.3D). Comparing DOPE:DSTAP to DOPE:DOTAP liposomes shows there is no significant difference in zeta potential of these formulations in both rehydration phases suggesting that changing in acyl chain of lipid and length of the lipid chain has no significant effect on surface charge of the liposomes ($P>0.05$), as would be expected.

To consider the role of the fusogenic lipid, DOPE was also replaced with a high transition temperature lipid DSPE. The effect of changing chain length and saturation of helper lipid in cationic liposomes and in the hydration media of PBS or distilled water was investigated. Results (Figure 3.3E) show the DSPE:DOTAP MLV size when hydrated in dH_2O was ≈ 900 nm and the lowest vesicle size for the liposomes achieved

after sonication was ≈ 128 nm with the zeta potential remaining constant (≈ 48 to 54 mV; Figure 3.3F).

Comparing DSPE:DOTAP to DOPE:DOTAP shows that neither the vesicle size nor the zeta potential were significantly different, suggesting that saturation of helper lipid has no significant effect. Interestingly, DSPE:DOTAP vesicles could not be formulated in PBS as the lipid film would not hydrate with PBS. This might be due to the effect of salt on critical packaging parameter (CPP) of the lipid. As the DSPE is a saturated form of DOPE, it has a longer chain and this can also affect the CPP and consequently influence forming of inverted hexagonal phase (H_{II}) of the liposomes leading to destabilising the bilayer and aggregation of the lipids in PBS (Regelin et al., 2000; Wasungu and Hoekstra, 2006)

3.2.3. Formulation of DRV and the influence of electrolytes

This work investigated the effect of salt on characterisation properties of different liposomes prepared in the DRV process. For this purpose, DRV liposomes of DOPE:DOTAP, DOPE:DSTAP and DSPE:DOTAP were prepared in both aqueous phases of distilled water and PBS and their size and zeta potential measured.

Figure 3.4A show the vesicle size of DOPE:DOTAP formed in dH_2O or PBS and in different forms of MLV, SUV and DRV. Results show presence of salt in the formulation did not effect the vesicle size of the DRV DOPE:DOTAP, as it is ≈ 570 nm in both hydration media (Figure 3.4A). The same trend has been shown for DOPE:DSTAP, as the vesicle size of DRV remains ≈ 750 nm for the liposome in each hydration media. This suggests that presence of salt does not influence on the size of DRV liposomes.

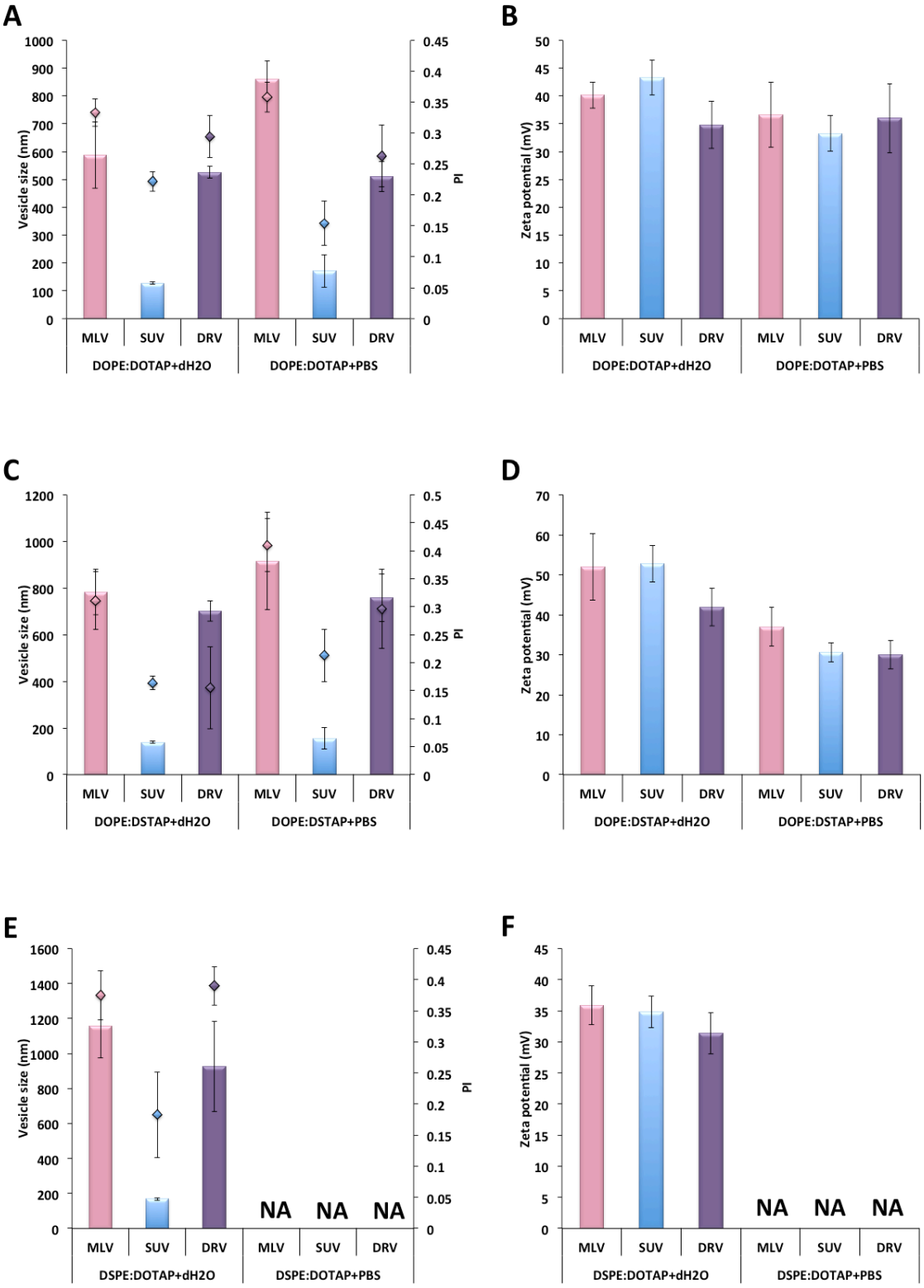

Figure 3.4: Comparison between MLV,SUV and DRV characteristics of DOPE:DOTAP**(A,B)**, DOPE:DSTAP **(C,D)** and DSPE:DOTAP **(E,F)** in PBS and dH_2O: **(A,C,E)** Particle size vs Polydispersity Index, **(B,D,F)** Zeta potential. Results represent mean ± SD, from 3 independent batches.

Results also reveal there is no significant change in the surface charge of liposomes due to change of the hydration media (Figure 3.4B and D). The zeta potential for DRV DOPE:DOTAP in both hydration media was ≈ 35 mV (Figure 3.4B) while it is 40 and 35 mV for DRV of DOPE:DSTAP formed in distilled water and PBS respectively (Figure 3.4D).

Investigating the effect of replacing DOTAP by DSTAP revealed that size and zeta potential of liposomes, when hydrated in dH_2O, has not changed significantly (Figure 3.4A & C). The same study was performed in the liposomes hydrated in PBS and as results in Figure 3.4A & B and Figure 3.4C & D show, there is no significant change in size or zeta potential of liposomes.

Comparison of size and zeta potential of DSPE:DOTAP and DOPE:DOTAP liposomes when prepared in distilled water demonstrated there is no significant change in vesicle size of MLV and SUV, although in DRV vesicle size significantly ($P<0.05$) increased from ≈525 nm in DOPE:DOTAP to ≈925 nm in DSPE:DOTAP. Zeta potential has decreased for all types of DSPE:DOTAP but it is not significant (Figure 3.4B and F).

The most notable change, which was observered in all of the studied formulations, was the increasing effect of freeze-drying and rehydration on vesicle size of the liposomes. This is mostly due to aggregation of the vesicles after rehydration of freeze-dried liposomes. The stability of liposomes depends on hydrogen bonds between water molecules and polar head groups of the liposomes. The drying phase of the freeze-drying process can affect these hydrogen bonds and may lead to vesicle fusion, aggregation, and loss of integrity of the liposomes. These all may lead to increasing vesicle size (Crowe et al., 1986), although freeze-drying of the liposome can improve

stability of the formulation (Darwis and Kellaway, 2001) and it has been shown to be more efficient *in vivo* for DNA delivery than SUV (Gregoriadis et al., 2002; Perrie et al., 2001).

3.3. Molecular packaging of lipids: the role of lipid structure and electrolytes

The Langmuir-Blodgett trough was used to investigate single and mixed lipid monolayers for their interactions within the monolayer in the aqueous sub-phase of either dH_2O or PBS, to consider how molecular packaging translates into liposomal systems. Pressure-area (π-A) isotherms for cationic liposome components are shown in Figure 3.5. The extrapolated (to zero pressure) area per molecule and collapse pressure for the individual lipids, in either a water or PBS subphase, and also for 1:1 lipid mixtures for all studied lipids are shown in Table 3.1. For the latter, the ideal extrapolated area per molecule was calculated based on taking the average area for the lipid combination, such that the calculated area could be compared to the actual area per molecule of the mixture. Deviations between the experimentally observed and the calculated ideal area may be considered as the measure of interactions between the mixed components, since the experimentally observed area depends on the intermolecular forces between the lipids in the mixed monolayer. Negative deviations (where the experimental area is less than the ideal calculated area per molecule) indicate attractive interactions occurring between the lipids, whilst positive deviations indicate repulsive interactions.

Considering the single component monolayers formed on dH_2O, the extrapolated area per molecule for each of the 4 lipids was in the order of DOTAP>DOPE>DSTAP>DSPE (Table 3.1), with the cationic lipids (DOTAP and

DSTAP) having a larger area per molecule than their comparable zwitterionic counterparts (DOPE and DSPE respectively; Table 3.1). The cationic lipids also formed liquid-expanded monolayers (Figure 3.5A) with lower collapse pressures than their PE counterparts (Table 3.1). Comparison between the saturated and unsaturated lipids, show that the saturated lipids are able to pack together closer in a solid monolayer than their unsaturated counterparts (Figure 3.5A) with DOTAP having approximately twice the measured molecular area compared to DSTAP (104 vs 53 A^2/molecule; Table 3.1). Due to their closer packaging arrangement, the saturated lipids also display a higher collapse pressure and a more rigid monolayer than their unsaturated counterparts (Table 3.1 and Figure 3.5A). Formation of these monolayers on PBS rather than dH_2O made no notable difference in the measured area per molecule, however this did result in an increased collapse pressure in the case of the cationic lipid monolayers, particularly in the case of DOTAP, which increased from 29.5 to 42.1 mN/m (Table 3.1 and Figure 3.5B). This resulted in their being no significant difference in collapse pressures between the cationic and zwitterionic lipid monolayers when formed in PBS (Table 3.1).

When prepared as mixed monolayers at a 1:1 molar ratio (as is commonly adopted in lipoplexes) the extrapolated area per molecule for the combinations was in the order of DOPE:DOTAP>DOPE:DSTAP ≈ DSPE:DOTAP>DSPE:DSTAP, with the combination of two unsaturated lipids giving the highest mean molecular area, whilst the fully saturated mixture (DSPE:DSTAP) had a smaller mean molecular area of 46 A^2 per molecule (Table 3.1), and formed a solid monolayer similar to the individual components (Figure 3.5). Of the 4 mixed monolayers, the DSPE:DSTAP monolayer also had the highest collapse pressure (52.9±1.3 mN/m; Table 3.1). When the dH_2O subphase was replaced with PBS, there was a notable increase in the extrapolated area per molecule for DSPE:DOTAP, suggesting the presence of buffer salts was inhibiting

the packaging of the monolayer, yet the collapse pressures were not influenced by the change in subphase (Table 3.1).

Considering the deviation from ideality (Table 3.1), which can be used to monitor molecular interactions between the molecules in the mixed monolayers, for those lipid monolayer containing either both saturated (DSPE:DSTAP) or both unsaturated (DOPE:DOTAP) lipids the deviation is minimal, irrespective of the choice of subphase (Table 3.1), suggesting there was no condensing effect occurring in either type of monolayer. In contrast, for the monolayers combining a saturated and an unsaturated lipid (DOPE:DSTAP or DSPE:DOTAP) there are large positive deviations from the calculated mean area, suggesting the lipids in these mixed monolayers packed in a more expanded arrangement than was predicted, particularly when the systems were in PBS as the deviation was > 30 % for both DOPE:DSTAP and DSPE:DOTAP (Table 3.1). However, these differences do not translate into changes in collapse pressures, with both mixed monolayers having the same collapse pressures in dH_2O as they did in PBS (Table 3.1).

Saturated long chain lipids often display strong attractive intermolecular interactions and this is supported by the small molecular area and high collapse pressure of the saturated monolayer and this might suggest that the DSPE:DSTAP combination could give a strong low permeability liposome system, as has previously been shown with water soluble drugs entrapped within vesicles (Hac-Wydro and Wydro, 2007; Hac-Wydro et al., 2004; Hac-Wydro et al., 2007). However, in the case of unsaturated lipids, these lipids are more bulky (as shown by their larger molecular area; Table 3.1) with a less densely packed arrangement, that can cause a more permeable liposome bilayer

with higher release profile of entrapped drug compared to saturated monolayers (Ali et al., 2010)

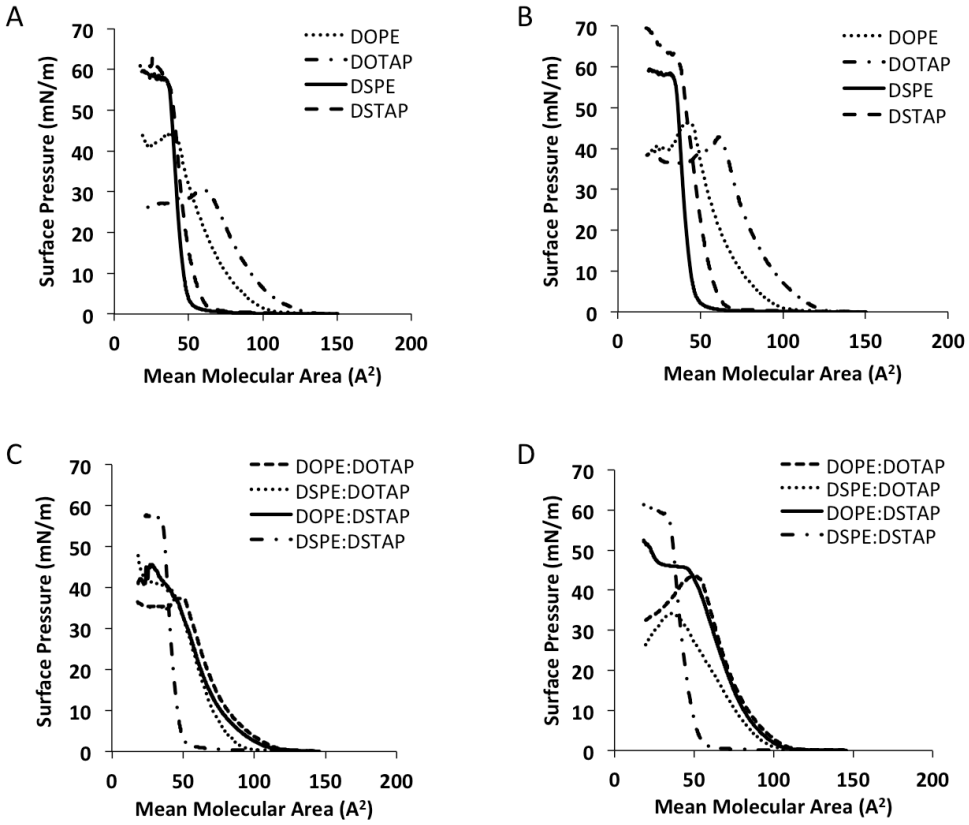

Figure 3.5: Compression isotherm studies of the single and mixture of lipid monolayers of DOPE:DOTAP, DSPE:DOTAP, DOPE:DSTAP and DSPE:DSTAP in deionised water (A and C) or PBS (B and D) at 20 °C. Results are expressed as the means of three experiments. SD has not shown for clarity.

From these results, it would suggest that the use of a fully saturated system promotes the higher packing density of lipids with high collapse pressure and which may promote a more rigid liposome system. In contrast, liposomes formed in PBS from lipid mixtures containing unsaturated lipid(s) in the mixture (either the helper lipid or the cationic lipid) could result in liposomes with less rigid bilayers (Table 3.1 and Figure 3.5). However, given lipoplexes require both stability on storage and fusogenic properties, the consideration of how such monolayer attributes translate into liposome formulation and transfection attributes was considered. In addition, this study was performed on

monolayers of the single and mixed lipids of DOPG and DSPG on dH_2O as a subphase. As with the cationic lipids, the molecular area of the saturated single lipid of DSPG is almost half of the molecular area for the unsaturated DOPG (44.9 Vs 80.1 A^2/Molecule; Table 3.1). Interestingly, the trend for collapse pressure of the anionic lipid monolayers follows the cationic lipids as saturated lipid of DSPG shows higher collapse pressure (52.8 mN/m) than unsaturated DOPG with the collapse pressure of 39 mN/m (Figure 3.6B and Table 3.1).

Table 3.1: The experimental extrapolated area and area compressibility of mixed and single monolayers at the air/aq media interface at 20 °C in dH_2O or PBS as sub-phase. Results denote mean ± SD, n=3.

Lipid	Extrapolated Area (A^2/Molecule)		Ideal Extrapolated Area (A^2/Molecule)		Deviation from Ideality (%)		Collapse Pressure (mN/m)	
	dH_2O	PBS	dH_2O	PBS	dH_2O	PBS	dH_2O	PBS
DOPE	71.9±6.0	70.6±7.7	-	-	-	-	42.3±0.4	42.2±2.6
DOTAP	104.3±12.9	93.4±10.1	-	-	-	-	29.5±1.5	42.1±0.9
DOPG	80.1±2.2	-	-	-	-	-	39.0±0.4	-
DSPE	47.6±0.5	45.7±2.3	-	-	-	-	55.7±0.5	53.5±1.0
DSTAP	53.2±2.5	53.0±2.1	-	-	-	-	50.3±3.1	55.9±0.8
DSPG	44.9±1.6	-	-	-	-	-	52.8±1.4	-
DOPE:DOTAP	89.7±6.5	81.3±5.3	88.1	82.0	+1.8	-0.9	38.4±1.5	42.7±0.9
DOPE:DSTAP	81.6±0.6	87.4±1.6	62.6	61.8	+30.5	+41.4	38.8±2.3	36.1±2.4
DSPE:DOTAP	80.6±1.5	91.7±1.4	75.9	69.5	+6.1	+31.9	37.7±0.3	34.5±0.7
DSPE:DSTAP	46.4±0.7	48.6±0.2	50.4	49.3	-7.9	-1.5	52.9±1.3	54.4±1.2
DOPE:DOPG	92.8±1.8	-	76.0	-	+18.1	-	45±0.14	-
DSPE:DSPG	54.6±1.2	-	46.3	-	+15.2	-	54.1±0.1	-

As it is shown in Figure 3.6C, the collapse pressure of the mixture of unsaturated lipids (DOPE:DOPG) are significantly lower ($P<0.05$) than saturated DSPE:DSPG and, as the cationic lipids, the molecular area of the mixture of unsaturated lipids (DOPE:DOPG) is nearly double the area for saturated lipid monolayers (92.8 A^2/Molecule Vs 54.6 A^2/Molecule; Table 3.1).

These results also show that irrespective of the charge of the lipid (anionic vs cationic), saturated lipids have a condensed structure with highly attractive intermolecular

interactions causing their small molecular area and high collapse pressure compared to the unsaturated lipids. However, in contrary to the mixture of fusogenic and cationic lipids, there is high positive deviation for DOPE:DOPG and DSPE:DSPG (+18 and +15 respectively), showing that in the case of anionic lipid, the helper lipid has more effect in changing the molecular packaging of the monolayers compared to the cationic lipids, making the monolayers more expanded rather than condensed. This may explain why it is possible to form vesicles from the DSPE:DSPG combination (Table 3.2) but not from DSPE:DSTAP (Section 3.2.2).

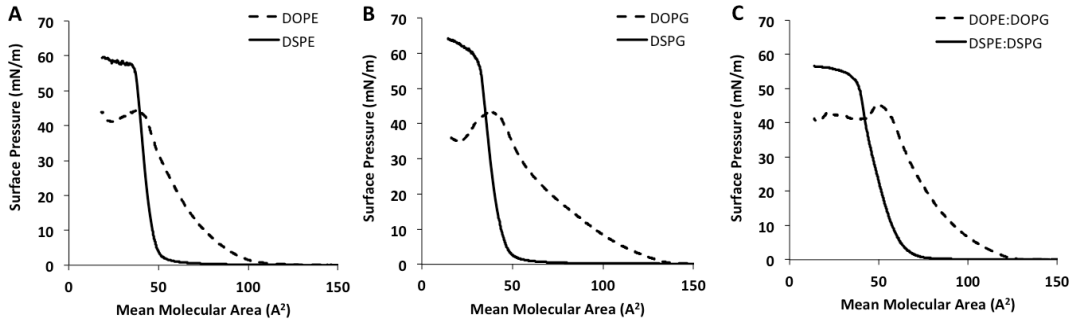

Figure 3.6: Compression isotherm studies of the **(A)** single neutral lipids of DOPE and DSPE, **(B)** single anionic lipids of DOPG and DSPG, and **(C)** mixture of lipid monolayers of DOPE:DOPG, DSPE:DSPG at 20 °C. Results are expressed as the means of three experiments. SD has not shown for clarity.

Table 3.2: Characteristic properties of the anionic liposomes of DOPE:DOPG and DSPE:DSPG both hydrated in distilled water and with the lipid ratio of (8:8 μmol).

Formulations	Vesicle size (nm)	Polydispersity Index	Zeta potential (mV)
DOPE:DOPG	80.9 ± 5.5	0.243 ± 0.007	-57.0 ± 2.55
DSPE:DSPG	88.8 ± 17.3	0.283 ± 0.034	-39.3 ± 14.9

3.4. The effect of alkyl chain and electrolytes on the characteristics of lipoplexes

To investigate the lipid properties on the lipoplex attributes, SUV liposomes prepared from DOPE:DOTAP, DOPE:DSTAP, DSPE:DOTAP and DSPE:DSTAP (all equimolar) and DRV liposomes of DOPE:DOTAP and DOPE:DSTAP (both equimolar) were formulated in distilled water or PBS and mixed with plasmid DNA (gWiz™ Luciferase) at a range of concentrations (2.5, 25, 50, 100, 200 and 1600 μg) for SUV liposomes and (2.5, 50, 200 and 1600 μg) for DRV liposomes.

As previously noted, of the four combinations, it was not possible to formulate liposomes from the combination of DSPE:DSTAP, suggesting that whilst this combination can form a closely packaged solid monolayer, this could not be translated into a liposomal bilayer. Similarly DSPE:DOTAP liposomes could only be formed in dH_2O and not in PBS. As mentioned, saturated lipids often display strong attractive intermolecular forces, which can make hydration and dispersion in water difficult (Regelin et al., 2000; Wasungu and Hoekstra, 2006), hence the difficulty in formulating liposomes form DSPE:DSTAP. The introduction of double bonds into the lipid tail (i.e. replacement of DSPE with DOPE or DSTAP with DOTAP) results in a less compact system, which is easier to disperse in water (Wasungu and Hoekstra, 2006). The inability of DSPE:DOTAP to form vesicles in PBS may be due to the effect of salt on critical packaging parameter (CPP) of the lipid which is dependent on length of hydrocarbon chain, volume of hydrophobic part and the surface area per molecule (Israelachvili and Mitchell, 1975; Israelachvili et al., 1977). Dispersion of lipid molecules in the water can lead to different structures such as micelles, inverted micelles, hexagonal, lamellar or cubic phase as well as liquid crystalline with CPP being a useful predictor of the structures formed. The desired CPP shape for lipids to form

liposomes is a truncated cone shape (a CPP of between ½ and 1); however, studies show that liposomes can form from lipids which individually do not have the truncated cone shape, but when combined are able to appropriately pack (Israelachvili and Mitchell, 1975; Israelachvili et al., 1977). In the case of DSPE:DOTAP, in water the molecular shape of DOTAP (with its large tail area and cationic head-group) may be able to compensate for the smaller DSPE molecular volume, however in the presence of buffer this can reduce the electrostatic nature of the DOTAP headgroup (Wasungu and Hoekstra, 2006), therefore changing the 'shape' of the molecule, which means it is not able to compensate for DSPE, thus prohibiting the formation of liposomes.

3.4.1. Characterisation studies of lipoplexes

In all formulations, vesicle size increased with increasing DNA concentration (Figure 3.7 and 3.8) suggesting aggregation of the system due to what has previously been attributed to a bridging effect (Ciani et al., 2004) and/or a re-organisation of the system (Elouahabi and Ruysschaert, 2005; Huebner et al., 1999; Tarahovsky et al., 2004; Weisman et al., 2004), which is highly dependent on the +/- charge ratio (Zuhorn et al., 2007) to larger sized constructs.

With the SUV DOPE:DOTAP formulation, the presence of the buffer salts in PBS made no significant difference in vesicle size, except for the highest DNA concentration tested; lipoplexes were around 300 nm if formulated in water compared to ~1800 nm when prepared in PBS (Figure 3.7A). In the case of the SUV DOPE:DSTAP lipoplexes, the presence of PBS was shown to significantly ($p < 0.05$) increase the size of the lipoplexes at all DNA concentrations and of the 4 SUV formulations, DOPE:DSTAP in PBS gave the largest lipoplexes (Figure 3.8B). Previous studies have also shown the size of the liposomes can increase to double the size in the presence of PBS, as the phosphate

group of the PBS can act as a glue and increase the vesicle size by bridging the cationic polar heads of the lipid (Ciani et al., 2007). Another study reveals cationic liposomes have a higher tendency to aggregate when there is salt in the formulation, due to the reduced electrostatic interactions between systems and consequently more aggregation and larger vesicles formed (Wasan et al., 1999). The difference in size between the formulations may be again due to the molecular packaging of the lipids, as more rigid assemblies, such as those formed from saturated cationic lipids, have been shown to preclude efficient re-organisation of this system, causing aggregation of large particles to form with lower transfection efficacy presumably due to reduced internalisation (Wasungu and Hoekstra, 2006). The same trend of results has been seen for DRV lipoplexes, where presence of salt in formulations caused a significant increase in the vesicle size of the lipoplexes at the highest DNA concentrations for both DOPE:DOTAP and DOPE:DSTAP (Figure 3.8A & B). Zeta potential studies of the systems were used to estimate the level of interaction between negative charges of DNA and positive charges of cationic lipid (Ma et al., 2007). As this electrostatic interaction is one of the basic components of DNA complexation, and the net charge of lipoplexes is important for overcoming cell barriers (Uyechi-O'Brien and Szoka, 2003; Wasungu and Hoekstra, 2006), zeta potential studies were performed to determine the surface charge of the lipoplexes.

Results illustrate that increasing the amount of DNA over the range used had little effect on the cationic nature of the lipoplexes when formulated in dH_2O, presumably due to the high cationic/anionic charge ratio (Figure 3.7 and 3.8). However, in PBS, the neutralising effect of the buffer electrolytes on the zeta potential can be seen, particularly for SUV DOPE:DSTAP, where the zeta potential reduces from ≈ 60 mV in distilled water formulations to ≈ 30 mV and less in PBS formulations (Figure 3.7E). For DRV

lipoplexes, zeta potential did not change significantly under the PBS effect or by having extra DNA in the formulation (Figure 3.8C & D).

The electrostatic interaction between positive charges of the cationic liposomes and negative charges of phosphate groups of DNA are reported as the main interaction in formation of lipoplexes (Ciani et al., 2007; Ciani et al., 2004). Some other researchers have added that packing properties of the lipids used in the formulation of lipoplexes may also play a role in condensation of the nucleic acid (Akao et al., 1996; Bennett et al., 1998). However, as it has been shown in Figure 3.7 and previously by Ciani et al. (2004) that the zeta potential of the lipoplexes are close to the zeta potential value of the pure liposomes at lower DNA concentrations, it can be concluded that half of cationic molecules of the liposomes are involved in the electrostatic interaction. These molecules are external lipids, which are located on the surface of the liposomes, and when the liposomes have been wrapped by DNA, the internal cationic lipids remain intact (Ciani et al., 2004). The effect of the buffer electrolytes in reducing the cationic charge of the lipoplexes can be due to the additional effect of phosphate polyanion in PBS, which reduces the positive charge of cationic lipids and in some cases results in their precipitation (Li and Hui, 1997), however a reduction in surface charge may be beneficial to allow appropriate DNA dissociation from the lipoplex after cellular uptake to allow the DNA to reach to the nucleus (Zhdanov et al., 2002).

All five of the liposome formulations gave high DNA complexation across the DNA concentration range tested (Figure 3.7G-I and 3.8E & F) with % DNA association being > 95 % in all cases, showing all systems were able to electrostatically interact with the DNA as would be expected. However, the effect this complexation had on the formed lipoplexes was dependent on the lipid combination and the choice of aqueous buffer (Figure 3.7 and 3.8).

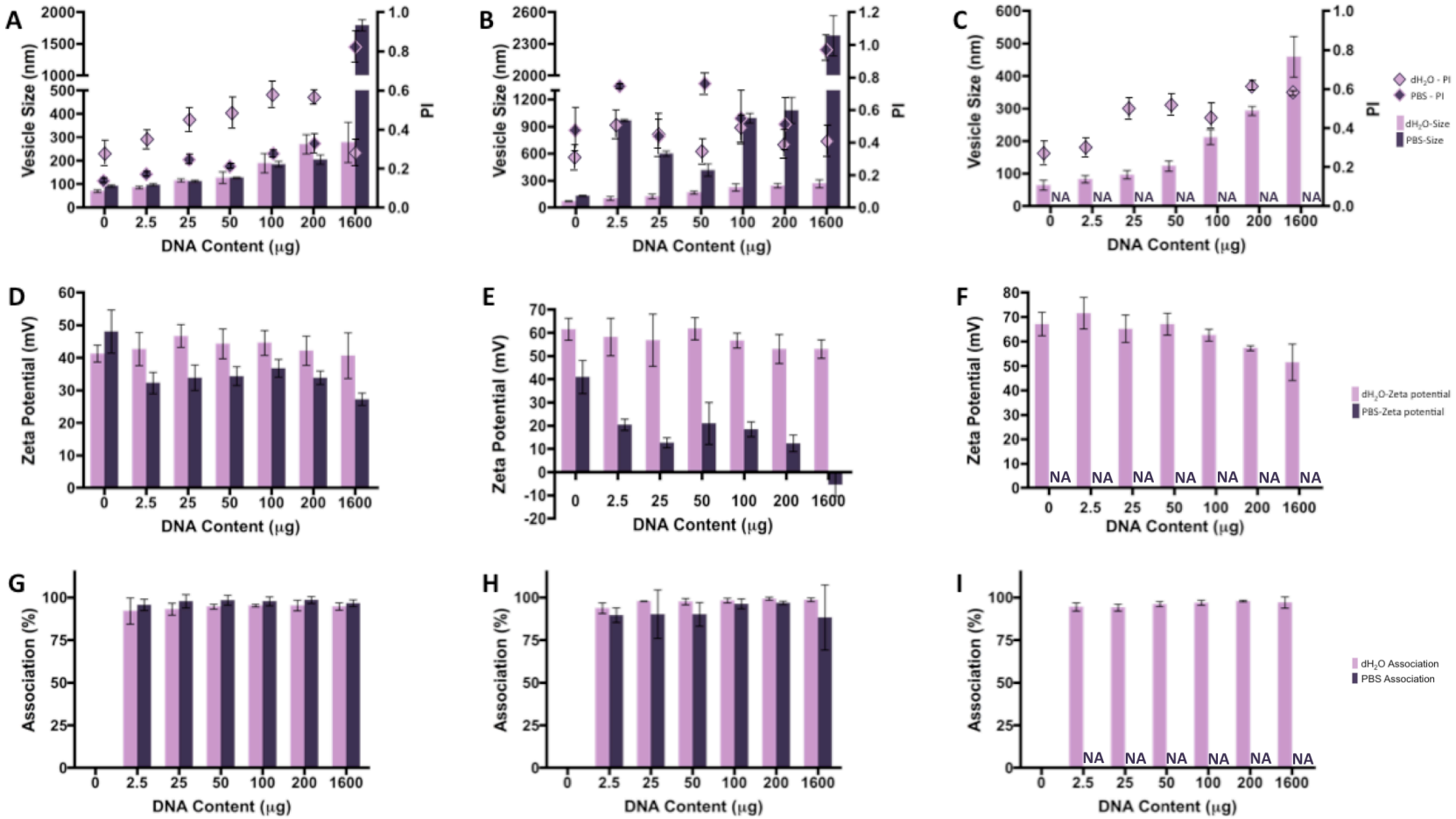

Figure 3.7: A,**B** and **C** demonstrate vesicle size and polydispersity index, **D**, **E** and **F** represent zeta potential and **G**, **H** and **I** show DNA association within SUV lipoplexes of DOPE:DOTAP, DOPE:DSTAP and DSPE:DOTAP hydrated in either dH_2O or PBS respectively. Results denote mean ± SD, from 3 independent batches.

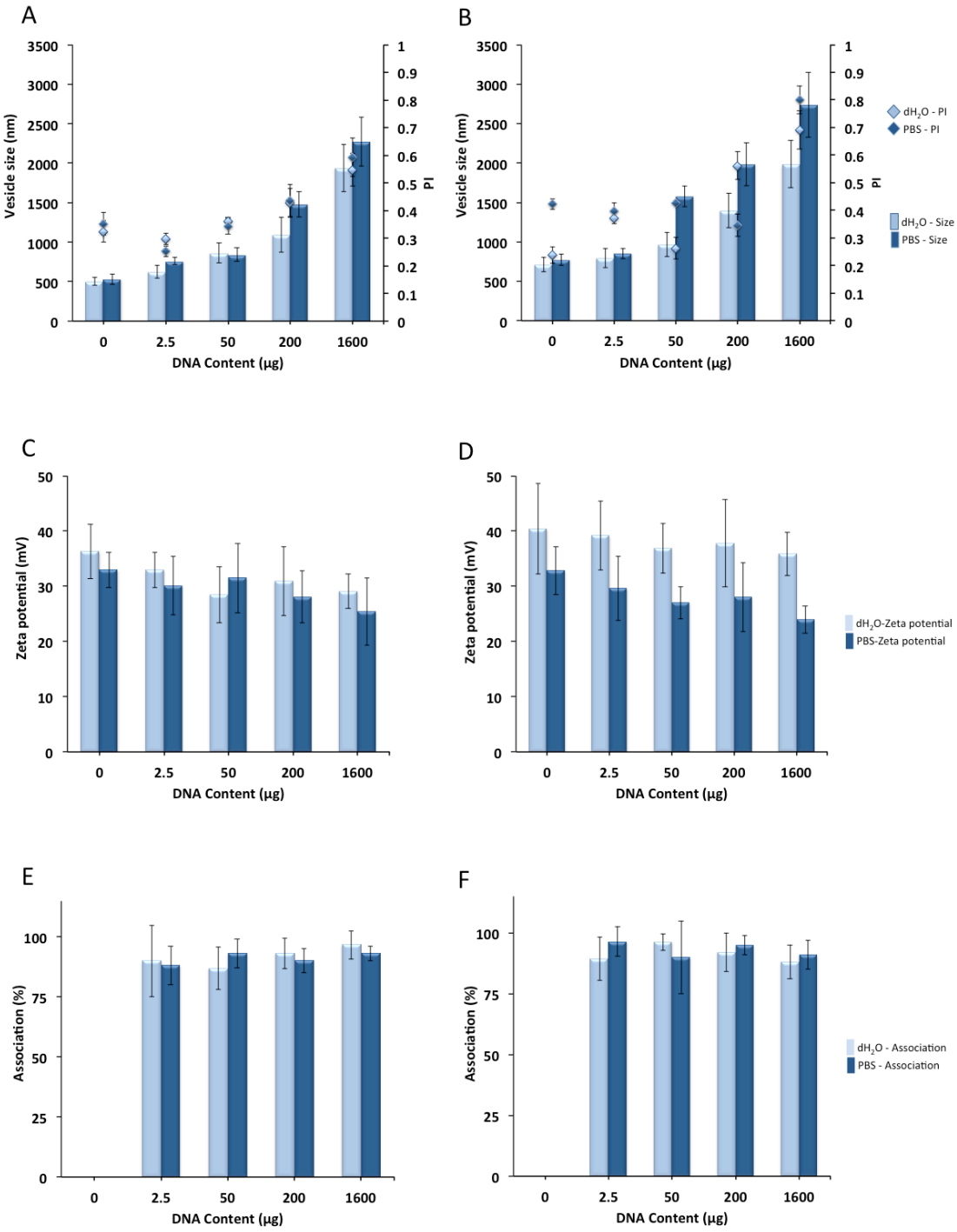

Figure 3.8: Characterisation studies of DRV lipoplexes of DOPE:DOTAP and DOPE:DSTAP in dH_2O or PBS.**A**,**C** and **E** demonstrate vesicle size and Polydispersity index, zeta potential and DNA association within DRV of DOPE:DOTAP lipoplexes respectively. Graphs **B**, **D** and **F** show the same information for DRV DOPE:DSTAP. Results denote mean ± SD, from 3 independent batches.

3.5. Conclusions

Whilst effectively considering only half a bilayer in a flat rather than curved structure, Langmuir-Blodgett monolayer studies on lipid mixtures have shown to offer a variety of applications to support our continued appreciation of (nano)mechanical properties of liposomes and further underpin our understanding of liposomal drug delivery systems.

Investigating the effect of lipid structure on liposome characteristics revealed that using two saturated lipids, such as DSPE and DSTAP, failed to form a liposome due to their highly compact molecular packaging. Monolayer studies on single lipids as well as mixture of lipids showed that saturated lipids have closer packaging arrangements than their unsaturated counterparts; whilst the use of lipids that form condensed monolayers may be beneficial in formulating low permeability bilayers, lipid combinations that form highly compact monolayers are not a suitable choice for the formulation of liposomes. Molecular packaging of the lipids also effects the size as the liposomes composed of saturated cationic lipids, which are more rigid, have been shown to cause aggregation of large particles and increase the vesicle size of the formulation.

Studying the effect of the hydration buffer on liposomal characteristics demonstrated that presence of salt in the hydration buffer might increase the vesicle size of the lipoplex, especially for SUV lipoplexes and in their higher DNA concentrations. This can be due to the reduced electrostatic interactions between systems. Also, the phosphate group of the PBS increases the vesicle size as it initiates the bridging of the cationic polar heads of the lipid. Moreover, presence of salt in the formulation, prevented DSPE:DOTAP to form a liposome as salt can reduce the electrostatic nature of DOTAP headgroup and change the critical packaging parameter of the lipid, which prohibits the formation of liposomes.

Chapter 4:

In vitro Characterisation of DNA liposomes

Papers relating to this chapter:

Moghaddam, B., McNeil, S.E., Zheng, Q., Mohammed, A.R., Perrie, Y., 2011. Exploring the Correlation Between Lipid Packaging in Lipoplexes and Their Transfection Efficacy. Pharmaceutics, 3, 848-864.

4.1. Introduction

Although it has been known for some time that direct injection of 'naked' DNA allows transgene expression in muscle (Vassaux et al., 2006), in most of the cases 'naked' DNA molecules are not able to enter cells efficiently due to their large size, negative charge and nuclease mediated degradation *in vivo* (Al-Dosari and Gao, 2009). Whilst this route of DNA delivery has been explored as a potential for DNA vaccination, the immune responses generated are low, as only a fraction of DNA is taken up by cells (McNeil et al., 2010). Therefore, a delivery vehicle (vector) must be used to carry the gene into the target cell to increase the gene transfection, and consequently increase immune responses to the DNA-encoded antigen. To achieve this, there are a range of possible vectors available, and within the non-viral systems, cationic liposomes have been heavily explored as a delivery vehicle due to their ability to protect DNA, and promote higher cell transfection (Gregoriadis, 1990).

It is clear there are many factors which contribute to the efficacy of such systems (McNeil et al., 2010); to develop liposome-DNA complexes with high transfection and low toxicity, several parameters should be taken into account such as: size, lipid/DNA ratio, the net positive charge of the lipoplexes, the chemical structure of cationic lipid and helper lipid, and finally the structure of the complex itself (Congiu et al., 2004; McNeil et al., 2010). However, given the dynamic nature of these structures, many factors, in addition to those already listed, can contribute to the resultant physicochemical attributes of the lipoplexes including the rate of mixing of the various components, the temperatures used and even the presence of electrolytes in the buffers used (Congiu et al., 2004; McNeil and Perrie, 2007). Whilst many of these factors have now been investigated in detail, the influence of electrolytes has received little attention, despite reports of its influence on cationic-DNA formulations efficacy both *in vitro* and

in vivo (Zhou and Huang, 1994b). Therefore, the aim of this chapter was to investigate the role of electrolytes in the *in vitro* activity of a commonly employed cationic liposome transfection agent (DOPE:DOTAP). To achieve this, the impact of the presence of electrolytes, in addition to the choice of cationic lipid and the type of liposome construct, on the liposomal-DNA formulation characteristics and their transfection efficiency were investigated.

4.2. Formulations investigated.

To investigate the characterisation and transfection efficiency of different lipoplexes, SUV and DRV lipoplexes containing 16 µmole lipid and 1.6 mg DNA were prepared, as previously outlined and discussed in Chapter 3. However, for reference, the data is summarised again in in Table 4.1, with specific reference to the DNA/lipid ratio used in the forthcoming studies.

Table 4.1: Characterisation study results including vesicle size, Polydispersity index, zeta potential, and loading efficiencies of SUV and DRV lipoplexes. Results denote mean±SD and n = 3 for three independently prepared batches.

Liposome Formulation	Aqueous Media	Vesicle Size (nm)	Polydispersity Index	Zeta Potential (mV)	Loading (%)
SUV DOPE:DOTAP	dH_2O	278.4 ± 86.1	0.319 ± 0.1	40.7 ± 6.9	94.7 ± 2.2
	PBS	1792.7 ± 87.4	0.809 ± 0.2	27.3 ± 1.9	96.6 ± 1.9
SUV DOPE:DSTAP	dH_2O	263.7 ± 44.8	0.405 ± 0.1	53.1 ± 3.9	98.6 ± 1.1
	PBS	2378.1 ± 193.2	0.948 ± 0.1	-5.4 ± 4.9	88.2 ± 19.1
SUV DSPE:DOTAP	dH_2O	458.9 ±62.4	0.579 ± 0.1	51.5 ± 7.5	97.3 ± 3.4
DRV DOPE:DOTAP	dH_2O	1937.2 ± 298.4	0.548 ± 0.1	29.1 ± 3.1	96.7 ± 5.9
	PBS	2272.7 ± 312.4	0.593 ± 0.1	25.4 ± 6.1	93.1 ± 3.2
DRV DOPE:DSTAP	dH_2O	1982.9 ± 310.2	0.593 ± 0.1	35.8 ± 3.9	88.1 ± 7.1
	PBS	2738.1 ± 412.8	0.801 ± 0.1	23.7 ± 2.5	91.3 ± 6.3

As noted in Chapter 3, the formulation of the liposomal-DNA constructs in PBS, rather than distilled water, resulted in larger constructs that are less cationic, presumably due to the condensing of the electrical double layer around the vesicles, resulting from the increased concentration of electrolytes present. However, this did not impact on the DNA loading of the vesicles, as would be expected; electrolytes do not impact on the surface potential of the vesicles, so would not necessarily hinder the binding of DNA to the cationic lipid head-groups, unless the degree of ionisation of the lipids was influenced, which would not occur in the pH range these formulations are in.

4.3. Considering the spatial location of DNA within various liposomal constructs.

Given that SUV are commonly considered to adsorb DNA to their surface, whilst DRV formulations have been reported to incorporate DNA within the vesicles (Gregoriadis et al., 2000; Perrie et al., 2001; Perrie and Gregoriadis, 2000), the spatial localisation of DNA within the cationic SUV and DRV lipoplexes was initially investigated by subjecting the formulations to gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) at 0.05 % concentration, below the critical micelle concentration of the surfactant. It was expected that the anionic SDS would be able to interact with the outer monolayer of the lipoplexes, electrostatically compete with DNA bound to the cationic surface charge and displace it from the surface of the vesicles, releasing the DNA into the medium (Perrie and Gregoriadis, 2000). The effect of electrolytes on the packaging of these liposomal DNA systems was also considered by preparing the formulations in either distilled water or phosphate buffered saline (PBS).

However, initial optimisation studies showed that having 0.05 % SDS in loading buffer was not enough to displace the DNA at the DNA/lipid ratios used (data not shown),

therefore 1.2% SDS was used for the main gel electrophoresis studies. Figure 4.1A shows that, on gel electrophoresis of SUV (1-5) and DRV (6-9) lipoplexes in the absence of anionic molecules, DNA remains within the well of the gel, bound to the cationic liposomes. In contrast, following electrophoresis in the presence of SDS, displaced DNA is seen to migrate towards the cathode (Figure 4.1B). Figure 4.1B also shows that more DNA was displaced from SUV lipoplexes rather than DRV preparations. Considering the lipid composition of the formulations, amongst the SUV lipoplexes, formulations that have DOTAP as their cationic lipid (Figure 4.1B Lanes 1-3) show more DNA loss compared to those containing DSTAP. However, comparison between liposomes of the same lipid composition, but hydrated in PBS instead of dH_2O, showed no notable difference in DNA retention in either 4.1A or B.

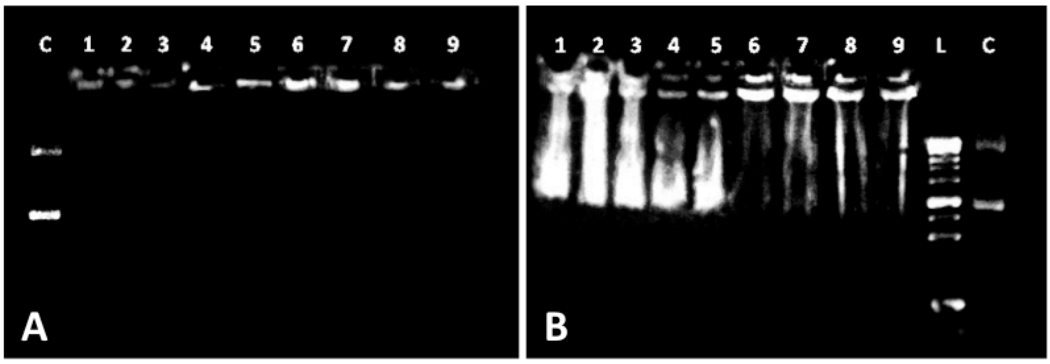

Figure 4.1: A) Gel electrophoresis of SUV lipoplexes of 1)DOPE:DOTAP+dH_2O, 2)DOPE:DOTAP+PBS, 3)DSPE:DOTAP+dH_2O, 4)DOPE:DSTAP+dH_2O, 5)DOPE:DSTAP+PBS, and DRV lipoplexes of 6)DOPE:DOAP+dH_2O, 7)DOPE:DOTAP+PBS, 8)DOPE:DSTAP+dH_2O and 9)DOPE:DSTAP+PBS. Lane (L) represents the DNA ladder and (C) shows the 'naked' DNA. **B)** As in A, but in the presence of 1.2 % SDS.

Having more displaced DNA for SUV liposomes, compared to DRV liposomes, in the presence of the anionic molecules of SDS suggests that most of the associated DNA with SUV lipoplexes are absorbed on the surface of the liposomes. However, for DRV lipoplexes, more DNA may be incorporated within closed bilayers and probably bound to the inner cationic charges of the liposomes, such that it is not accessible for

displacement by SDS. These observations are similar to those previously shown with SUV and DRV lipoplexes formed by DOTAP and DC-Cholesterol (Perrie et al., 2004; Perrie and Gregoriadis, 2000).

However, spatial location of the DNA was not the only controlling factor in these DNA retention studies; SUV formulations composed of DOPE:DSTAP lipoplexes (Figure 4.1B lanes 4,5) show reduced DNA displacement compared to DOPE:DOTAP and DSPE:DOTAP formulations (Figure 4.1B lanes 1-3). Previously, Perrie et al. showed PC:DOPE:DOTAP lipoplexes have more DNA displacement than PC:DOPE:DC-Cholesterol lipoplexes in the presence of anionic molecules of SDS (Perrie et al., 2004). This could be due to the fact that DOPE causes some disability to the formulations, as it promotes membrane fusion to change the packing parameter of the liposomes (Farhood et al., 1995; Wrobel and Collins, 1995). Therefore, the bilayer system made from the combination of DOPE and DOTAP may be less resistant to the destabilisation compared to the lipoplexes containing DSTAP (Figure 4.1B) or DC-Cholesterol (Perrie et al., 2004).

Given that the two hydration media tested showed no notable effect on DNA displacement, this suggests that this parameter may not affect DNA release or transfection efficacy for formulations under investigation. Based on this information, it can be predicted DRV formulations may be better placed to deliver higher DNA loads to cells compared to their SUV counterparts and that DOPE:DSTAP (with both hydration media) formulations may be preferable compared to the lower transition temperature DOTAP formulations. This will be discussed more in section 4.5.

4.4. DNA protection from extracellular enzymes

As mentioned earlier, injection of 'naked' DNA would not cause high cell transfection due to DNA degradation by nucleases such as deoxyribonuclease I (DNase I), so delivery systems such as cationic liposomes are required to protect DNA from hydrolysis and degradation (Gregoriadis et al., 2002; Gregoriadis et al., 1996; Patil et al., 2005). Previously, Gregoriadis et al. (1996) have shown liposomes can protect DNA from degradation by entrapping the plasmid DNA within the liposomes and, as a result, degrading enzymes such as DNase I do not have access to the DNA, so the plasmid DNA would be protected from the enzymes and can perform its therapeutic duty (Gregoriadis et al., 2000; Gregoriadis et al., 1997; Gregoriadis et al., 1996).

To investigate the ability of studied SUV and DRV liposomes to protect the plasmid DNA from degradation by digestive enzymes, as explained in section 2.8, plasmid DNA was incubated with DNase I as either 'naked' (Figure 4.2A-D Lane C) or entrapped within cationic liposomes (Figure 4.2A-D lane 1-9) followed by agarose gel electrophoresis to test DNA integrity of the preparations. Figure 4.2 shows 'naked' DNA, which was not exposed to the DNase I (Figure 4.2 A and B, lane 'C') and the digested 'naked' DNA (Figure 4.2 C and D, lane 'C'). The band of migrated anionic DNA towards the cathode is demonstrated for undigested 'naked' DNA in absence (Figure 4.2A, lane C) and presence of SDS (Figure 4.2B, lane C). However, when 'naked' DNA exposed to the DNase I, there is no visible band on the agarose gel (Figure 4.2C and D lane C) showing the 'naked' DNA was digested by DNase I.

For all the liposomes formulations tested, in the absence of SDS (Figure 4.2A lane 1-9 and Figure 4.2C, lane 1-9), DNA migration is not seen as plasmid DNA remains associated with the liposomes and is retained within the wells of the gel. In the presence

of the competitive anionic molecules of SDS, the liposomes can be disrupted and the plasmid DNA released (Figure 4.2B, lane 1-9). With the liposomes that were exposed to DNase I (Figure 4.2D, lane 1-9), plasmid DNA can still be seen, demonstrating that both the SUV and DRV liposomes were able to protect the DNA from degradation (in contrast to the 'naked' DNA, which is rapidly digested). This is in line with previous studies of different groups (Fenske et al., 2002; Gregoriadis et al., 1996; Wong et al., 2001).

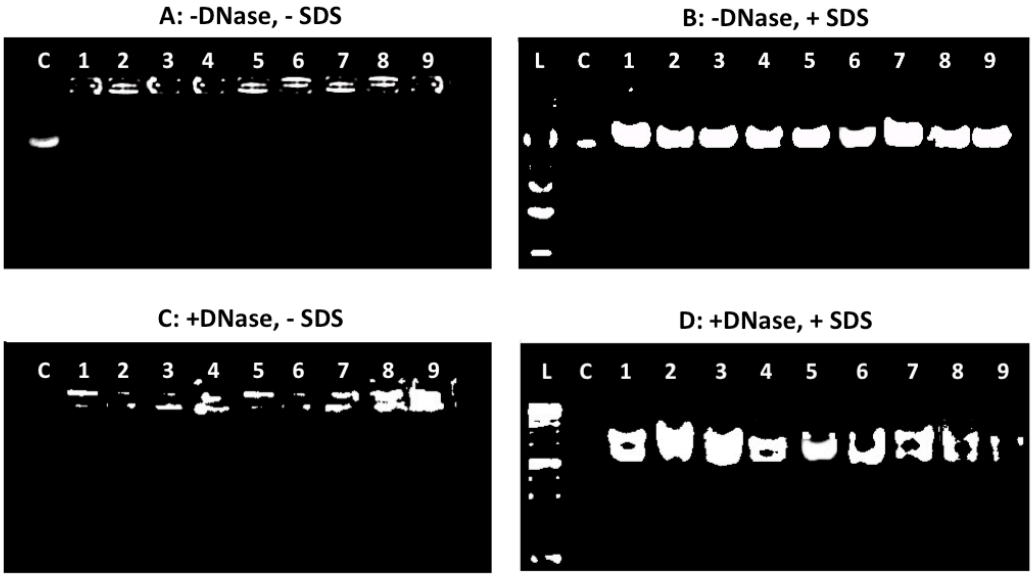

Figure 4.2: Gel electrophoresis of free and liposome entrapped plasmid DNA before and after exposure to digestive enzyme, DNase I. A) Gel electrophoresis of 1) SUV DOPE:DOTAP+dH_2O, 2) SUV DOPE:DOTAP+PBS, 3) SUV DSPE:DOTAP+dH_2O, 4) SUV DOPE:DSTAP+dH_2O, 5) SUV DOPE:DSTAP+PBS, 6) DRV DOPE:DOAP+dH_2O, 7) DRV DOPE:DOTAP+PBS, 8) DRV DOPE:DSTAP+dH_2O and 9) DRV DOPE:DSTAP+PBS before exposure to DNase I with no SDS, B) same as A but in the presence of 2.5% SDS (rather than 1.2 % as in Figure 4.1), C) same as A but after exposure to DNase I with no SDS, D) Same as C but in the presence of 2.5% SDS. Lane (L) represents the DNA ladder and lane (C) represents 'naked' DNA.

As it is shown by Figure 4.2D, this protection role is similar for SUV (lane 1-5) and DRV (lane 6-9) liposomes. In addition, differences in physicochemical characteristics of the lipoplexes such as size, charge, hydration media and the transition temperature of the cationic lipids, are not seen to have an impact on DNA protection from DNase I

digestion. This is a result of the electrostatic interactions of the cationic headgroups of the liposomes interacting with the anionic plasmid DNA, resulting in its condensation (Gregoriadis et al., 2002). This in turn blocks the ability of DNase I to interact with the plasmid and digest it, therefore, in terms of protection, there is no notable difference between surface adsorbed and entrapped formulations.

4.5. *In vitro* DNA release

The release of DNA from different cationic liposomes was investigated in PBS, pH 7.4, 37 °C over an 8 day period (192 hours). Figure 4.3 shows the release profile for SUV (Figure 4.3A) and DRV (Figure 4.3B) formulations. Results show an initial burst release of 30% (of total entrapped DNA) after 4 hours for SUV lipoplexes of DOPE:DOTAP and DOPE:DSTAP in both hydration media. After 48 hours, the amount of released DNA reached to ≈55% for DOTAP SUV lipoplexes, and ≈65% for DSTAP SUV lipoplexes. Beyond this point, DNA release from the lipoplexes appeared to plateau out, with no further significant increase in release. In terms of total release over the time period, DOPE:DSTAP gave an overall higher release than DOPE:DOTAP, and those formulated in dH_2O tended to have lower release rates (Figure 4.3A). In contrast, with SUV lipoplexes, which had a burst release as soon as 4 hours, the DRV formulations showed a more sustained release profile: Figure 4.3B shows the DNA release for all DRV formulations after 24 hours was ≈20% (of total entrapped DNA) and their burst release occurred after 48 hours, with ≈55% of DNA for DRV DOPE:DOTAP lipoplexes (in both hydration media) and DRV DOPE:DSTAP formed in dH_2O being released. Again, comparing between the formulations there was no notable differences in the choice of lipid composition used to prepare the liposomes, however the trend was similar to that of SUV (i.e. DSTAP>DOTAP and PBS>dH_2O), in terms of total release (Figure 4.3B).

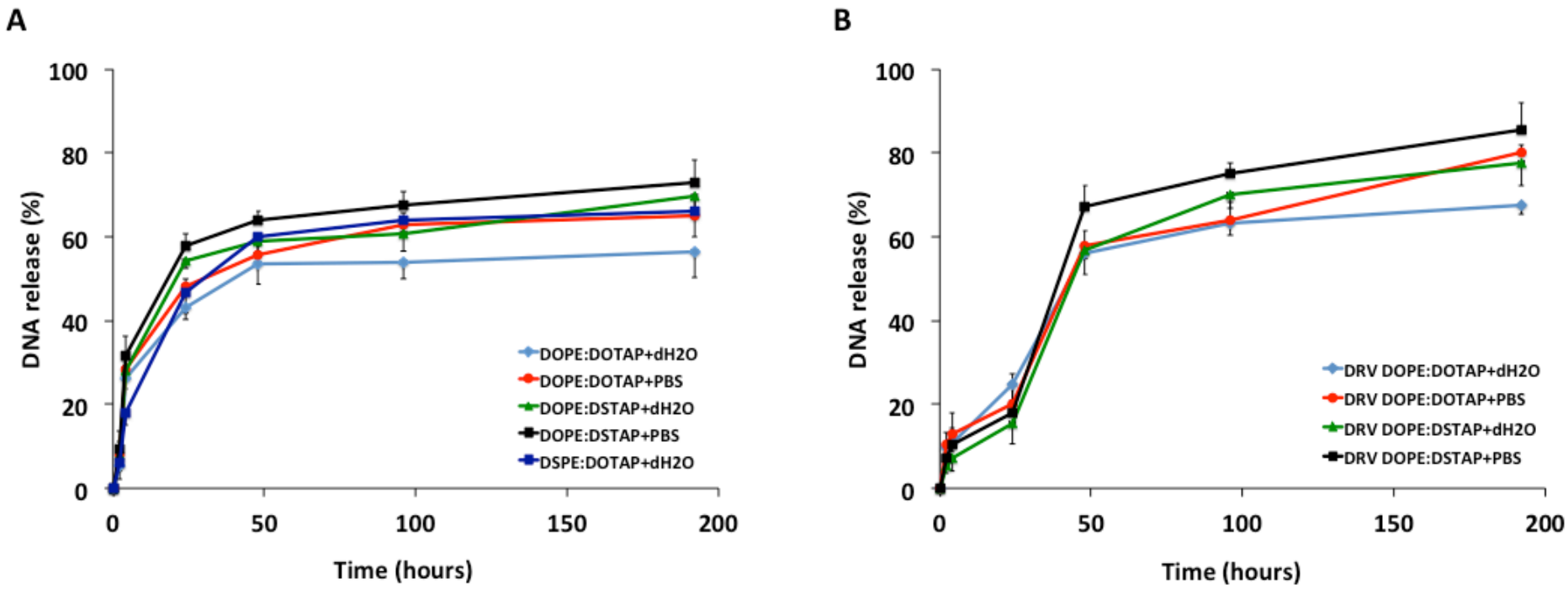

Figure 4.3: DNA release of **A)** SUV lipoplexes of DOPE:DOTAP, DOPE:DSTAP both hydrated in dH_2O or PBS and DSPE:DOTAP in dH_2O and **B)** DRV lipoplexes of DOPE:DOTAP and DOPE:DSTAP in dH_2O or PBS, pH 7.4; at 37 °C at time points of 2, 4, 24, 48, 96 and 192 hours. Results represent percentage release initially loaded DNA expressed as mean±SD, n=3 for three independently prepared batches.

The difference in release profiles between the SUV and DRV could be due to the fact that DNA will be entrapped within the bilayers for DRV formulations, however, for SUV lipoplexes, DNA molecules are likely to be adsorbed on the surface of the liposomes and easier to be detached from the liposomes, therefore the DNA release occurs sooner for SUV lipoplexes. In terms of the differences between the lipid composition, whilst it might be expected that the higher transition temperature DSTAP formulations would have lower release rates (Gregoriadis, 1990), monolayer studies (Section 3.3) showed that having saturated lipids in the formulation makes the bilayer structure more rigid, which may actually reduce liposome-DNA interactions, resulting in higher release rates (Zuhorn et al., 2002) as seen in Figure 4.3.

4.6. *In vitro* transfection efficiency

To investigate the transfection efficiency of different lipoplexes, SUV and DRV lipoplexes containing 16 μmole lipid and 1.6 mg DNA were prepared as outlined in Table 4.1. Performing luciferase assay transfection efficiency of each formulation on COS-7 cell line was compared to the transfection produced by the marketed transfection reagent Lipofectin™.

Figure 4.4A shows the transfection efficiency of each formulation compared to the 'naked' DNA and Lipofectin™ with SUV lipoplexes of DOPE:DSTAP in PBS showing the highest transfection levels. Transfection efficiency of SUV lipoplexes formulated in water were in the order of DOPE:DSTAP > DOPE:DOTAP ≈ DSPE:DOTAP and DOPE:DSTAP > DOPE:DOTAP when formulated in PBS. Among DRV liposomes, DSTAP based formulations in each hydration media have higher transfection efficiency than DOTAP lipoplexes of similar hydration media (Figure 4.4A). All of the lipoplexes have given higher transfection efficiency than Lipofectin™. Cell viability of all

lipoplexes was performed using MTS assay and the cells were exposed to the lipoplexes for 24 hours. Results show the cell viability for all lipoplexes was high and at the concentrations tested there was no significant difference between the formulations (Figure 4.4B).

Among SUV liposomes, DOPE:DSTAP formulated in distilled water produces transfection levels 25 times higher than Lipofectin™ and 21 times higher than DOPE:DOTAP+dH_2O. When DOPE:DSTAP was hydrated in PBS rather than distilled water, transfection efficiency was reduced but was still 15 times higher than Lipofectin™ and 6 times higher than DOPE:DOTAP+PBS (Figure 4.4A).

Considering DRV lipoplexes, DOPE:DSTAP formulated in dH_2O produced 7 fold higher transfection than DOPE:DOTAP formed in dH_2O. The same trend has been shown for DRV lipoplexes formulated in PBS (Figure 4.4A). These results demonstrate that DSTAP lipoplexes no matter if formulated as a SUV or DRV, and in distilled water or PBS, have higher transfection efficiency than the other formulations tested.

Comparing the transfection efficacy of SUV lipoplexes to DRV formulations show there is not an obvious trend for all of the formulations. Transfection levels of DOPE:DOTAP+dH_2O as a SUV lipoplex is significantly lower ($P<0.05$) and almost half of its DRV formulation. However, when DOPE:DOTAP formed in PBS, there is no significant difference in their transfection efficiencies (Figure 4.4A). In contrast, for DSTAP formulations and in both hydration media, SUV lipoplex show more transfection than DRV (Figure 4.4A). In general, decreasing the hydrocarbon chain length has been reported to increase transfection efficiency (McNeil et al., 2010; Writer et al., 2006). However, given both lipids have the same carbon chain length, this case

cannot be a reason for the variation in transfection efficiency between DOTAP and DSTAP liposomes.

Equally, the size, charge and cationic lipid/DNA ratio have all been attributed to play a role in controlling transfection (Aljaberi et al., 2007; Caracciolo et al., 2007). However, in the case of DSTAP and DOTAP SUV, neither the size nor the charge of the system seem to correlate to its higher transfection efficacy, given it is neither notably different in size (e.g., DOPE:DOTAP and DOPE:DSTAP in dH_2O are similar in size (Table 4.1) but have large differences in transfection efficacy), nor cationic nature than the other formulations. Similarly, both systems contain the fusogenic lipid DOPE that may enhance intracellular delivery of DNA (Farhood et al., 1995; Israelachvili and Mitchell, 1975; Israelachvili et al., 1977). The theory of using helper lipid comes from the original research of Felgner et al., (1987). The authors demonstrated that the transfection activity of DOTMA when formulated with DOPE is more than when is formulated with DOPC and it has been proposed the ability of DOPE to promote the transition from lamellar phase to an inverted hexagonal phase (Uyechi-O'Brien and Szoka, 2003) thereby promoting the conversion of the lamellar lipoplex phase into a non-lamellar structure due to the inverted cone-shaped structure of DOPE (Aljaberi et al., 2007; Caracciolo et al., 2007; McNeil and Perrie, 2007; McNeil et al., 2010). After endosomal uptake of the lipoplexes, the presence of DOPE in the formulation is suggested to aid disruption of endosomal membrane, allowing the release of the DNA from the endosome and the lipoplex, leaving it free to enter the nucleus (Ciani et al., 2004; McNeil et al., 2010). However, in this study, lipoplexes formulated with DOPE:DOTAP and DSPE:DOTAP showed no significant difference in transfection levels, suggesting that DSPE may be equally suitable as a helper lipid for unsaturated cationic lipids but not useful when combined with saturated lipids.

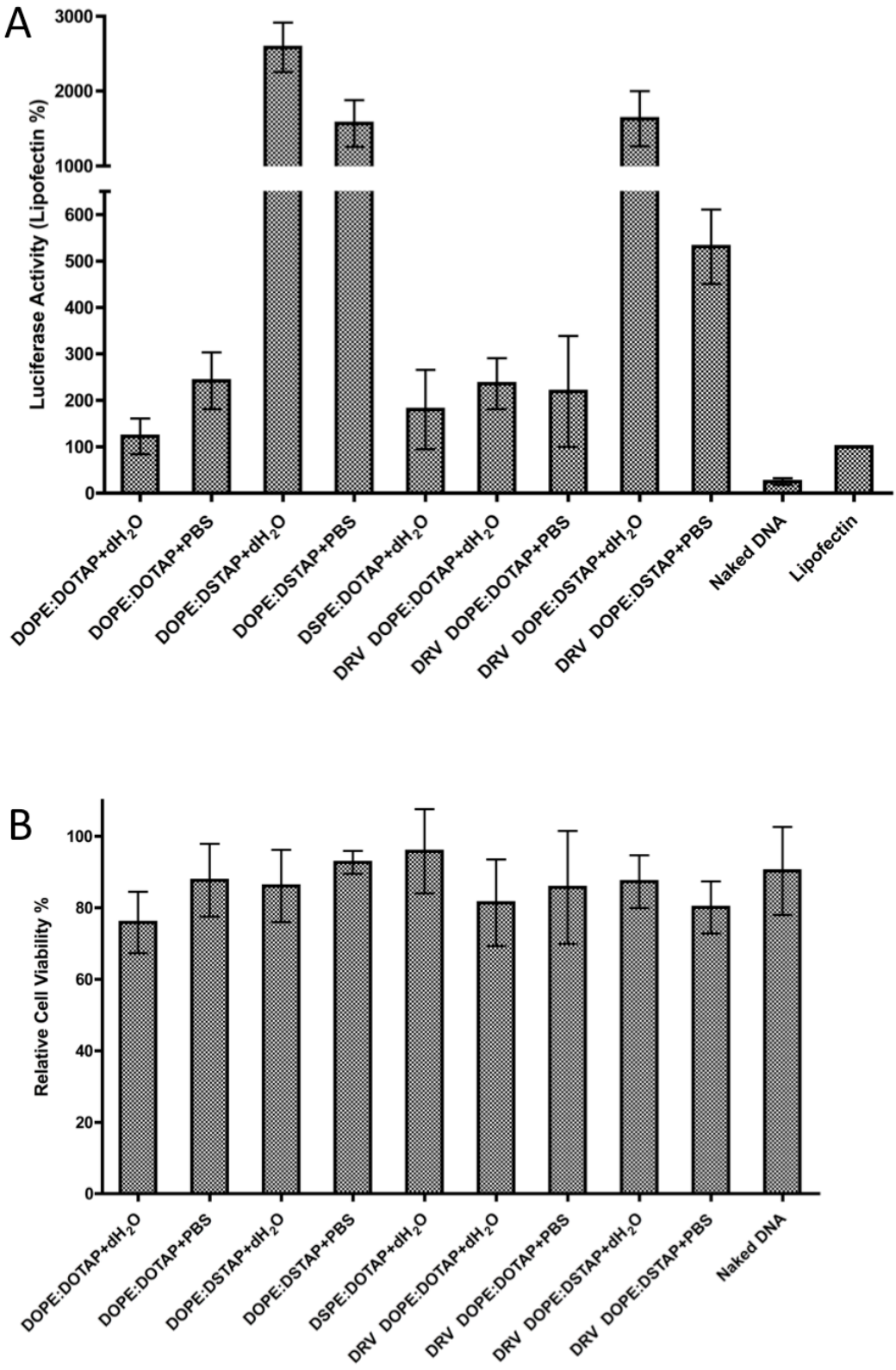

Figure 4.4. A) Comparison of transfection efficiency of five SUV lipoplexes and four DRV lipoplexes on COS-7 cell line by applying luciferase assay. Positive control is Lipofectin™ and negative control is 'naked' DNA. **B)**Comparison of cell viability of five SUV lipoplexes and four DRV lipoplexes on COS-7 cell line and by applying MTS assay. Results denote mean±SD and n = 3 for three independently prepared batches.

It has been reported that saturated cationic lipids make rigid and packed bilayers (Maurer et al., 2001; Ulrich, 2002), and hence show lower transfection efficiency than their unsaturated analogues (Kudsiova et al., 2011; Wasungu and Hoekstra, 2006). However, in this current study, saturated DSTAP has been shown to produce higher transfection efficiency compared to unsaturated DOTAP. Equally, in another study considering cellular uptake of lipoplexes by macrophage cells, DOPE:DSTAP uptake was shown to be higher than DOPE:DOTAP (Filion and Phillips, 1997). As discussed earlier, all the characteristic properties studied for DOTAP and DSTAP based lipoplexes for this study were shown to be similar. In terms of their thermodynamic nature, a previous study (Lobo et al., 2002) has shown that although DSTAP bilayers are rigid and closely packed, addition of DNA to the bilayers, increases their fluidity while decreasing their stability. This may mean that, whilst DSTAP formulations would form more rigid structures, the addition of plasmid DNA may fluidise these bilayers, again mitigating this attribute. Adding the fusogenic effect of DOPE to the system also makes the bilayers more fluidic, allowing the bilayers to be instable enough to be able to produce a comparable *in vitro* transfection. Indeed, Regelin et al., (Regelin et al., 2000) have shown DOPE:DSTAP lipoplexes are highly unstable compared to DOPE:DOTAP. This suggests that, although DSTAP monolayer studies (Section 3.3, Chapter 3) show a high rigidity as a result of their high transition temperature (Lobo et al., 2002; Regelin et al., 2000), addition of DNA could make the lipoplex structure flexible enough to promote high transfection efficiency.

Correlation between monolayer studies and transfection efficiencies of cationic liposomes has been reported earlier (Savva et al., 2005). Moreover, monolayer studies in this lab revealed that the mixture of DOPE:DSTAP in either of subphases (PBS or dH_2O) produced a significantly ($P<0.001$) higher positive deviation compared to

DOPE:DOTAP monolayers (Section 3.3, Chapter 3). It has been reported that positive deviation is related to the less attractive interactions between film-forming molecules (Hac-Wydro and Wydro, 2007; Hac-Wydro et al., 2007), which offers instability to the bilayers and enhances their *in vitro* transfection efficiency. This might explain the higher transfection efficiency of DSTAP-based lipoplexes as shown in Figure 4.4 and also previous research which has shown that lipoplexes formulated with the saturated cationic lipid DPTAP in lipoplex formulation created stronger transfection compared to unsaturated DOTAP (McNeil et al., 2010).

In this current study, considering the effect of presence of salt in the lipoplex formulations did not show an obvious trend for all of the formulations. Previous studies have shown that the impact on transfection of electrolytes present in lipoplex suspensions, is an outcome of the liposomes size, which is influenced by electrolytes, as formation of DNA lipoplex under physiological salt concentration formed large (1 μm) vesicles and liposomes with these large salt-induced aggregates, induced 10-100 fold higher *in vitro* transfection efficiency (Ogris et al., 1998). It has also been shown that increases in vesicle size can enhance transfection efficiency of liposomes (Eastman et al., 1997; McNeil et al., 2010). The correlation of particle size versus transfection in this study have been considered and are shown in Figure 4.5, which demonstrated a very low R^2 equal to 0.015 showing a very poor correlation between vesicle size and transfection efficiency of the formulations.

Among the DOTAP-based SUV formulations, presence of salt in the liposomes hydration media caused an increase in transfection efficiencies of SUV DOPE:DOTAP (Figure 4.4). This is line with the above findings as PBS significantly increased the vesicle size of SUV DOPE:DOTAP (Table 4.1). In contrast, for DRV DOPE:DOTAP,

the presence of salt did not have impact on vesicle size and, as a result, the transfection efficiency of both formulations are similar (Figure 4.4A). This trend is not detectable for DSTAP formulations, as presence of salt caused increase in vesicle size of the liposomes (Table 4.1) but this not only did not increase the transfection of the liposomes but also significantly decreased their transfection efficiency (Figure 4.4A). This shows the vesicle size is not the only decisive parameter and biological or biophysical properties such as mechanism of action for the lipoplexes on their endosmal release may have a role in their transfection efficiencies.

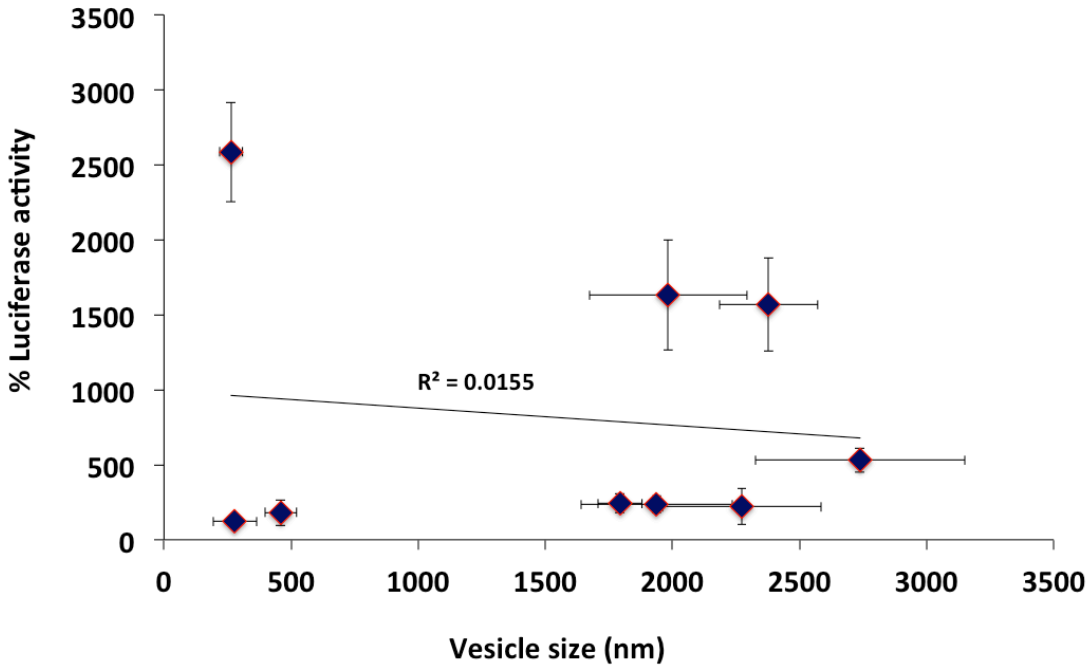

Figure 4.5: Correlation between vesicle size and luciferase activity percentage of tested formulations. Results denote mean±SD and n = 3 for three independently prepared batches.

4.7. Conclusion

Previously in chapter 3, the monolayer studies revealed saturated lipids such as DSTAP would make a condensed monolayer and using this type of lipid may lead to having a bilayer with low permeability. The effect of molecular arrangements on the physicochemical properties of the liposomes was also considered. In theory, it would be expected that monolayer studies along with characterisation studies could help to show correlation with transfection efficacy of the liposomes. However, in terms of transfection efficacy, neither considering the molecular packaging of the lipids with DNA-liposome constructs, nor their basic physicochemical attributes (size, cationic nature, DNA release profiles), have been shown to correlate with transfection efficacy with several general assumptions being shown to be misleading in our studies. Similarly, the role of electrolytes in lipoplex formulations is shown to be dependent on the formulation, with PBS diminishing the transfection of DSTAP systems yet enhancing DOTAP based lipoplexes. Therefore, there remains no clear physicochemical screening that can be adopted to predict *in vitro* efficacy. Combining this with the lack of *in vitro* and *in vivo* efficacy that plagues non-viral delivery systems suggests that for the continued development of non-viral transfection agents, new tools are needed to rationalise these differences.

Therefore, whilst there is not clear link between characterisation and *in vitro* performance, formulations to progress as potential DNA delivery systems *in vivo* had to be selected. Thus, based on *in vitro* studies, the formulations which were shown to be more effective were selected. SUV DOPE:DOTAP and SUV DOPE:DSTAP in both hydration media were chosen, along with the most efficient DRV, which was DOPE:DSTAP+dH_2O, to investigate their potential as delivery systems for DNA vaccines.

Chapter 5:

In vivo studies on DNA Vaccine Formulations

5.1. Introduction

Vaccination remains one of the most effective ways of supporting the health of a population, with several effective vaccines playing a key role in global healthcare. Indeed the WHO vaccination campaign against Smallpox, initiated in 1967 (Fenner, 1993) ensured the complete eradication of this disease. However, there remains a pressing need for the development of vaccines for new diseases e.g. tumour associated diseases, and older-poorly controlled diseases e.g. HIV, TB and malaria.

In terms of options for vaccines, live vaccines generally offer the strongest protection; however, they tend to have higher adverse events associated with them. In contrast, sub-unit vaccines tend to have a good safety profile, but are generally only weakly immunogenic. DNA vaccines are also being investigated as a potential option: DNA vaccines can be designed to encode bacterial, viral or tumour antigens and may offer several advantages, as they are easy to produce, potentially more stable than subunit vaccines (e.g. heat stability), easy to manipulate and can mimic viral infections, yet there is no risk of reversion to pathogenicity (Gregoriadis, 1998; Henke, 2002). Like protein subunit vaccines, DNA vaccines are considered to be both safe and cost effective (Henriksen-Lacey et al., 2011c), however, both subunit and DNA vaccines suffer from poor immunogenicity, rapid clearance from the body and degradation by the host immune system (Perrie et al., 2001; Singh and O'Hagan, 2002). Hence, there is an urgent need for safe, cost effective and efficient vaccine adjuvants and delivery systems to overcome these issues (Gregoriadis et al., 1999; Watson et al., 2012).

Cationic liposomes have been extensively studied as potential vaccine adjuvants to increase the immune response and their role as antigen delivery systems in vaccines is well recognised (Henriksen-Lacey et al., 2011c; Kaur et al., 2012a; Watson et al., 2012).

Comparing to other vaccine adjuvants, liposomal adjuvant systems have several advantages: they are safe, relatively easy to produce and show low reactogenicity (Herzog et al., 2009; Watson et al., 2012). In addition, any type of antigen such as proteins, peptides, nucleic acids, carbohydrates and small molecule haptens can be incorporated in to the liposomes due to their versatility. For example, cationic liposomes can electrostatically interact with anionic proteins and nucleic acid allowing these antigens to be absorbed onto the surface of cationic liposomes and be carried to the antigen presenting cells (APCs) (Kaur et al., 2012a; Watson et al., 2012). In contrast, although microspheres have also been described as potential adjuvants for antigens producing both humoural (O'Hagan et al., 1991) and cellular immunity (Audran et al., 2003), they have failed to initiate immune responses at comparative levels to the liposomes due to the difference in their characteristic parameters (Kirby et al., 2008).

Given the ability of cationic lipids/liposomes to protect plasmid DNA against degradation and promote gene expression, it is not surprising that these systems have been applied for the delivery of DNA vaccines, with cationic liposome systems having been shown to effectively induce humoural and cellular immune responses against antigen-encoding plasmid DNA (Jiao et al., 2003; Lay et al., 2009; Morrey et al., 2011; Perrie et al., 2001). In general, these studies have considered the choice of the lipid/liposome construct in a bid to enhance immune responses. However, a study by Yan and Huang (2009) also investigated the effect of salt on the physicochemical and immunogenicity of the protein based vaccines formulated in cationic lipids. This study reported that presence of small amounts of salt (30 mM) within cationic liposome formulations could enhance immune performance of the vaccine. This was attributed to the interference of salt with the electrostatic interactions between the cationic lipid and the antigen, which facilitates the antigen release from the carrier and at the same time

activates the antigen presenting cells. Therefore, within this chapter, the aim of the work was to consider the application of the liposomal formulations developed within Chapter 3 as possible DNA vaccine delivery systems. From the *in vitro* studies carried out in chapter 4, a select few liposome formulations were chosen for further investigation as possible vaccine delivery systems. Since there was no significant difference in transfection efficiencies of SUV and DRV liposomes, the *in vivo* study will focus on the SUV lipoplexes prepared from DOPE:DOTAP and DOPE:DSTAP lipoplexes in both hydration media. However, the best DRV liposome in terms of *in vitro* transfection efficiency (Chapter 4) was also selected (DRV DOPE:DSTAP with no electrolyte in its hydration media).

When developing vaccines, immunisation strategies have commonly been based on homologous regimens, which involves giving the same vaccines multiple times. However, some studies (Carstens et al., 2011; Yang et al., 2008) suggest that prime-boost strategies, which use a combination of DNA-encoded antigen as a primer immunisation and subunit vaccines of the same antigen as a booster, can enhance humoural and cellular immune responses. Indeed, studies investigating different sequences of prime and boost injections have shown that two prime immunisations of plasmid DNA, following by one boost injection of protein (DDP), is more effective than a DNA (D), protein (P), protein combination or homologous injections of DD or P only (Deshmukh et al., 2007; Yang et al., 2008). Therefore, based on these finding, within this current study the DDP regimen has been adopted.

5.2. Physicochemical characteristics of DNA lipoplexes and protein-liposome complexes.

5.2.1. Radio-labelling of protein antigen

As explained in details in section 2.6.2, for entrapment studies, HBsAg was radiolabelled with the radioisotope Iodine-125 (^{125}I). The labelled protein was separated from the free, unincorporated ^{125}I via Sephadex G-75 gel chromatography. 0.25 mL aliquots were collected and the radioactivity of ^{125}I and the absorbance (A_{280}) for protein content within each aliquot was determined. As shown in Figure 5.1A, both high levels of ^{125}I and protein content are found in samples 11 and 12, with protein content in these samples being confirmed by the BCA assay (Figure 5.1B). From Figure 5.1A it can also be seen that the second peak in radioactivity does not have associated protein content, and is typical of free iodine (Figure 5.1A).

5.2.2. Liposomal delivery systems for DNA or sub-unit antigens

Given that previous studies have shown the presence of electrolytes within the aqueous media of cationic liposome suspensions can impact the vesicle characteristics (Section 3.4, Chapter 3), the impact of electrolytes on liposomal-protein antigen system was also briefly investigated, as these formulations would be used as the 'booster' for the *in vivo* vaccine studies. Therefore, initially the two cationic lipid formulations containing the fusogenic lipid DOPE, combined with either DOTAP or DSTAP, were formulated in either a high electrolyte buffer (PBS) or non-electrolyte (sucrose 10%) aqueous media (Table 5.1).

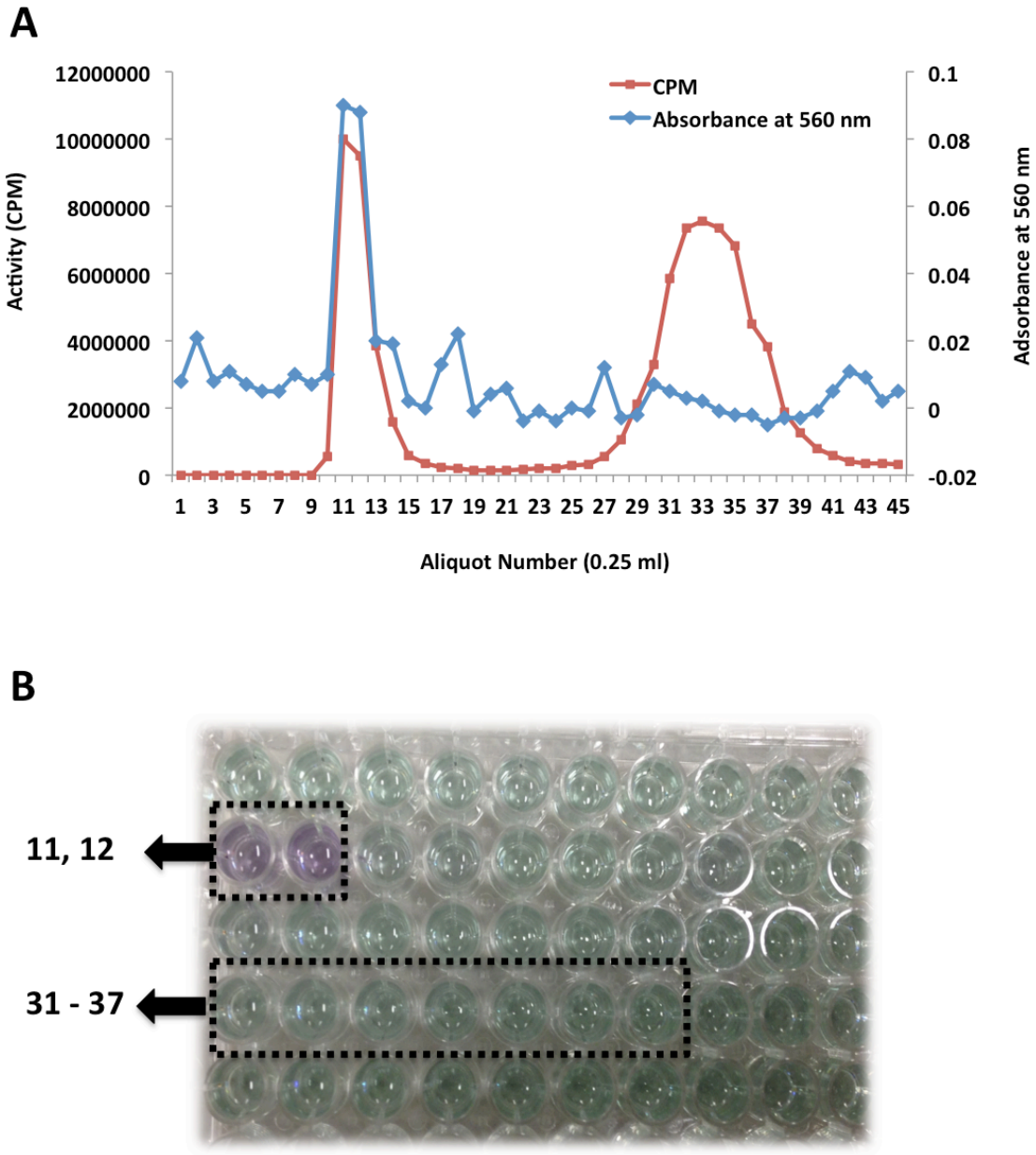

Figure 5.1: A) Determination of radioactivity for iodine labelled HBsAg samples eluted through Sephadex column, as well as their absorbance at 560 nm. **B)** Confirmatory BCA assay results for protein labelled samples. A colour change from green to purple indicated the presence of protein for samples 11 and 12.

Results in Table 5.1 show that both formulations, when complexed with plasmid DNA showed a significant increase ($p<0.05$) in vesicle size and significant reduction ($p<0.05$) in zeta potential, when formulated in the presence of electrolytes as previously reported in Chapter 4 and summarised in Table 5.1. However, this effect was not specific to lipoplexes, with liposomal-subunit complexes also demonstrating a similar outcome (Table 5.1). This is to be expected as the drop in zeta potential for the cationic systems is attributed to the condensing of the electrical double-layer. The increase in vesicles size measured may be attributed to the phosphate groups within PBS bridging the cationic polar heads of the lipid, thereby promoting aggregation (Ciani et al., 2007; Wasan et al., 1999). Therefore, these physicochemical changes would apply to the cationic lipids irrespective of what anionic antigen was adsorbed (DNA or protein) (Table 5.1).

Table 5.1: Characteristics of cationic liposomes. Size and zeta potential measured by Malvern Zetasizer Nano-ZS. Results represent mean±SD of triplicate independent experiments.

Liposome Formulation	Antigen	Aqueous Media	Vesicle Size (nm)	Polydispersity Index	Zeta Potential (mV)	Loading (%)
SUV DOPE:DOTAP	-	Sucrose	69.6 ± 5.4	0.351 ± 0.02	61.3 ± 2.6	0.0
	-	PBS	91.9 ± 4.7	0.198 ± 0.01	48.1 ± 6.6	0.0
	DNA	Sucrose	241.4 ± 76.3	0.375 ± 0.02	58.4 ± 1.3	86.3 ± 4.5
	DNA	PBS	658.4 ± 32.1	0.465 ± 0.04	38.7 ± 1.3	88.1 ± 5.7
	Protein	Sucrose	113.3 ± 19.9	0.274 ± 0.01	54.5 ± 2.1	85.6 ± 5.6
	Protein	PBS	167.3 ± 19.7	0.368 ± 0.04	30.5 ± 1.6	69.3 ± 6.4
SUV DOPE:DSTAP	-	Sucrose	69.2 ± 4.1	0.332 ± 0.03	61.6 ± 4.7	0.0
	-	PBS	130.0 ± 10.3	0.493 ± 0.02	41.0 ± 7.1	0.0
	DNA	Sucrose	497.6 ± 51.3	0.621 ± 0.03	45.7 ± 0.8	91.4 ± 3.8
	DNA	PBS	827.5 ± 70.7	0.464 ± 0.04	19.5 ± 3.6	89.9 ± 4.1
	Protein	Sucrose	142.5 ± 11.2	0.263 ± 0.02	46.8 ± 1.2	93.8 ± 4.9
	Protein	PBS	398.2 ± 24.6	0.402 ± 0.06	49.6 ± 3.4	86.3 ± 3.2
DRV DOPE:DSTAP	-	Sucrose	710.4 ± 93.4	0.238 ± 0.03	56.4 ± 8.2	0.0
	DNA	Sucrose	769.6 ± 65.3	0.767 ± 0.06	48.2 ± 2.1	92.5 ± 6.4
	Protein	Sucrose	637.3 ± 39.5	0.402 ± 0.03	50.2 ± 2.7	82.4 ± 5.3

5.3. Investigating the depot-formation of cationic liposome-based DNA vaccines

To investigate how the electrolyte-induced changes in physicochemical characteristics translated to *in vivo* performance, the ability of these lipoplexes to carry and deliver DNA-encoded antigen after intramuscular injection was investigated. It has been previously reported that cationic liposomes can induce a depot effect at the site of injection (Henriksen-Lacey et al., 2010c), causing a prolonged deposition of the antigen and adjuvant at the site of injection, and this may be responsible for the enhanced immune responses promoted by some cationic formulations. Since cationic liposomes are retained for a longer period at the site of injection, the adsorbed antigen (DNA or protein) will also have longer retention time leading to an increase exposure of the antigen presenting cells (APC) to antigen at the site of injection. This is shown to be beneficial for induction of Th1 immune responses, since this increases the production of IFN-γ (Christensen et al., 2012; Henriksen-Lacey et al., 2010c). Therefore, the biodistribution of both lipid compositions and their associated DNA, formulated in the presence and absence of electrolytes was investigated after intramuscular injection.

5.3.1. Monocyte influx to the site of injection

Given the ability of pontamine blue to be taken up by macrophages *in vivo* (Tilney, 1971a) it can be applied to identify the lymph nodes and give an indication to the ability of liposomes to induce innate immune cell influx to the injection site (Henriksen-Lacey et al., 2010b). However, given that it is also known that inflammation can occur at the site of injection, due to the tissue damage caused by injection and the inflammatory mediators potentially promoted by cationic lipids (Filion and Phillips, 1997), visualisation of monocyte influx can be tracked by pre-injecting mice with pontamine blue. Therefore, to determine the level of infiltrated immune cells to the site of injection,

pontamine blue dye was injected subcutaneously 3 days prior to the start of the biodistribution study.

Figure 5.2A shows the monocyte influx to the site of injection on day 8 p.i for SUV formulations and 'naked' DNA. Prior to the intramuscular injection of these formulations, and at early time-points, no staining was noted at the site of injection (data not shown). However, as time progressed an increase level of blue staining was noted at the injection site for mice injected with the liposomal DNA formulations, with high levels shown at day 8 (Figure 5.2A; samples 1 to 4), yet this was not the case with mice injected with the DNA alone where no staining was noted over the period of the study (Figure 5.2A; leg 5). Whilst not quantitative, these results suggest that cationic liposomes are able to enhance recruitment of circulating monocyte to the injection site whilst DNA alone does not.

5.3.2. Determination of vaccine components at the injection site.

The concentration of the liposomal carrier, and the DNA antigen was measured at site of injection (SOI), the local draining lymph node (PLN), liver, kidney, spleen and lung were investigated at day 1, 4 and 8, post injection (p.i.); of these sites, only the site of injection and the PLN had detectable levels of liposomes and/or DNA and therefore only this data is presented (Figure 5.2).

From these results, it can be seen that plasmid DNA injected without a liposomal carrier was quickly cleared, with only 10% of DNA detected at the site of injection 24 h after administration. This reduced significantly ($p < 0.05$) to 3% on day 4 and to less than 1% on day 8 p.i (Figure 5.2B). In contrast, DNA associated with any of the tested liposome formulations gave enhanced retention at the injection site, with approximately 40 to

60% of the liposome-associated DNA remaining at the injection site after 24 h (Figure 5.2B). Whilst the choice of cationic lipid (DOTAP vs DSTAP) made no significant impact on retention rates after 24 h, the higher transition DSTAP based lipoplexes promoted significantly ($p<0.05$) higher DNA retention at the injection site over the longer periods (Figure 5.2B) with DNA retention on day 8 p.i for DOPE:DSTAP formed in sucrose being double compared with DOPE:DOTAP hydrated in same medium. The same trend was shown for the formulations made in PBS with DNA retention for the DSTAP based lipoplex being 30%, compared with less than 3 % for DOPE:DOTAP formed in PBS (Figure 5.2B; day 8). This was mirrored by movement of the liposomes, with no significant differences being noted at day 1 and 4, however by day 8 the DOPE:DSTAP formulations tended to show higher levels at the injection site after 8 days compared to the DOTAP formulations (Figure 5.2C).

Interestingly, despite the presence of electrolytes making a significant impact on the vesicle characteristics prior to injection, this did not translate to measurable differences in either DNA or liposome movement from the injection site (Figure 5.2B & C); for DOPE:DOTAP formulated in either sucrose or PBS there was no significant difference in DNA (Figure 5.2B) or liposome retention (Figure 5.2C) at the site of injection despite the vesicles being twice the size when formulated in PBS (Table 5.1). This was also reflected with the DOPE:DSTAP formulations, with the difference in vesicle size and zeta potential having no impact on clearance rates (Figure 5.2B,C). However, it is important to consider that whilst differences in vesicle size are noted prior to injection, it is likely that on injection vesicle aggregation will occur due to the presence of interstitial proteins, which would negate any difference in vesicle sizes prior to injection. This can be supported by recent work by Kaur et al (Kaur et al., 2012b), which demonstrated that

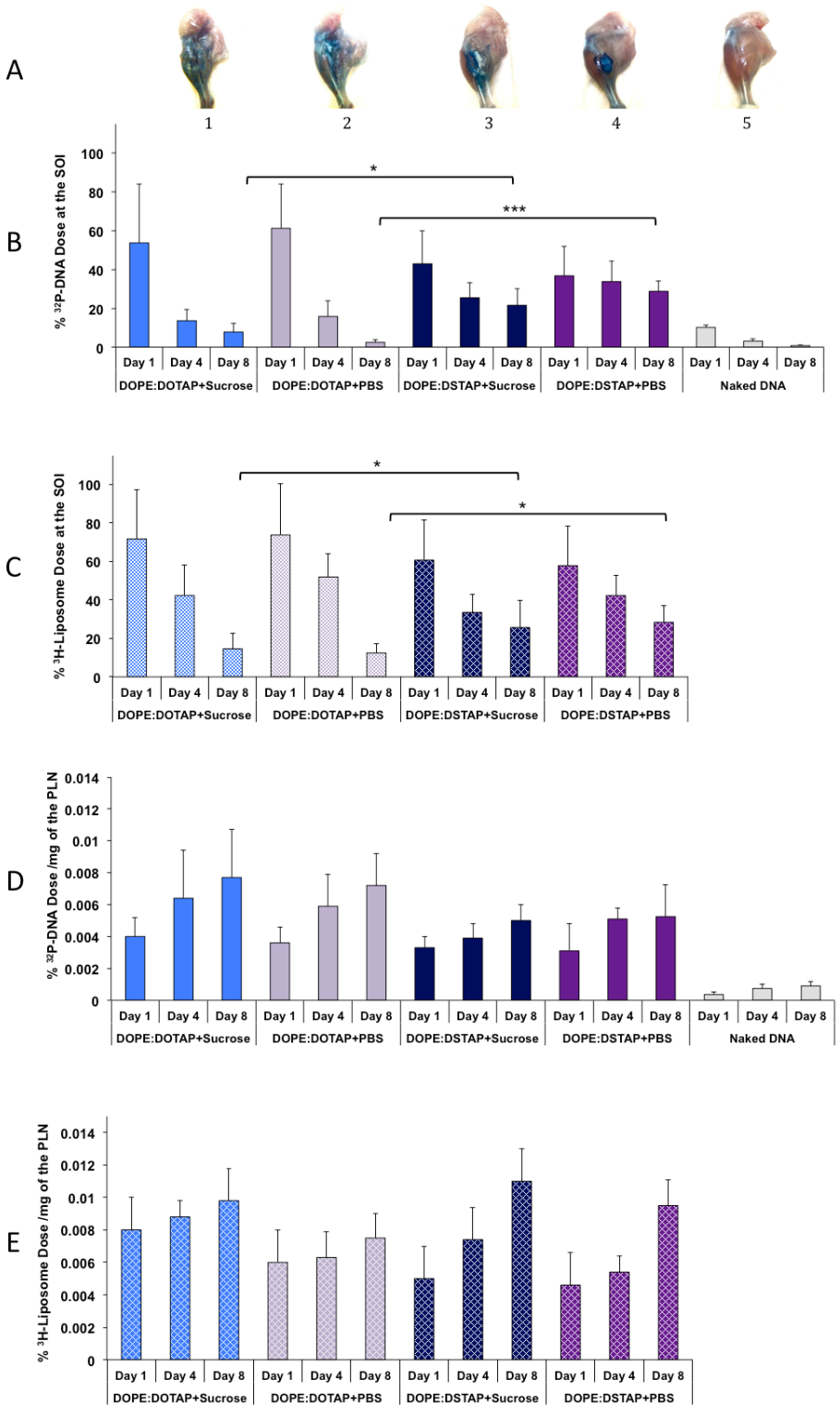

Figure 5.2: A) Pontamine blue staining on day 8 p.i of the injection site (quadriceps muscle) after i.m. injection with pDNA lipoplexes of 1)DOPE:DOTAP+Sucrose, 2)DOPE:DOTAP+PBS, 3)DOPE:DSTAP+Sucrose, 4)DOPE:DSTAP+PBS and 5)free DNA. B-C) pDNA and liposome detection at the site of injection and D-E) draining lymph nodes (PLN) following i.m. injection. pDNA and liposome quantification were determined at days 1,4 and 8 p.i. using radiolabel counting methods. Data represents mean ± SD of 4 mice and is presented % dose at the SOI (B,C) or the % dose per mg PLN harvested (D,E). Significance measured by one-way ANOVA and Tukey's post test, it is shown by *$p<0.05$, and ***$P<0.001$.

pegylation of cationic vesicles, which was able to block aggregation of vesicles, promoted enhance drainage from the injection site.

Tracking the distribution of vaccine to the draining PLN (Figure 5.2D and E) indicates that the amount of detectable DNA and lipid in all of the formulations at the PLN increased over the time. DNA and lipid retention at PLN did not show any significant difference between different formulations and therefore was not influenced by size nor liposome composition. However, all four liposome formulations gave significantly ($p<0.05$) high DNA delivery to the PLN compared to 'naked' plasmid DNA.

To investigate the effect of different vesicle types, SUV and DRV of DOPE:DSTAP were also compared. Figure 5.3A shows the comparison between the two different liposome preparations of the DSTAP formulation (DRV vs SUV), with no difference on monocyte infiltration between these two liposome preparations being notable. Considering the distribution of the liposomal formulation and the plasmid DNA, there was no significant difference between DNA retention of SUV 8 days after immunisation, with approximately 30% of DNA dose retention at the injection site compared with ~20% for the DRV formulation (Figure 5.3B). In terms of liposome retention at the injection site, the choice of liposome preparation made no significant difference (Figure 5.3C).

Measuring the DNA and lipid content at the draining PLN shows the amount of drained DNA and lipid increased over the time, however generally there was no notable difference between the SUV and DRV formulation (Figure 5.3D and E).

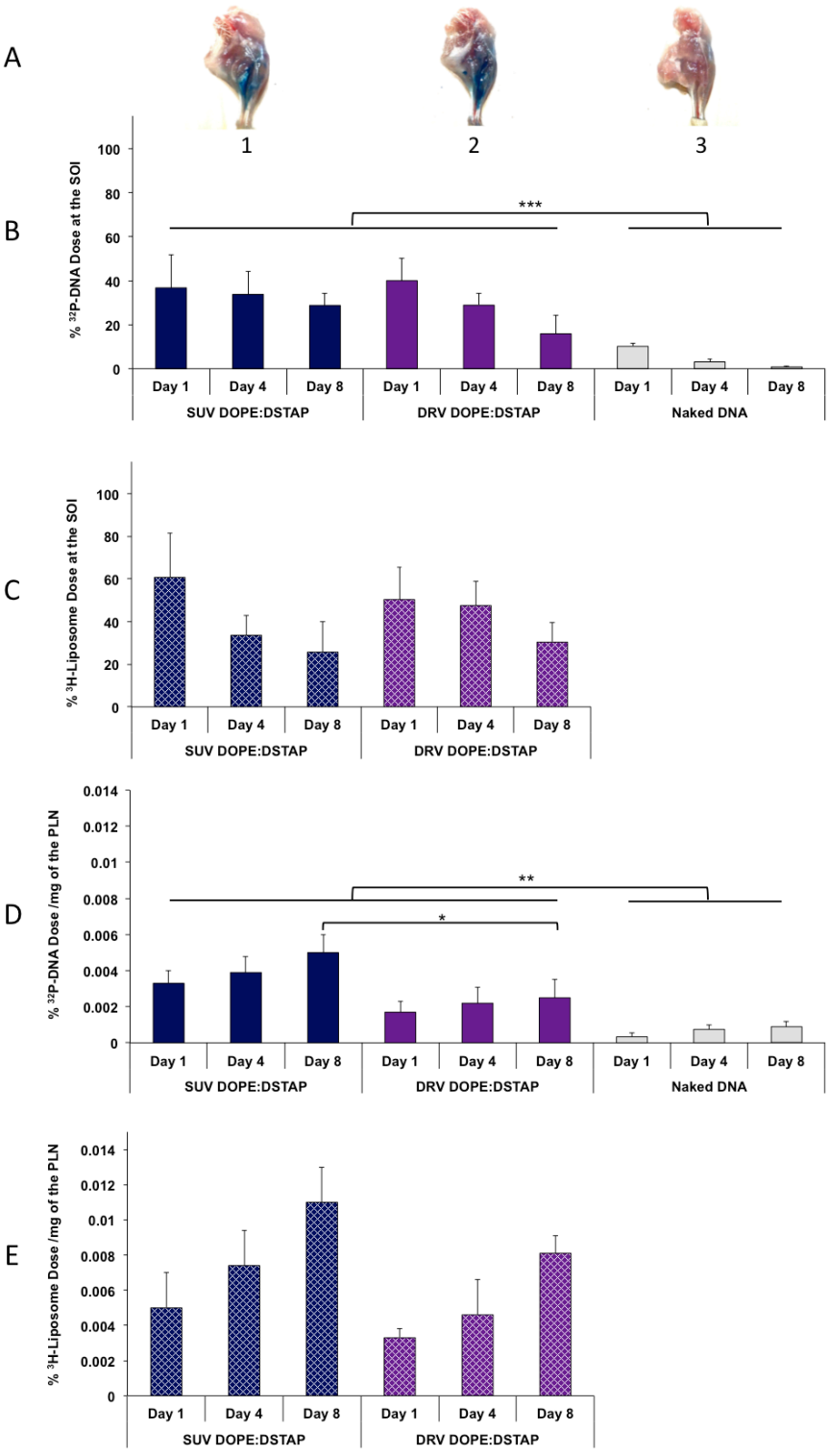

Figure 5.3: A) Pontamine blue staining at day 8 p.i of the injection site (quadriceps muscle) after i.m. injection with pDNA lipoplexes of 1) SUV DOPE:DSTAP+Sucrose, 2) DRV DOPE:DSTAP+Sucrose and 3)free DNA. B-C) pDNA and liposome detection at the site of injection and D-E) draining lymph nodes (PLN) following i.m. injection. pDNA and liposome quantification were determined at days 1,4 and 8 p.i. using radiolabel counting methods. Data represents mean ± SD of 4 mice and is presented % dose at the SOI (B,C) or the % dose per mg PLN harvested (D,E). Significance measured by one-way ANOVA and Tukey's post test, it is shown by *p<0.05, **p<0.01, and ***P<0.001.

These results (Figure 5.2 and 5.3) suggest that the key feature in the clearance rate of these vesicles, and their loaded DNA, from the site of injection may be the transition temperature of the cationic lipid used rather than vesicle size and antigen location; DSTAP, with its higher transition temperature, presents as a more rigid bilayer vesicle (Lobo et al., 2002; Regelin et al., 2000) compared to DOTAP formulations, whose more fluid bilayers allows more lipid clearance from the injection site (Christensen et al., 2012; Henriksen-Lacey et al., 2011a).

Monolayer studies showed that, compared to DOTAP, the cationic lipid of DSTAP has a more rigid structure due to its saturated alkyl chain and higher T_c; however, incorporation of DOPE to the DSTAP reduced the rigidity of the DSTAP and both DOPE:DOTAP and DOPE:DSTAP monolayers had similar monolayer properties (Section 3.3, Chapter 3). From another perspective, differential scanning calorimetry (DSC) studies (Regelin et al., 2000) reported a higher transition temperature for DSTAP and showed that although incorporation of helper lipid DOPE within DSTAP in an equimolar ratio decreased the transition temperature, it was still high enough to make rigid bilayers.

Considering the cationic liposomes as adjuvant systems, they are able to form a depot effect at the injection site, and are then taken up by the infiltrating antigen presenting cells (APCs) and move slowly to the lymph nodes and consequently promote more antibody production (Henriksen-Lacey et al., 2010b; Richards et al., 2004). Recent studies have suggested that the more rigid the liposome formulations are, the more depot they form at the site of injection (Christensen et al., 2012; Henriksen-Lacey et al., 2011a), which is in line with results of this study. Henriksen-Lacey et al, compared the depot formation of DDA, DC-Chol and DOTAP-based liposomes and reported that

more rigid DDA and DC-Chol-based liposomes showed significantly higher antigen localisation at the site of injection compared to fluidic DOTAP-based liposomes (Henriksen-Lacey et al., 2011a). Furthermore, it has been reported that upon i.v, s.c or i.m injection of liposomal vaccines, formulations with rigid bilayers show more localisation (for s.c and i.m) and improved circulation (i.v) and ultimately show stronger immune responses (Frezard, 1999; Storm and Crommelin, 1998; Yasuda et al., 1977).

5.4. Investigating the immunogenicity of cationic liposomes

To consider the potential of these various liposome systems as vaccine delivery systems, formulations including SUV liposomes of DOPE:DOTAP and DOPE:DSTAP formulated in either sucrose or PBS were investigated and the effect of presence of electrolytes, as well as the impact of the transition temperature of the cationic lipid on immunogenicity of the formulations were studied. Separately, the ability of DRV formulations of DOPE:DSTAP to immunise the mice were compared with SUV liposomes of DOPE:DSTAP. Based on a study performed by Yang et al. (2008), the chosen immunisation strategy for this study was a heterologous prime-prime-boost, which included two i.m immunisations of liposomal-pDNA (DD), followed by one i.m immunisation of liposomal-subunit antigen (P) injected at two week intervals (combined to DDP; Figure 5.4).

The immunogenicity of heterologous DDP immunisation was compared to the homologous vaccination regimens of DNA only (DD-) and sub-unit protein only (--P) using DOPE:DSTAP liposomes, to allow the measurement of the impact of the two plasmid DNA immunisations and the additional impact of the boost with protein. Table 5.2 shows the immunisation plan for this study. With regards to the heterologous

immunisation regimen, several sequences for the injection of the prime and booster have been proposed. Most of reviewed studies show 3 injections including 2 DNA and 1 protein injection (DDP) (Carstens et al., 2011; Yang et al., 2008). However, the sequences of DP (Wang et al., 2008), DDDP (Wierzbicki et al., 2002) and DDDPP (Vaine et al., 2010) have also been reported. Some studies have investigated different sequences of prime and boost injections and showed that DDP is more effective compared to DPP or homologous injections of DD or P only (Deshmukh et al., 2007; Yang et al., 2008). Also, it has been reported that no synergy has been seen upon injection in the sequence of PDD (Yang et al., 2008).

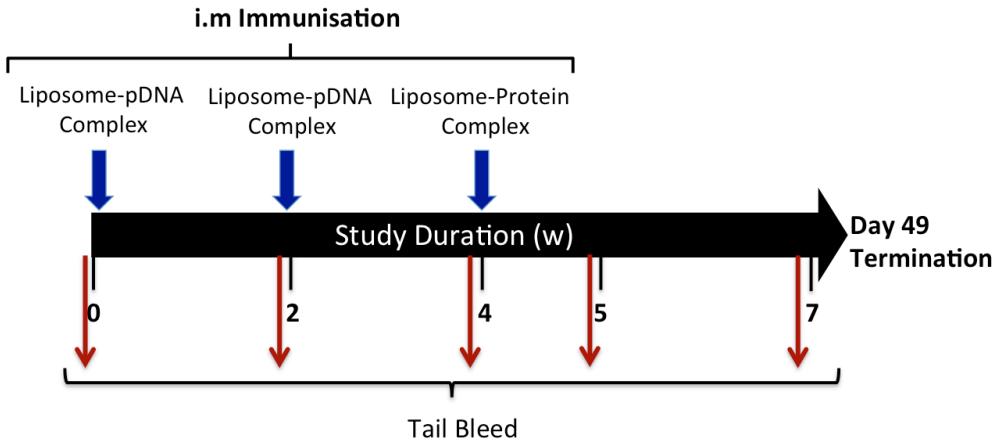

Figure 5.4: Schematic diagram of immunisation plan for the prime-boost heterologous vaccination study. Two prime liposome-pDNA complex i.m injections followed by a liposome-protein complex i.m injection on a two week intervals. One day before each injection and one week and three weeks after last injection tail bleed was performed on groups of 5 female C57BL/6Jico mice. Mice were terminated on day 49 post first injection and the spleen and site of injection was dissected and processed for immunological studies.

Table.5.2: Immunisation plan for DNA vaccine study.

	Formulation	Injection 1 (D)	Injection 2 (D)	Injection 3 (P)
1	**DOPE:DOTAP + Sucrose**	pDNA-Liposome	pDNA-Liposome	HBsAg-Liposome
2	**DOPE:DOTAP + PBS**	pDNA-Liposome	pDNA-Liposome	HBsAg-Liposome
3		pDNA-Liposome	pDNA-Liposome	HBsAg-Liposome
4	**DOPE:DSTAP + Sucrose**	pDNA-Liposome	pDNA-Liposome	----------
5		----------	----------	HBsAg-Liposome
6		pDNA-Liposome	pDNA-Liposome	HBsAg-Liposome
7	**DOPE:DSTAP + PBS**	pDNA-Liposome	pDNA-Liposome	----------
8		----------	----------	HBsAg-Liposome
9	**DRV DOPE:DSTAP + Sucrose**	pDNA-Liposome	pDNA-Liposome	HBsAg-Liposome
10	**Free DDP**	pDNA in PBS	pDNA in PBS	HBsAg in PBS
11	**Free Antigen**	----------	----------	HBsAg in PBS
12	**Free DNA**	pDNA in PBS	pDNA in PBS	----------
13	**Alhydrogel**	----------	----------	(HBsAg)Alhydrogel
14	**Naïve Group**	----------	----------	----------

5.4.1. Exploring the effect of vaccine formulation on antibody responses against HBsAg in a heterologous immunisation schedule

Considering first the responses from groups that received the heterologous immunisation schedule (1st and 2nd immunisation with DNA, third with sub-unit protein; groups 1,4,7 and 8) using SUV as delivery systems the ability of DOPE:DOTAP and DOPE:DSTAP liposomes to induce IgG (total), IgG1 and IgG2 antibody isotypes was studied using ELISAs. As explained in section 2.12.2 and shown in Figure 5.4 blood was collected on days 0, 13, 28, 36 and 49 and mice were injected with vaccines on days 1, 14 and 29.

5.4.1.1. Investigating the effect of electrolyte and transition temperature on antibody responses

Investigating the total IgG levels produced by cationic liposomes (Figure 5.5A) shows only liposomes formulated from DOPE:DOTAP (Group 1; Figure 5.5A) and DOPE:DSTAP (Group 3; Figure 5.5A) suspended in sucrose, were able to induce the antibody production on day 36, and additionally were able to induce significantly ($p<0.01$) higher levels of IgG relative to free DDP (Group 10; Figure 5.5A) on day 49. However, DOPE:DOTAP (Group 2; Figure 5.5A) and DOPE:DSTAP (Group 6; Figure 5.5A) formed in PBS, showed levels of IgG equal to free DDP (Group 10; Figure 5.5A), 'naked' HBsAg subunit antigen (Group 11; Figure 5.5A), and alum-HBsAg (Group 13; Figure 5.5A) on day 49. Indeed, free DNA (Group 12; Figure 5.5A) showed backgrounds levels of IgG similar to the naïve group (Group 14; Figure 5.5A) and remarkably lower than other immunisation groups (Figure 5.5A).

Comparing between the two cationic lipids, replacing DOTAP (Group 1 and 2; Figure 5.5A) with DSTAP (Group 3 and 6; Figure 5.5A) in the liposome formulations showed no significant change in total IgG levels by day 49. However, the impact of the presence of electrolyte in the formulation, antigen-specific IgG production was significantly ($P<0.05$) lower for DOPE:DOTAP and DOPE:DSTAP when formed in PBS (Group 2 and 6 respectively; Figure 5.5A) compared to sucrose (Group 1 and 3 respectively; Figure 5.5A). This trend was also seen in Th2 responses of mice that were immunised with formulations prepared in PBS rather than sucrose; however, in contrast, Th1 responses were significantly higher ($P<0.05$) in mice immunised with DOPE:DSTAP formulated in PBS (Group 6) compared with all other liposome systems (Figure 5.5B). This adds to the previous knowledge that liposomes containing DOTAP promote lower Th1 responses compared to other cationic lipids (Henriksen-Lacey et al., 2011a).

As controls, a series of options were considered: free DDP (Group 10), free sub-unit protein (group 11), free DNA (Group 12) and a sub-unit protein formulated with Alum (Group 13). Despite the free DDP and sub-unit group formulations promoting total IgG and IgG1 levels similar to liposomal formulations, alum and free DNA could not induce any detectable level of IgG2b at this stage (Figure 5.5B). Alum is one of the oldest vaccine adjuvants, despite its inability to produce cellular responses (Davidsen et al., 2005). This explains the failure of the alhydrogel to induce detectable levels of IgG2b. In addition, this shows the ability of the cationic liposomes to induce high levels of both humoural and cellular immune responses (Figure 5.5B).

Results show that, compared to free DDP group, which is composed of two free DNA and one free subunit antigen injections, liposomal vaccines can induce significantly higher ($P<0.05$) levels of IgG and IgG1 (Figure 5.5). However, the free DDP group, is the only non-liposomal group showing detectable levels of IgG2b (Figure 5.5B). This might be the effect of prime-boost immunisation, which will be discussed later in section 5.4.1.3. HBsAg is known to be a moderately strong immunogen, as it contains both T and B cell epitopes (Schirmbeck et al., 1994a; Schirmbeck et al., 1994b); however, its injection as a free subunit antigen does not induce high antibody response (Group 12, Figure 5.5). Previous studies also demonstrated that giving free HBsAg as a booster after prime injections of plasmid DNA increases the IgG production significantly; however, upon encapsulation of HBsAg protein within liposomes, further (3 times higher, $P<0.01$) enhancement was observed (Yang et al., 2008). This demonstrates the adjuvant effect of cationic liposomes, which is in line with the findings of this current study.

The influence of cationic lipid content within the liposome formulation on antibody production, may be explained by the impact of liposomal depot effect at the site of

injection, and hence draining of the plasmid DNA into the PLN, on antibody production. It has been reported that upon entrance of the antigens to the lymph node, antigen presenting cells (APCs), such as dendritic cells (DCs), will present the antigen to the MHC class II molecules which activate $CD4^+$ T cells and induce antibody production (Bramwell and Perrie, 2005a; McCullough and Summerfield, 2005; Medzhitov, 2007). Biodistribution results (Figure 5.2, Section 5.3.2) showed that, although DSTAP-based formulations show more localisation at the injection site, DNA drainage rate is similar for both kinds of DOTAP and DSTAP liposomes. This was followed by similar IgG and IgG1 production levels for all formulations, emphasising the effect of DNA drainage to the PLN on humoural immune response and antibody production. In contrast, lower IgG2b levels produced by DOTAP formulations reflects the difference in intracellular pathways, which leads to induction of either Th1 or Th2 responses. Given that IgG2b production is related to Th1 responses implies that DOTAP based formulations show less Th1 responses when they are compared to DSTAP liposomes.

Having a more rigid structure due to the higher transition temperature, DSTAP liposomes have been shown to produce more depot effect at the site of injection. Their rigid structure may have caused slower localisation of DNA to the local draining lymph nodes and this gives more opportunity to APCs to be in contact with the plasmid DNA and activate the pathways, which leads to enhanced Th1 activation. This is in line with the findings of a previous study (Henriksen-Lacey et al., 2011a), which compared the effect of different cationic lipids on immunogenicity of the liposomes and demonstrated that cationic lipids of DDA and DC-chol, which have a more rigid structure, produce higher Th1 response compared to DOTAP with fluidic bilayer structure. The difference

in structures of the DOTAP and DSTAP has previously been discussed in details in chapter 3 (Section 3.3).

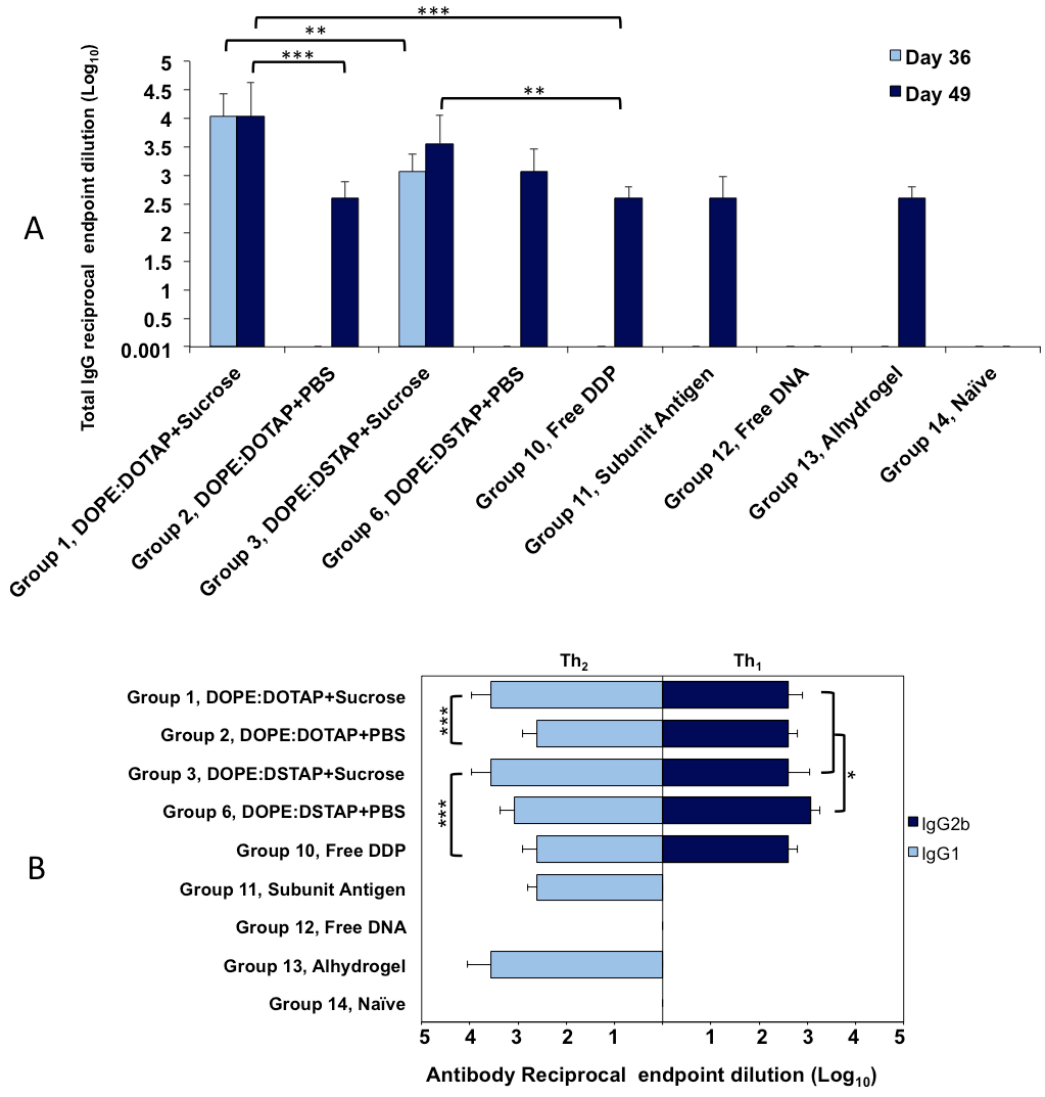

Figure 5.5: HBsAg specific antibody titres. A) Total IgG for all of the tested formulations in heterologous immunisation regimen, serum taken from day 36 and 49, B) IgG_1(light blue bars) and IgG_{2b}(dark blue bars) for all of the tested formulations in heterologous immunisation regimen, serum taken from day 49. Results show the mean ± SD reciprocal endpoint dilution (log_{10}) of 5 mice. Significance measured by one-way ANOVA and Tukey's post test, it is shown by *p<0.05, **p<0.01 and ***P<0.001.

5.4.1.2. Comparing the ability of SUV and DRV liposomal vaccines to induce antibody responses

Figure 5.6 compares the antibody production in mice immunised with the heterologous strategy using SUV and DRV liposomes of DOPE:DSTAP, so to consider if entrapping the antigen with the system could further boost the immune responses.

Results demonstrate SUV liposomes induce significantly ($P<0.05$) higher IgG (Figure 5.6A) and IgG1 responses (Figure 5.6B) than DRV, while IgG2b levels are similar for both SUV and DRV formulations (Figure 5.6B). Given the similar depot effect for both formulations, higher humoural response of SUV formulations could be due to the smaller vesicle size compared to DRV. Characterisation studies (Table 5.1) showed DRV formulations own significantly larger vesicles than SUV liposomes. Moreover, *in vitro* transfection studies demonstrate significantly higher cell transfection for SUV DOPE:DSTAP+dH_2O compared to DRV of the same formulation (Section 3.4, Chapter 3). These data imply that having smaller size, SUV formulations have more chance than DRV to be uptaken by APCs and hence they induce stronger humoural immunity. In agreement to this, another study (Carstens et al., 2011) showed that although larger vesicles produce stronger depot effect at the injection site, the induced immune response of smaller cationic liposomes was significantly stronger and suggested that liposomal DNA vaccines do not benefit from strong depot effect and other factors such as vesicle size have a role in immune performance of the vaccine.

These results are in line with the results of a recent study (Milicic et al., 2012) which has compared the immune response of MLV, SUV and DRV of DDA:TDB liposomes and showed total IgG production of SUV formulation was higher than MLV and DRV formulations.

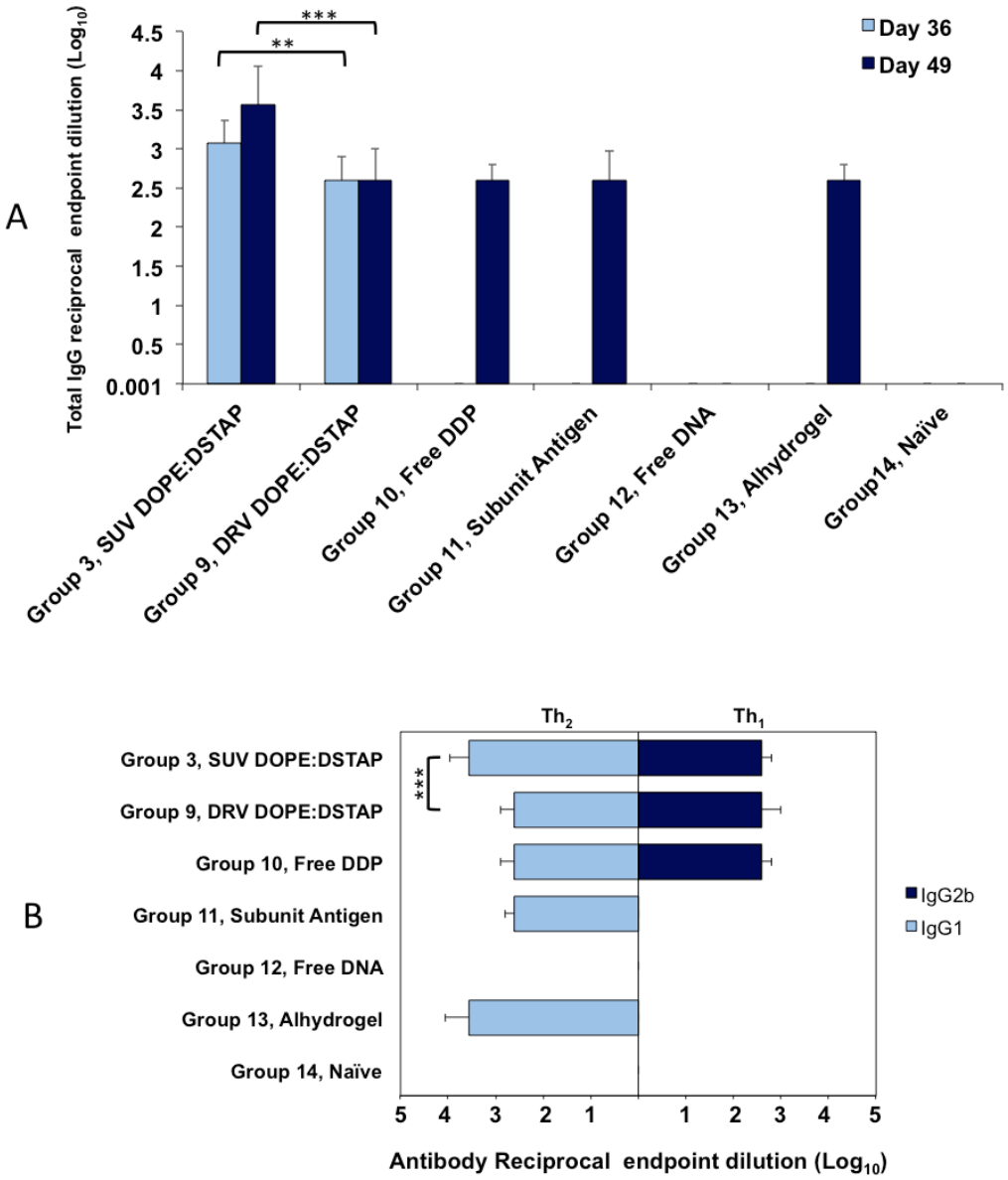

Figure 5.6: HBsAg specific antibody titres A) Total IgG for SUV and DRV of DOPE:DSTAP+Sucrose, serum taken from day 36 and 49, B) IgG1(light blue bars) and IgG2b(dark blue bars) for SUV and DRV of DOPE:DSTAP+Sucrose, serum taken from day 49. Results show the mean ± SD reciprocal endpoint dilution (log10) of 5 mice. Significance measured by one-way ANOVA and Tukey's post test, it is shown by **p<0.01 and ***P<0.001.

5.4.1.3. Considering the immunisation regimen on antibody production levels induced by DOPE:DSTAP liposomes

Given DSTAP based formulations elicit high antibody levels for all three studied antibodies (Figure 5.5), SUV liposomes of DOPE:DSTAP were studied to show the effect of immunisation regimen on their ability to induce antibody (Figure 5.7);

therefore, two homologous and one heterologous vaccination strategies were tested using DOPE:DSTAP hydrated in sucrose or PBS.

Results show no detectable levels of HBsAg-specific antibodies found in the serum when mice received two immunisations of liposomal-DNA vaccines (Group 4 and 7; Prime-Prime (DD-); Figure 5.7), whilst mice that received a single liposomal-sub-unit antigen dose (Group 5 and 8; Boost (--P); Figure 5.7) gave measurable responses on day 49 for all antibodies tested.

The heterologous strategy (prime-prime-boost; DDP), promoted high antibody responses from both formulations (Group 3 and 6; Figure 5.7) and comparing to the --P regimen demonstrated significantly ($P<0.05$) higher levels of total IgG and IgG1 for DOPE:DSTAP when formulated in sucrose (Figure 5.7). In contrast, for IgG2b there was no significant change between DDP and --P immunisation strategies (Figure 5.7B). Comparing the single immunisation of liposomally formulated sub-unit antigen, the formulation in PBS versus sucrose made no significant difference. These results suggest that the two liposomal DNA prime immunisations were unable to elicit antigen-specific humoural antibody responses, and that the addition of a protein booster immunisation was required. Furthermore, the DDP strategy was not significantly better than a single liposomal-protein immunisation in terms of Th1 responses (Figure 5.7B).

Given the low immunogenicity of DNA vaccines (Li et al., 2012), several studies have shown that DNA vaccines have the ability to prime the immune system for responses to other vaccines which are known as boost (Lu, 2009; Park et al., 2003; Richmond et al., 1998; Robinson et al., 1999).

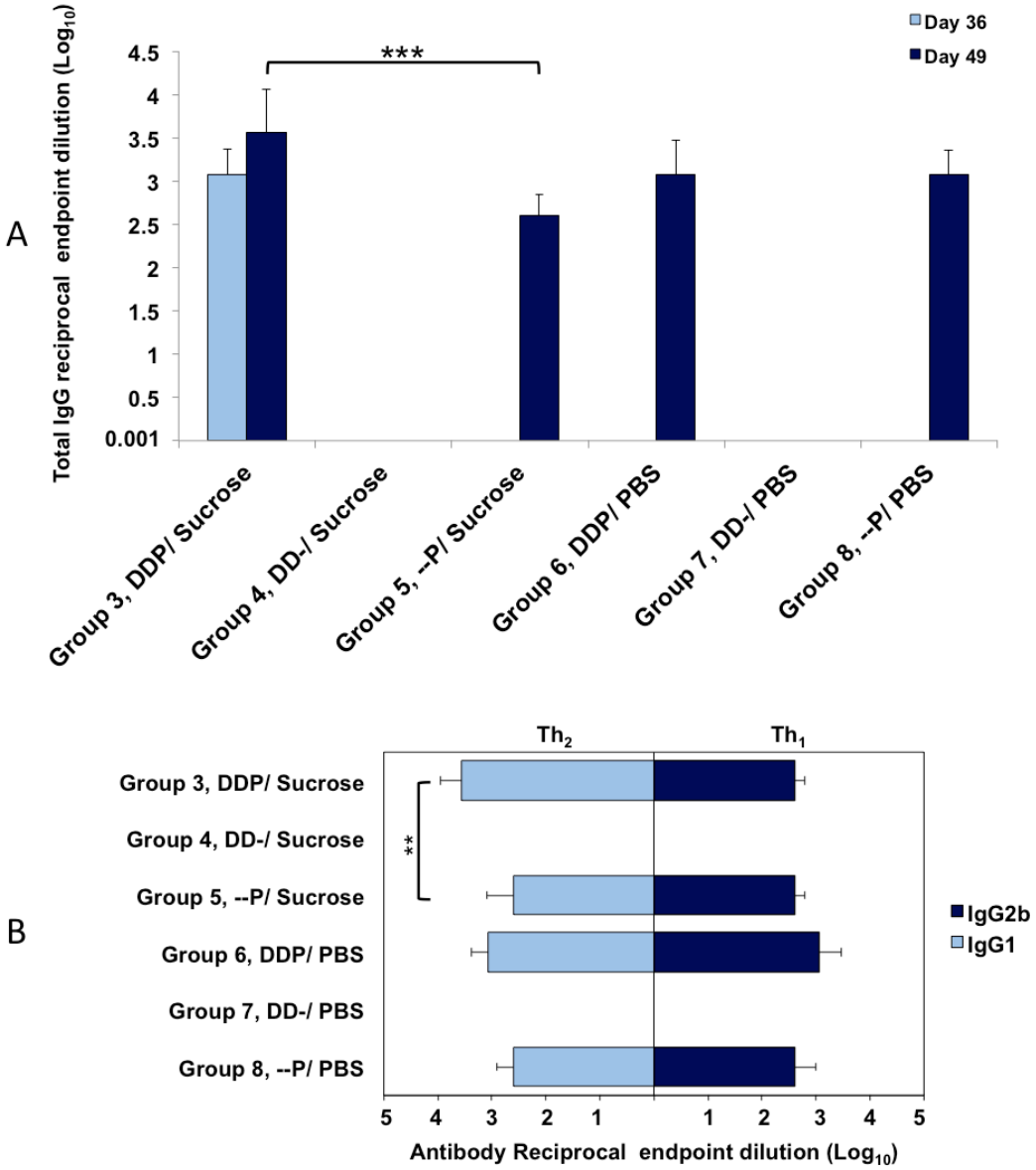

Figure 5.7: HBsAg specific antibody titres. A) Total IgG to compare heterologous and homologous immunisation of DOPE:DSTAP formed in sucrose or PBS, serum taken from day 36 and 49, B) IgG1(light blue bars) and IgG2b(dark blue bars) to compare heterologous and homologous immunisation of DOPE:DSTAP formed in sucrose or PBS, serum taken from day 49 Results show the mean ± SD reciprocal endpoint dilution (log_{10}) of 5 mice. Significance measured by one-way ANOVA and Tukey's post test, it is shown by **P<0.01 ***P<0.001

It is also shown that when the contents of prime and boost immunisations are different, which is called heterologous immunisation, the immune response is remarkably higher than using the same vaccine different times (homologous immunisation) (Lu, 2009; Mazumder et al., 2011). Although the mechanism of this technology is still unknown (Li et al., 2012; Liu, 2011), it is believed that as DNA vaccines only encode and present

the antigen of interest, this makes it easier for initial immune responses to only focus on the key antigen during the prime immunisation (De Mare et al., 2008). Furthermore, the lower antigen expression by DNA vaccines compared to protein subunit vaccines, may prime T helper cell responses and subsequently humoural response will be boosted by the protein boost immunisation, so the antibody production level in prime boost regimen will be higher than when it is seen with DNA alone (Otten et al., 2005; Stambas et al., 2005). Particularly, Yang et al. (2008) showed that immunisation of C57BL/6 mice with heterologous prime/boost immunisation of HBsAg as either free antigen or liposomal vaccines induced the antibody production. These findings are in line with the results of this study and explain why (DDP) antibody responses for all antigens are significantly higher than (DD-) immunisation (Figure 5.7).

5.4.2. Investigating the ability of cationic liposomal vaccines to promote cytokine production in response to heterologous immunisations with HBsAg

In order to further examine the role of different cationic liposomal vaccines on both cell mediated and humoural immune responses following immunisation, supernatants from HBsAg restimulated splenocyte cultures were removed and cytokine levels (IL-2, IL-5, IL-6, IL-10 and IFN-γ) determined using ELISA.

Furthermore, tissue from the SOI was excised, digested and analysed for the presence of the pro-inflammatory cytokine IL-1ß. Production of IL-1ß has been related to uptake of particulate adjuvants by dendritic cells (Henriksen-Lacey et al., 2011b; Sharp et al., 2009) and will therefore be useful to see how vesicular vaccine adjuvants promote antigen uptake at the SOI.

5.4.2.1. Studying the effect of bilayer composition of liposomal vaccines on their cytokine production efficiency

Figures 5.8 and 5.9 show the cytokine production level for all studied formulations in the presence of media alone (negative control) and 5μg/mL Con A (positive control), respectively. Media results show the background levels of cytokine production were low (Figure 5.8) and Con A results show Con A simulated splenocytes produced relatively high levels of cytokines measured, confirming the viability of the model (Figure 5.9).

Figure 5.8A shows the production of IL-2 from splenocytes in the absence of a stimulant, which is in a range between 20 to 60 pg/mL for all the immunisation groups. Upon re-stimulation of splenocytes with HBsAg there were significant increases in IL-2 production for all the groups except naïve and free antigens and alum groups that showed the baseline levels (Figure 5.10A). Between the liposome injected groups, there was no notable trend; for DOTAP based liposomes (Group 1 and 2), liposomes formed in sucrose (Group 1; Figure 5.10A) show a significantly ($P<0.05$) higher IL-2 production (with 700 pg/mL) compared to the DOPE:DOTAP formulated in PBS (with 350 pg/mL) (Group 2; Figure 5.10A). In contrast, for DSTAP based liposomes (Group 3 and 6), the vaccine formulations formed in PBS (Group 6; Figure 5.10A) show significantly ($P<0.05$) higher cytokine production with approximately 900 pg/mL compared to 550 pg/mL IL-2 produced under the effect of DOPE:DSTAP hydrated in sucrose (Group 3; Figure 5.10A). In addition, replacing DOTAP with DSTAP does not result in a significant difference in cytokine responses for the liposomes made in sucrose. However, DOPE:DSTAP+PBS (Group 6; Figure 5.10A) produced significantly higher levels of IL-2 compared to its DOTAP analogue (Group 2; Figure 5.10A).

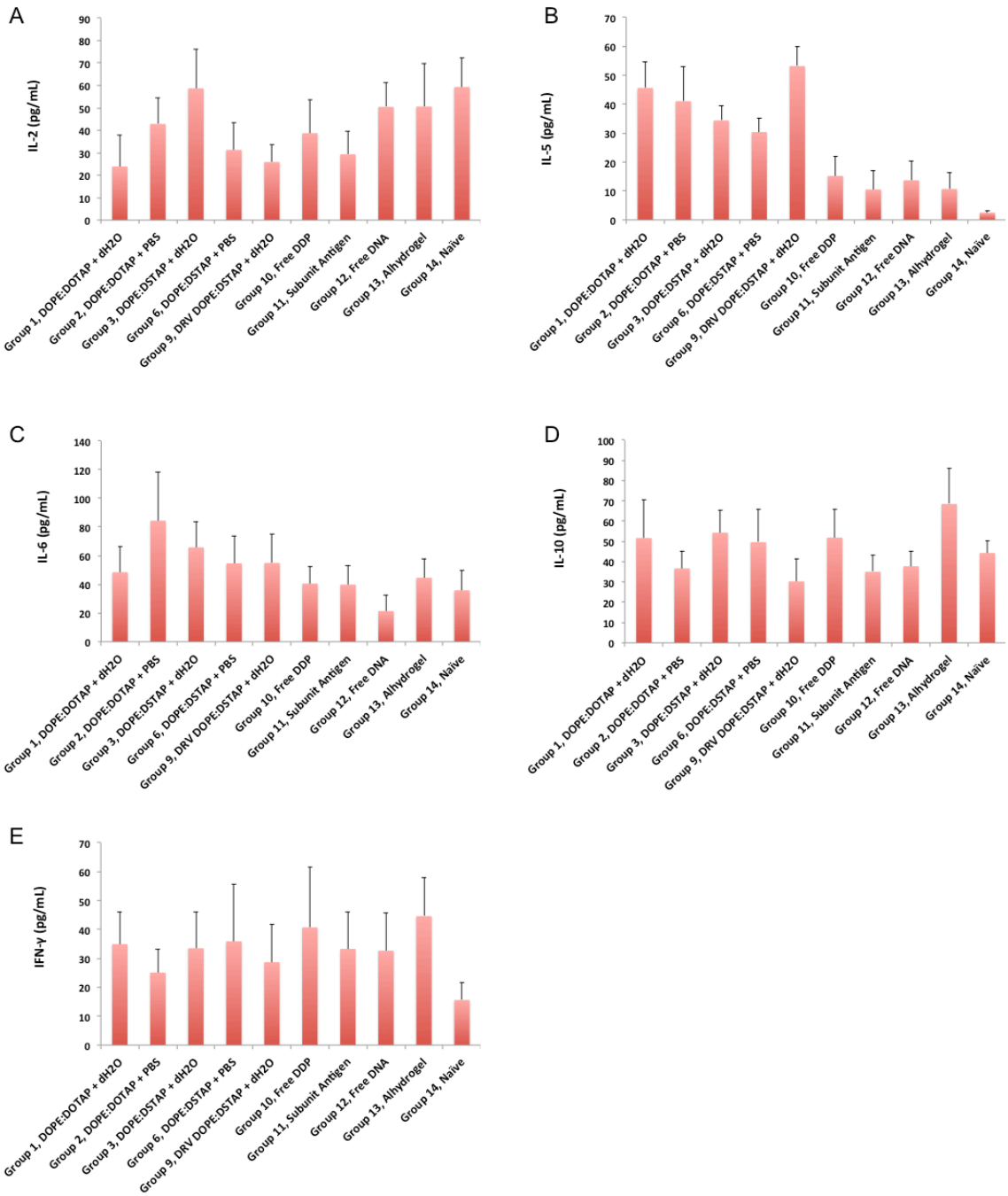

Figure 5.8: Cytokine production showing IL-2 (A), IL-5 (B), IL-6 (C), IL-10 (D), IFN-γ (E) from unrestimulated splenocytes derived from immunised mice or naïve group. Cytokines were measured from splenocyte or muscle supernatants using sandwich ELISAs.

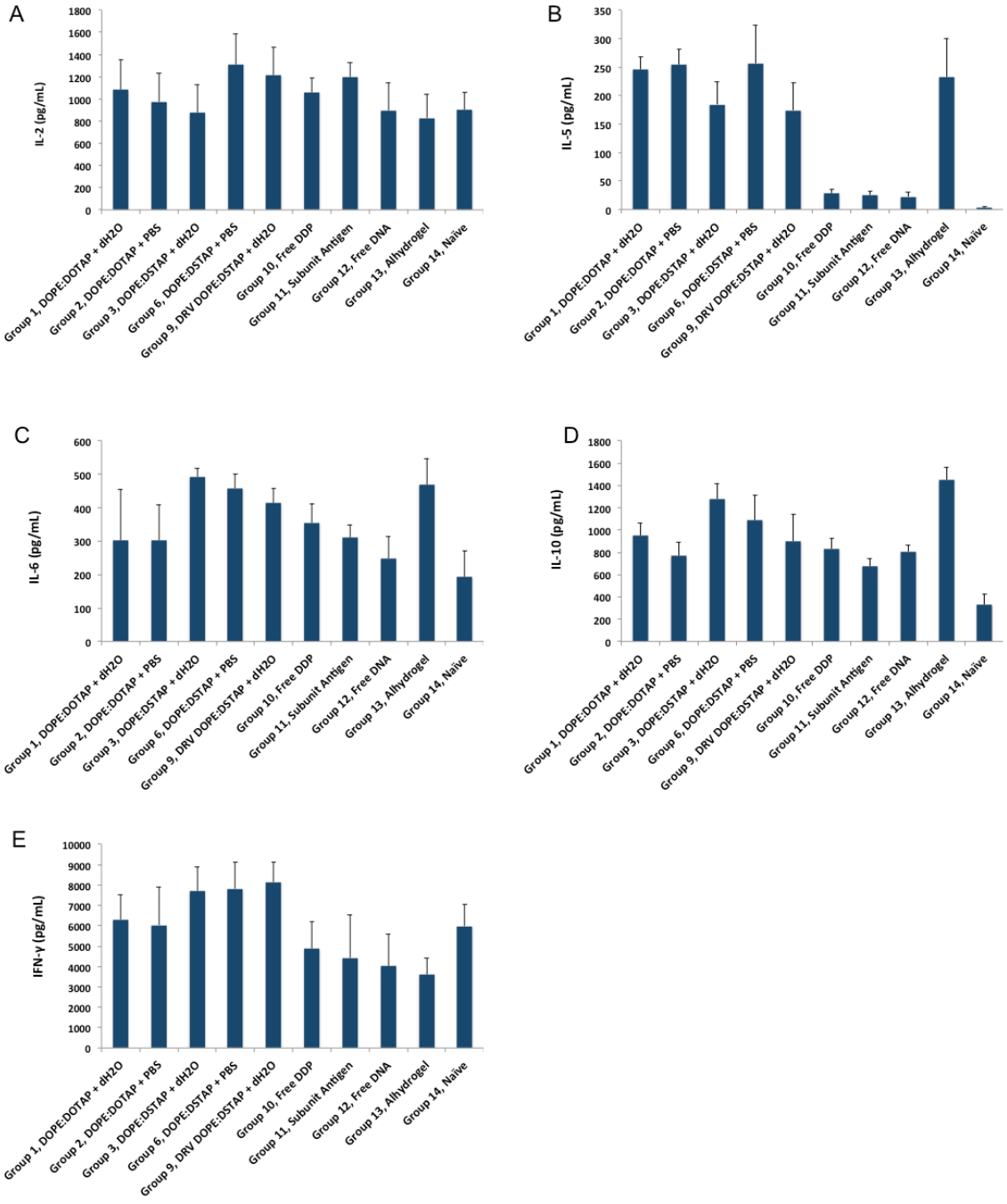

Figure 5.9: Cytokine production showing IL-2 (A), IL-5 (B), IL-6 (C), IL-10 (D), IFN-γ (E) from restimulated splenocytes with 5μg/mL Con A derived from immunised mice or naïve group. Cytokines were measured from splenocyte or muscle supernatants using sandwich ELISAs.

IL-2 is known as a growth and expansion factor for T helper cells and influences the production of T-cell derived cytokines. Its major function is to promote proliferation of both $CD4^+$ and $CD8^+$ T-cells and promotes production of NK-derived cytokines such as TNF-α and IFN-γ (Gaffen and Liu, 2004). It is known as a Th1 mediating cytokine and plays a role in cellular immune response (Christensen et al., 2012; Henriksen-Lacey et al., 2011a). Considering IL-2 as a Th1 indicator cytokine, the low IL-2 production of alum proves the poor ability of this adjuvant, which only induces Th2 responses, to present HBsAg to the T cells.

Figure 5.8B shows IL-5 production from splenocytes in the absence of a stimulant; these results show background levels in a range between 10 to 60 pg/mL. In the presence of HBsAg the IL-5 production level increased significantly ($P<0.05$) for the liposomes formed in sucrose (Group 1 and 3; Figure 5.10B); however, the presence of electrolytes in the liposomes caused a lower IL-5 production for DOTAP and DSTAP liposomes (Group 2 and 6; Figure 5.10B). Cytokine levels for alhydrogel (Group 13; Figure 5.10B) increased significantly ($P<0.05$) from 10 pg/mL (Figure 5.8B) to 60 pg/mL when it is restimulated with HBsAg (Figure 5.10B). Con A stimulation induced higher IL-5 production from splenocytes derived from all liposome injected groups and alum group in a range between 200 to 250 pg/mL (Figure 5.9B). IL-5 is known as a Th2 indicator (Christensen et al., 2009; Kaur et al., 2012b), which promotes B cell proliferation and explains the reason alum has higher cytokine production than DOTAP and DSTAP liposomes formed in PBS (Group 3 and 6; Figure 5.10B). IL-5 production levels suggest, regardless of the transition temperature of the cationic lipids, liposomes when formulated in PBS show lower antibody production as Th2 cells induce humoural immune responses, which are responsible for antibody production.

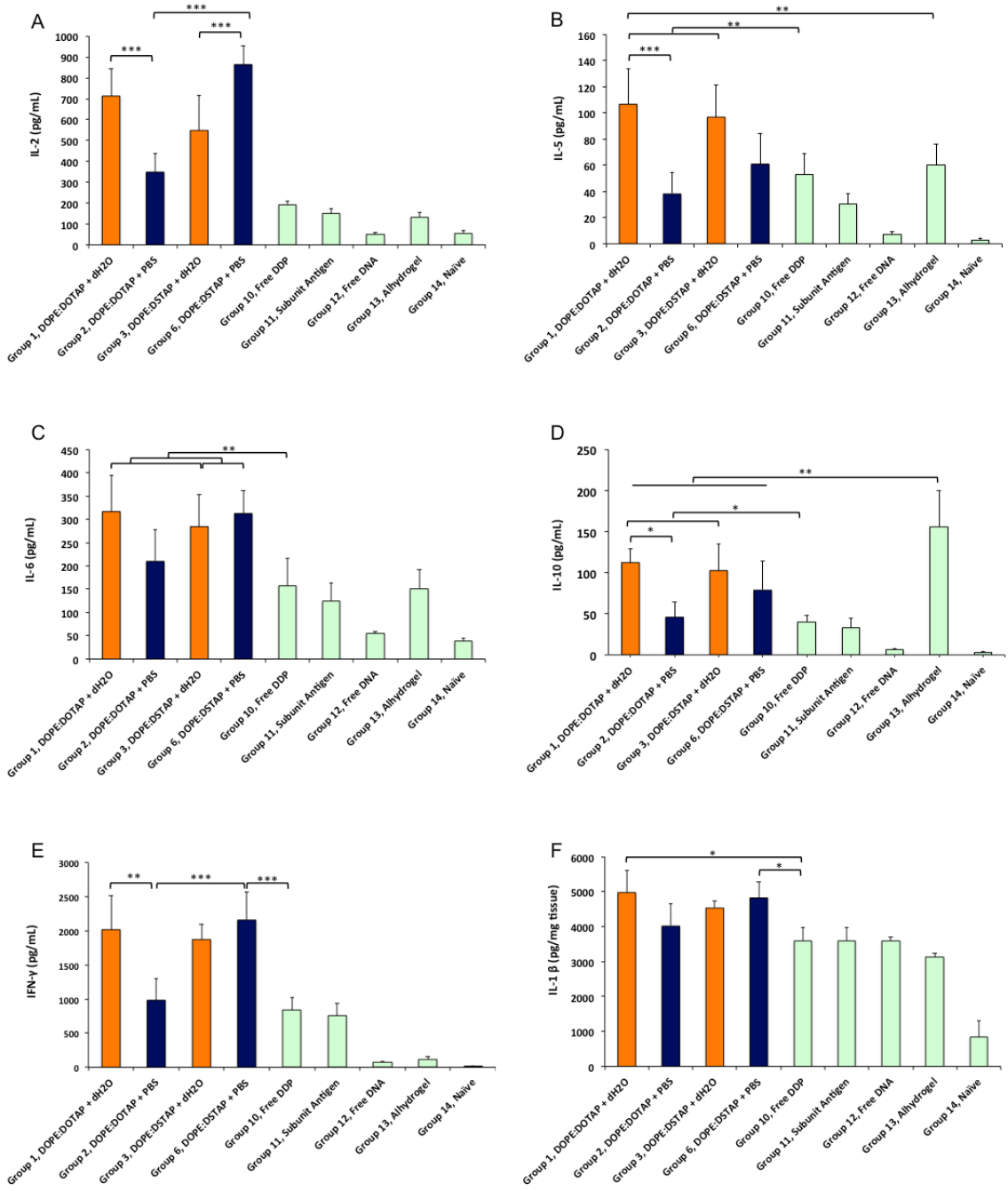

Figure 5.10: IL-2, IL-5, IL-6, IL-10 and IFN-γ cytokine production from splenocytes (A–E) and IL-1β production from excised leg muscle from the SOI (F) derived from mice immunised with DOPE:DOTAP or DOPE:DSTAP pDNA-lipoplex or subunit protein liposome formed in sucrose or PBS and in heterologous prime boost strategy. Splenocytes and muscle from the SOI were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 48 h in the presence of HBsAg (5 μg/ml). Leg muscle was excised, digested and homogenised. Cytokines were measured from splenocyte or muscle supernatants using sandwich ELISAs. Significance measured by one-way ANOVA and Tukey's post test, it is shown by *p<0.05, **p<0.01 and ***P<0.001.

IL-6 is another cytokine which represents Th2 responses, however this cytokine also promotes differentiation of Th17 cells with production of IL-17 (Dienz and Rincon, 2009). This cytokine has been reported to detect both cellular and humoural responses (Diehl and Rincon, 2002; Henriksen-Lacey et al., 2011b). Non-stimulated splenocyte IL-6 production was between 20-80 pg/mL with no significant difference between immunisation groups (Figure 5.8C). These background levels remained for free DNA and Naïve group when restimulated with HBsAg; however, alum and free antigen groups showed significant increase when restimulated with HBsAg (Figure 5.10C). Considering the liposome injected groups, whilst no trend was noted between the liposomal groups, all groups responded to HBsAg restimulation, producing levels of IL-6 approximately 4 to 6 fold ($P<0.05$) higher than free DDP and alum group (Group 10 and 13 respectvely; Figure 5.10C). Results show no significant difference between IL-6 production levels of liposomal groups when restimulated by HBsAg (Figure 5.10C) or Con A (Figure 5.9C).

Figure 5.8D demonstrates IL-10 production from splenocytes from unstimulated immunisation groups, which is in a range of 30 to 70 pg/mL showing no trend or significant difference between vaccination groups. Restimulation of the immunisation groups with HBsAg led to a significantly higher IL-10 production level for alum group (Group 13; Figure 5.10D) compared to any liposomally dosed group. Amongst the liposomally immunised groups, a similar trend to IL-5 production was seen, since the liposomes formed in sucrose (Group 1 and 3; Figure 5.1D) show higher IL-10 production levels than those mice which received the liposomes with electrolyte in their hydration media (Group 2 and 6; Figure 5.10D). The use of Con A as a positive control resulted in IL-10 production of 1000 to 1400 pg/mL for all the injected groups except from naïve groups with 400 pg/mL IL-10 production (Figure 5.9A). IL-10 is known to

be a Th2 indicator (Henriksen-Lacey et al., 2011b) same as IL-5 and this explains high IL-10 production of alum as it tends to induce humoural immune response.

The next cytokine investigated was IFN-γ, which is critical for innate and acquired immune systems. IFN-γ is produced by natural killer (NK) cells and CD4 Th1 cells and is indicative of cell mediated immune response (Henriksen-Lacey et al., 2011b; Trinchieri, 1997). Figure 5.8E displays IFN-γ production from unstimulated splenocytes derived from all immunised groups, which is between 20 to 40 pg/mL. Exposure of HBsAg to naïve, free DNA and alum groups also showed background levels of IFN-γ, while free HBsAg group as well as all liposomal groups show high IFN-γ production levels (Figure 5.10E). It should be noted that all liposomal groups produce significantly ($P<0.05$) higher IFN-γ than free DDP (Group 10; Figure 5.10E). Similar to IL-2 production, IFN-γ levels of DOTAP liposomes when formed in sucrose (Group 1; Figure 5.10E) are significantly ($P<0.05$) higher than the group which formed in PBS (Group 2; Figure 5.10E); in contrast, presence of electrolyte in the formulation does not influence produced IFN-γ levels in response to DSTAP based liposomes (Group 3 and 6; Figure 5.10E) and both of the DSTAP formulations show high IFN-γ production near to 2000 pg/mL. Con A restimulation produced higher levels of IFN-γ in a range 5000 to 7000 pg/mL (Figure 5.9E).

The last studied cytokine was IL-1ß. It is a potent proinflammatory cytokine whose production is related to the uptake of particulate adjuvants by dendritic cells (DCs) (Henriksen-Lacey et al., 2011b; Sharp et al., 2009); hence, as liposomal vaccines promote antigen uptake at the SOI, it would be of interest to investigate the production levels of IL-1ß at the injection site. Figure 5.10F shows that not only the liposome formulations (Group 1, 2, 3 and 6), but also DDP (Group 10), free subunit antigen

(Group 11), free plasmid DNA (Group 12) and alum (Group 13), are able to activate dendritic cells to induce the innate immune response; however, the level which is shown by liposomal groups is significantly ($P<0.05$) higher than non-liposomal injected groups (Groups 10-13; Figure 5.10F). Results also display that there is no significant difference between the liposomal formulations, as they all of them produce high levels of IL-1ß in a range between 4000 to 5000 pg/mg of the injection site (Figure 5.10F).

Considering Th1 cell mediated immune responses, DOPE:DOTAP+Sucrose and DOPE:DSTAP+PBS induce the highest cytokine (IL-2 and IFN-γ) levels (Figure 5.10A and E). Investigating the effect of electrolytes in the formulation, this does not give any convincing conclusion; DOPE:DOTAP incorporating PBS caused significant ($P<0.01$) decrease in the cytokine production level for both IL-2 and IFN-γ. Even though for DOPE:DSTAP, the presence of electrolyte in the formulation has led to an increase in cytokine production (Figure 5.10A and E). Furthermore, replacing the cationic lipid DOTAP with DSTAP caused no notable difference in Th1 response when sucrose was used for hydration of the lipid film. In contrast, DOPE:DOTAP+PBS liposomes showed significantly ($P<0.001$) lower IL-2 and IFN-γ production comparing to DOPE:DSTAP+PBS (Figure 5.10A and E), showing that DSTAP formulations induce high cell mediated immune response without being limited by nature of hydration media.

Alhydrogel formulation is an aluminium salt and is known to be predominate activator of Th2 biased immunity (Davidsen et al., 2005). This is clearly shown in the results as the level of alhydrogel in IL-5 and IL-10 is comparable with tested liposomes and particularly the IL-10 level of alhydrogel significantly higher ($P<0.001$) than any of the four liposomes in this study (Figure 5.10D). However, the level of produced IL-2 and

IFN-γ by alum is similar to the naïve group results (Figure 5.10A and E), showing that alum is not able to produce cell mediated immune responses.

Considering humoural immune responses, it is shown that liposomes formulated in sucrose give the highest Th2 response, as both DOPE:DOTAP (Group 1; Figure 5.10B and D) and DOPE:DSTAP (Group 3; Figure 5.10B and D) formed in sucrose give 100 pg/mL and 110 pg/mL for IL-5 and IL-10, respectively (Figure 5.10B and D). It is also shown that IL-5 and IL-10 production level for DOPE:DOTAP+PBS (Group 2; Figure 5.10B and D) are significantly ($P<0.05$) lower than the same formulation hydrated in sucrose (Group 1; Figure 5.10B and D). The same trend has been seen for DOPE:DSTAP, however, the difference is not significant (Group 3 and 6; Figure 5.10B and D).

From these results, it appears that both DOTAP and DSTAP liposomal vaccines are able to induce comparable levels of Th1 and Th2 responses; however, DSTAP formulations showed more stability in inducing immune response in both hydration media and for all studied cytokines. Previously in section 5.4.1.1., the impact of these formulations on antibody production was described and it was concluded that both DOTAP and DSTAP formulations show similar IgG and IgG1 production levels due to their similar levels of DNA drainage to the local draining lymph nodes at the site of injection. The results also showed higher IgG2 production for DSTAP-based vaccines, which was as a result of higher transition temperature of DSTAP. With regards to the Th1 and Th2 antibody responses, both formulations show similar levels of Th2 responses and DSTAP liposomes show higher Th1 responses. Considering the results from cytokine study, the results show the same trend as DSTAP-based liposomes with higher cellular immune responses compared to DOTAP formulations, while both

formulations show similar innate (based on IL-1ß results) and humoural immune responses. This shows the higher transition temperature and more rigidity of DOPE:DSTAP structure, which caused higher *in vitro* transfection efficiency for this formulation compared to DOTAP liposomes (Section 4.6, Chapter 4 of this thesis), is the key factor in higher Th1 immune response.

Previous studies (Henriksen-Lacey et al., 2011a) have also compared the immunogenicity of different cationic lipids and demonstrated that lipids with higher transition temperature like DDA and DC-Chol show higher cellular immune response than DOTAP, which has a Tc below room temperature, and its bilayer structure is fluidic. They suggest that the higher transition temperature caused more antigen retention at the site of injection resulting in stronger antigen recall and consequently higher IFN-γ production level, which indicates DDA and Dc-chol liposomes, induce higher cell mediated responses. In addition, a recent study (Christensen et al., 2012) compared saturated DDA to its unsaturated analogue DODA, and results showed more antigen retention at the injection site for DDA resulted in higher expression of co-stimulatory molecules CD40 and CD86 and this led to higher cellular immune response for DDA-based liposomes.

These results are in agreement with the findings of this current study, suggesting a similar mechanism caused higher IL-2 and IFN-γ production for saturated DSTAP liposomes compared to unsaturated and fluidic DOTAP formulations. Finally, this study suggests that bilayer composition has a major role in induced immune response by liposomal vaccines.

5.4.2.2. Exploring the effect of vesicle morphology on cytokine production levels of cationic liposomal vaccines in a heterologous immunisation schedule

The impact of the liposomal vaccine type (DRV vs SUV) on cytokine production was investigated using DOPE:DSTAP. Results (Figure 5.11) show no significant difference in producing Th1 response, with both SUV and DRV inducing similar cytokine production for IL-2 (Figure 5.11A) and IFN-γ (Figure 5.11E). IL-6 production also followed the same trend, although, it is shown that IL-5 and IL-10 production levels of SUV formulations are higher than their DRV analogue (Figure 5.11B and D). This shows SUV liposomes may be better at inducing humoural immune responses compared to DRV liposomal vaccines.

In vitro transfection results (Section 4.6, Chapter 4) showed significantly ($P<0.05$) higher transfection efficiency for SUV DSTAP based liposomes compared to DRV formulations when they formed in the absence of electrolytes. However, biodistribution investigations revealed no notable differences between the two types of liposomes (Section 5.3.2; Figure 5.3), yet antibody production was reduced when DRV liposomes were used (Section 5.4.1.2; Figure 5.6). This could also explain the significantly ($P<0.05$) lower IL-5 levels for DRV liposomes; IL-5 is characteristic of humoural responses. A recent study of Milicic et al (Milicic et al., 2012) demonstrated that SUV liposomal vaccines of DDA:TDB induced higher IgG levels than DRV formulations. This study also demonstrates that SUV liposomes induce stronger $CD4^+$ and $CD8^+$ T cell responses as well as antibody production compared to DRV. More activation of $CD4^+$ T cells by MHC II class molecules may have caused higher humoural response for SUV liposomes, which is in line with the results of this current study and according to Carstens et al., it might be due to the smaller vesicle size of the SUV liposomes than DRV formulations and consequently higher APC uptake of SUV (Carstens et al., 2011).

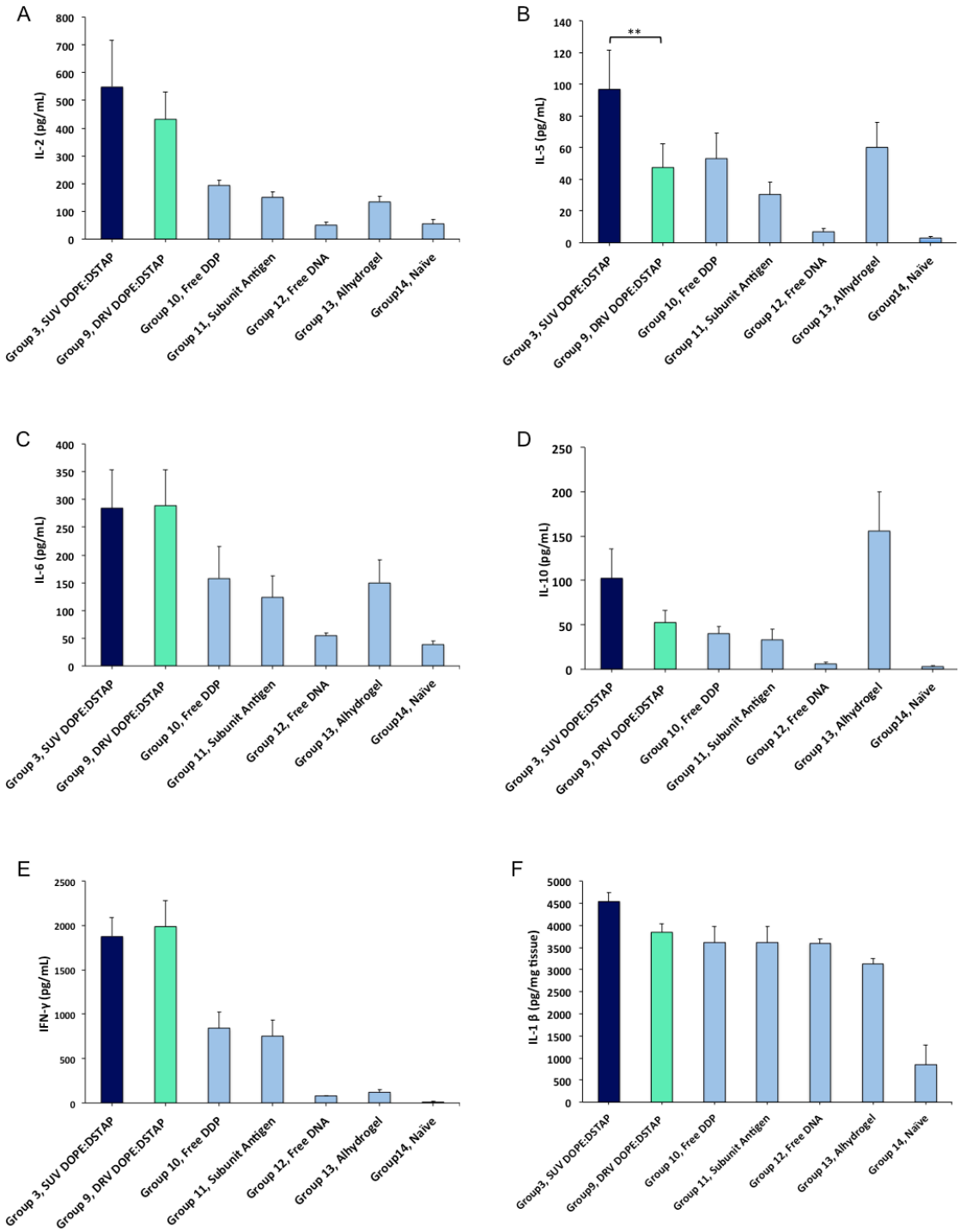

Figure 5.11: IL-2, -5, -6, -10 and IFN-γ cytokine production from splenocytes (A–E) and IL-1β production from excised leg muscle from the SOI (F) derived from mice immunised with SUV DOPE:DSTAP or DRV DOPE:DSTAP pDNA-lipoplex or subunit protein liposome formed in sucrose and in heterologous prime boost strategy. Splenocytes and muscle from the SOI were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 48 h in the presence of HBsAg (5 μg/mL). Leg muscle was excised, digested and homogenised. Cytokines were measured from splenocyte or muscle supernatants using sandwich ELISAs. Significance measured by one-way ANOVA and Tukey's post test, it is shown by **$p<0.01$.

5.4.2.3. Studying the impact of heterologous vaccination strategy on cytokine production of DOPE:DSTAP liposomal vaccines

Although both DOTAP and DSTAP based liposomes showed the ability of producing high levels of cytokine and inducing both humoural and cell mediated immune responses, SUV DOPE:DSTAP promoted higher responses in both hydration media for all of the cytokines. Hence DOPE:DSTAP were investigated to compare the cytokine responses for prime-boost vaccination (DDP) compared to the homologous immunisations of two DNA primes (DD-) or protein boost only (--P) (Figure 5.12).

As it is shown in figure 5.12, DDP immunisation caused a significantly ($P<0.001$) higher cytokine response in splenocytes compared to prime only immunisation (DD-). However, it was slightly different when the heterologous immunisation was compared to the homologous boost only (--P) immunisation. In many formulations there was not a significant difference between cytokine production levels in response to these immunisation strategies. This was not limited to one hydration media and was seen with both sucrose and PBS formed liposomes. For IL-2 and IL-6 cytokines the prime-boost immunisations responses to the DOPE:DSTAP+sucrose are higher than their boost immunisation responses, but the difference is not significant (Figure 5.12A and C, orange bars). Similar results have been seen in IL-5, IL-6 and IL-10 for DOPE:DSTAP+PBS (Figure 5.12B,C and D, dark blue bars). However, IFN-γ responses to both DOPE:DSTAP liposomes that are formulated in sucrose or PBS show the prime boost immunisation induces significantly higher ($P<0.05$) cytokine production than boost only immunisation (Figure 5.12E). Overall, considering the Th1 and Th2 responses, the results show both cellular and humoural responses were enhanced by the effect of heterologous immunisation.

Tissue excised from the injection site of mice immunised with three different immunisation strategies show no significant difference between the vaccination regimens in both hydration media (Figure 5.12F), as all of the responses were near to (5000 ng/mg tissue) suggesting that any kind of liposomal immunisation has the ability to stimulate pro-inflammatory responses.

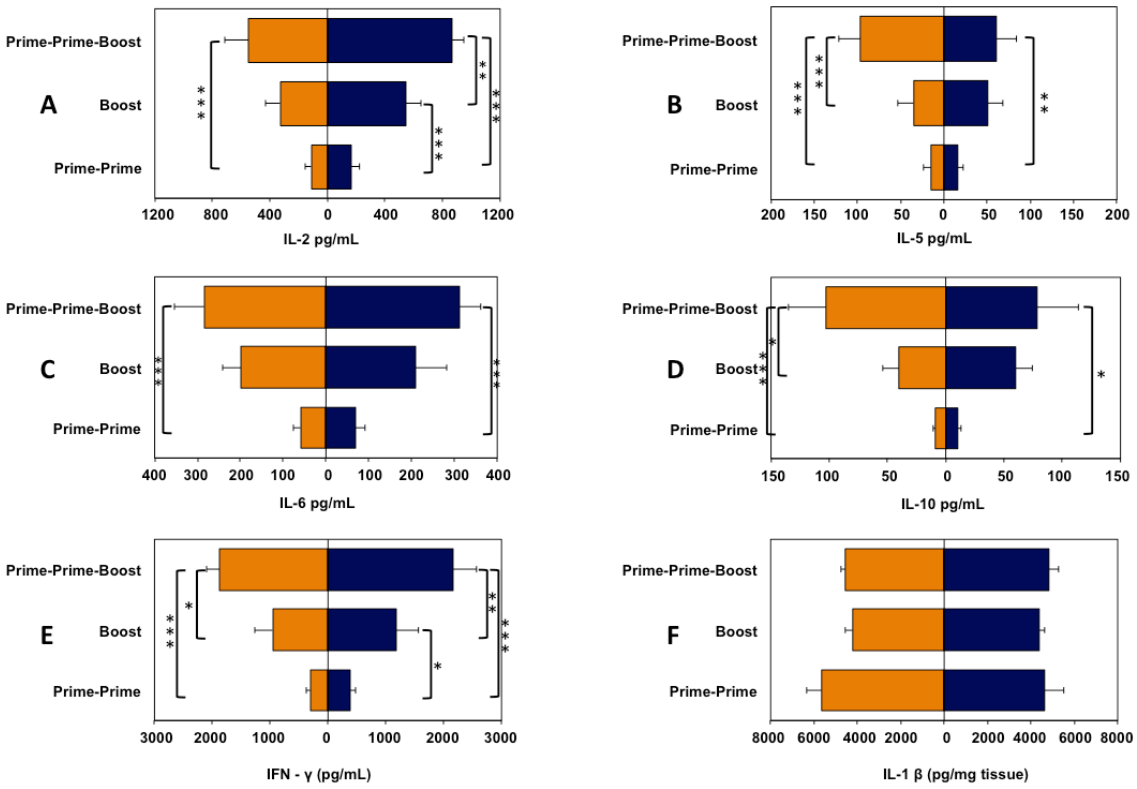

Figure 5.12: IL-2, IL-5, IL-6, IL-10 and IFN-γ cytokine production from splenocytes (A–E) and IL-1β production from excised leg muscle from the SOI (F) derived from mice immunised with SUV DOPE:DSTAP pDNA-lipoplex or subunit protein liposome formed in sucrose (orange bars) or PBS (dark blue bars). Immunisation strategies varied between heterologous prime-boost, homologous pDNA prime and homologous protein boost. Splenocytes and muscle from the SOI were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 48 h in the presence of HBsAg (5 μg/mL). Leg muscle was excised, digested and homogenised. Cytokines were measured from splenocyte or muscle supernatants using sandwich ELISAs. Significance measured by one-way ANOVA and Tukey's post test, it is shown by *p<0.05, **p<0.01 and ***P<0.001.

Taken together, this data (Figure 5.12) suggests that the subunit protein vaccination (--P) provides immunisation responses similar to the produced levels by prime-boost vaccination composed of two DNA vaccines and one subunit protein vaccine (DDP).

A similar study by Yang et al (Yang et al., 2008), showed prime-boost immunisation induces higher humoural immune responses and also increases the $CD8^+$ T cell activity. This caused an increased IFN-γ production suggesting that heterologous immunisation can induce stronger cell mediated immune responses. Their results show the synergic effect of prime-boost immunisation has caused a significantly higher Th1 and Th2 responses compared to homologous immunisations; however, in this current study, heterologous immunisation of DOPE:DSTAP vaccines did not produce significantly higher response than single i.m injection of DOPE:DSTAP-subunit. Comparing these two studies shows the only major difference is in the route of the administration of protein boost immunisation. Yang et al. (2008) study included 2 i.m injection of DNA-liposomes and a single i.n. injection of boost HBsAg; however in this study all the injections were applied as an i.m injection. This indicates the higher synergic effect of heterologous immunisation could be as a result of mucosal immune response, which was activated by the i.n. injection.

5.4.3. Splenocyte proliferation

In addition to the cytokine study, liposomal vaccines were investigated for their ability to initiate antigen specific spleen cell proliferation (Figure 5.13-5.16). Splenocytes were restimulated with 0.05, 0.5 and 5 μg/mL HBsAg antigen, in addition to the positive control stimuli Con A (5 μg/mL). Figure 5.13 shows the proliferative ability of splenocytes of all immunised groups in response to Con A; all the groups showed

response to Con A as the ^{3}H-thymidine uptake was shown to be between 30000 to 40000 CPM (Figure 5.13).

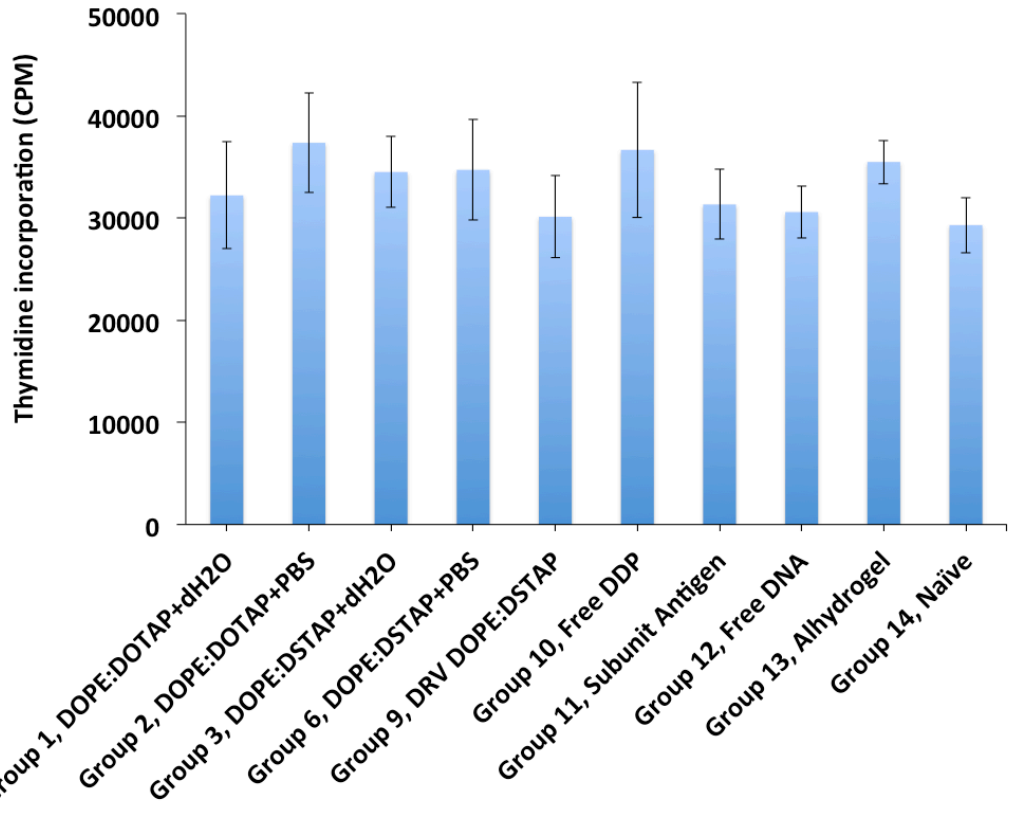

Figure 5.13: Splenocyte proliferation of each immunisation group and naïve group in response to stimulation with 5 µg/mL Con A. Splenocytes were derived on day 49 of the study and restimulated *ex vivo* with Con A as a positive control. After a further 24 hrs cells were harvested and the proportion of ^{3}H-thymidine incorporated in the cells measured using standard scintillation counting.

Studying the splenocytes, when exposed to HBsAg antigen, shows that increasing the amount of HBsAg concentration increased the splenocyte proliferation in all of the immunised groups tested with the exception of groups 10, 11, 12 and 14 (Figure 5.14).

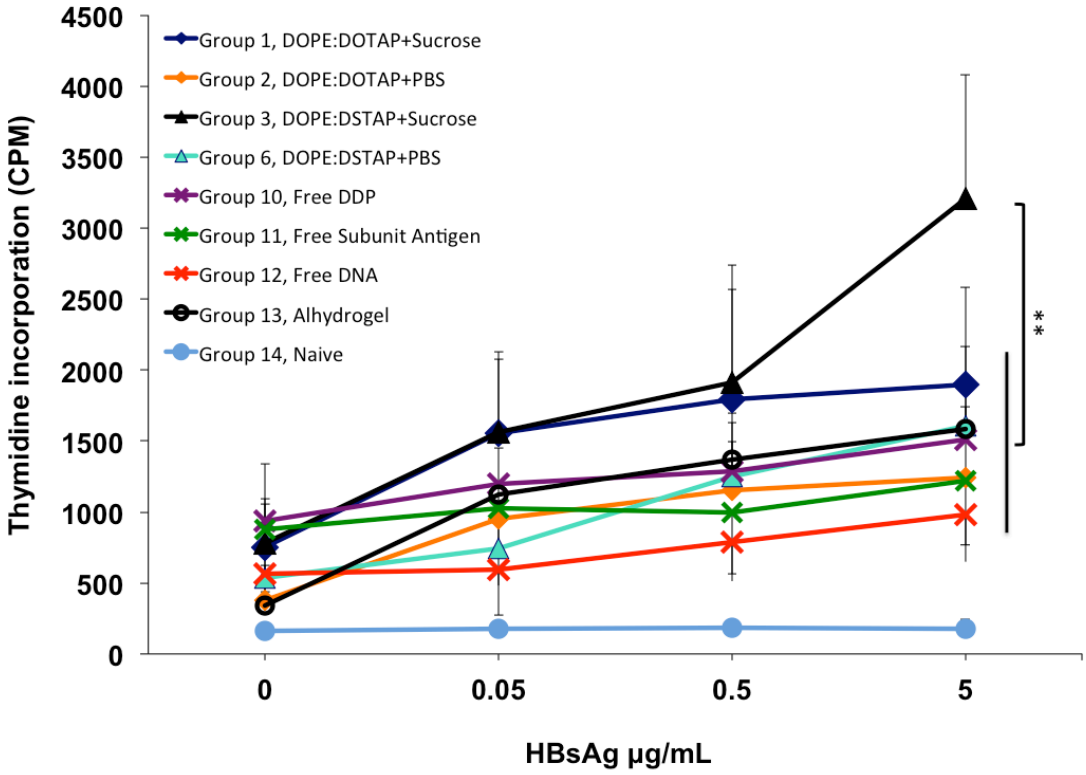

Figure 5.14: Splenocyte proliferation of DOPE:DOTAP and DOPE:DSTAP both hydrated in sucrose or PBS and injected under heterologous regimen, Alum, free antigen, free pDNA and naïve group. Splenocytes were derived on day 49 of the study and restimulated *ex vivo* with range of 0 to 5 µg/mL HBsAg .After a further 24 hrs cells were harvested and the proportion of ^{3}H-thymidine incorporated in the cells measured using standard scintillation counting. Significance measured by one-way ANOVA and Tukey's post test, it is shown by **p<0.01.

Comparing to the naïve group, all other groups show a significantly (P<0.05) higher proliferation for all concentrations of HBsAg. Considering the effect of presence of electrolytes in the formulation, results show both liposomes where formulated with PBS showed lower proliferation compared to the liposomes hydrated in sucrose (Groups 2 vs 1 and 4 vs 3 respectively; Figure 5.14). It is also shown in antigen concentrations below 5 µg/mL there is no significant difference in proliferation of mice immunised with DOTAP or DSTAP liposomes and the only notable change is in 5 µg/mL concentration of HBsAg, where mice immunised with DOPE:DSTAP+sucrose (Group 3) show a significantly (P<0.05) higher splenocyte proliferation than any other formulation (Figure 5.14). In the mice immunised with alum (Group 13; Figure 5.14), proliferation

was shown to be similar to mice receiving the liposome formulations and higher than free antigens, with only the group which received DOPE:DSTAP/sucrose (Group 3; Figure 5.14) giving significantly ($p<0.05$) higher responses.

Figure 5.15 shows the comparison between SUV and DRV liposomes of DOPE:DSTAP in splenocyte proliferation and it demonstrates that SUV liposomes show significantly ($P<0.05$) higher cell proliferation than DRV liposomes in all of HBsAg concentrations (Figure 5.15).

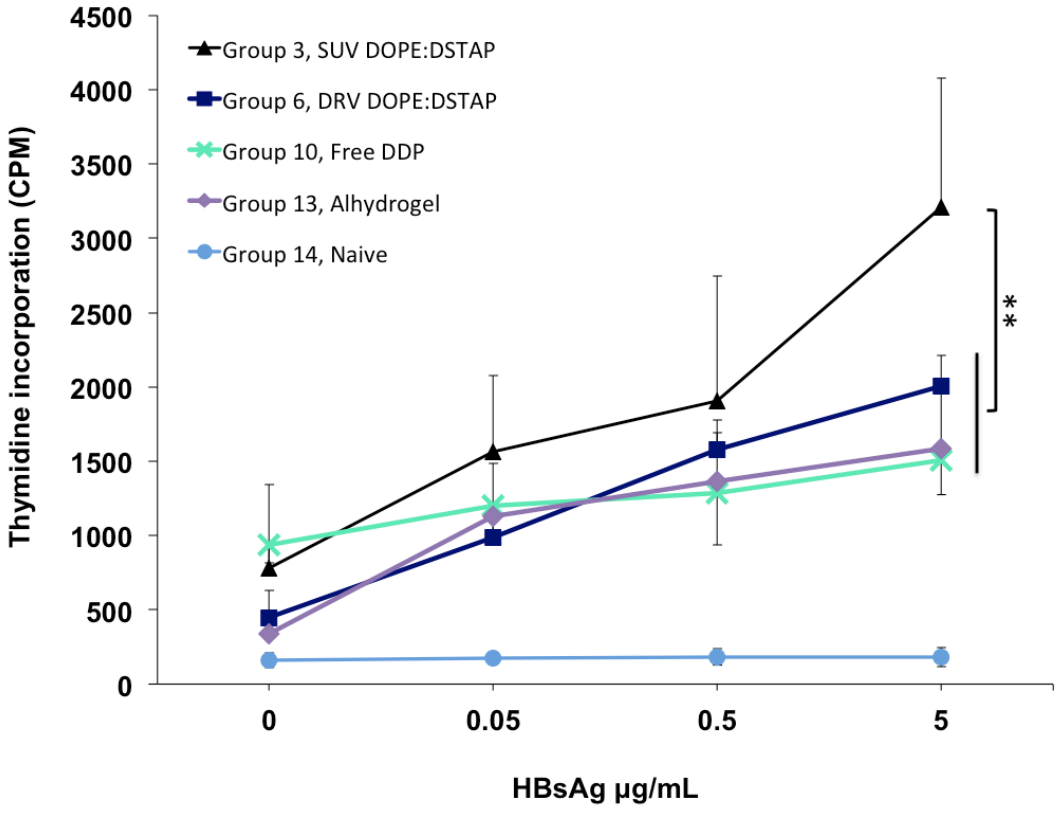

Figure 5.15: Comparing splenocyte proliferation of SUV DOPE:DSTAP and DRV DOPE:DSTAP both formed in sucrose. Splenocytes were derived on day 49 of the study and restimulated *ex vivo* with range of 0 to 5 µg/mL HBsAg .After a further 24 hrs cells were harvested and the proportion of ^{3}H-thymidine incorporated in the cells measured using standard scintillation counting. Significance measured by one-way ANOVA and Tukey's post test, it is shown by **$p<0.01$.

Comparing the impact of different immunisation strategies on splenocyte proliferation shows the heterologous prime-boost immunisation of DOPE:DSTAP+sucrose has the highest proliferation level, which is significantly higher (P<0.001) than homologous immunisations at the 5 µg/mL concentration of HBsAg (Figure 5.16). However, there is no significant difference between heterologous and homologous immunisations of DOPE:DSTAP+PBS.

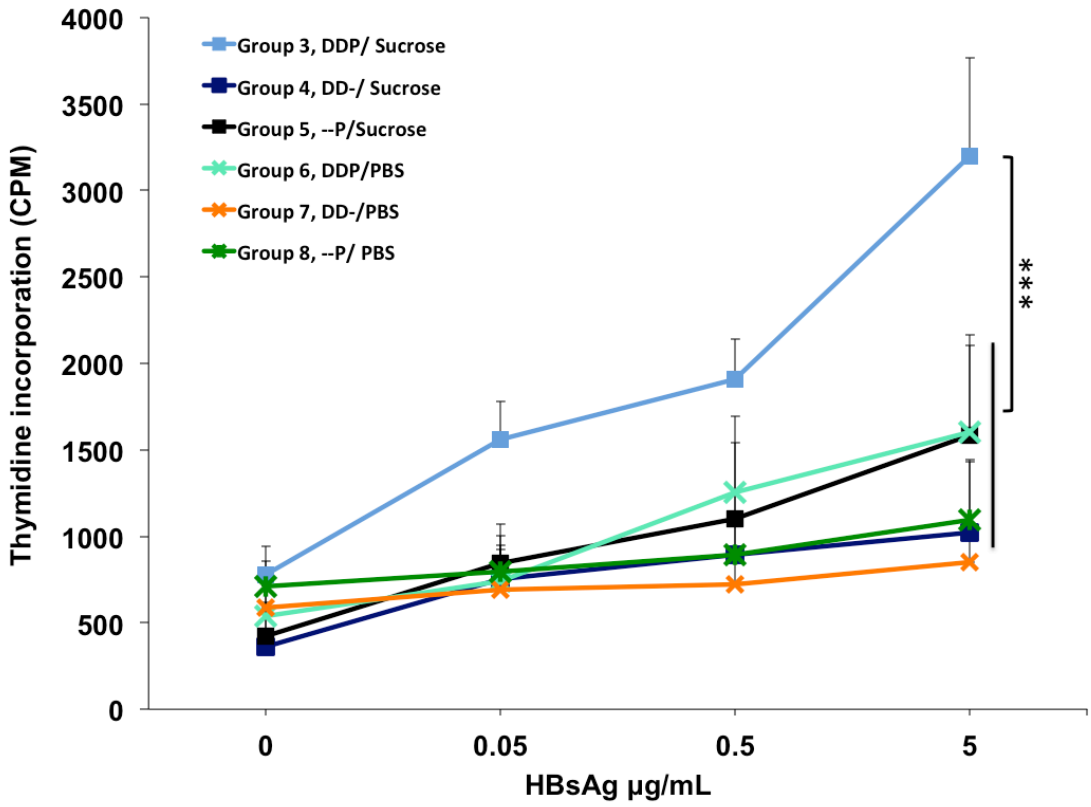

Figure 5.16: Comparing splenocyte proliferation of heterologous prime boost immunisation to homologous pDNA prime and protein boost for DOPE:DSTAP+Sucrose and DOPE:DSTAP+PBS. Splenocytes were derived on day 49 of the study and restimulated *ex vivo* with range of 0 to 5 µg/mL HBsAg .After a further 24 hrs cells were harvested and the proportion of ^{3}H-thymidine incorporated in the cells measured using standard scintillation counting. Significance measured by one-way ANOVA and Tukey's post test, it is shown by ***p<0.001.

Taken together, these results show that, apart from DSTAP formulations when made in sucrose, other liposomal vaccines show a similar splenocyte proliferation rate regardless of their cationic lipid, hydration media or immunisation strategy.

5.5. Conclusion

The studies in this chapter aimed to investigate the ability of liposomal formulations as possible DNA vaccine delivery systems and to consider the impact of their physicochemical properties on their immunogenicity. Furthermore, induced immune responses resulting from a heterologous immunisation schedule were compared to two different homologous vaccinations.

In general, all liposomal formulations tested showed significantly higher cellular and humoural immune response compared to the all free antigens and naïve group, as expected (Gregoriadis, 1990; Gregoriadis et al., 1997; Henriksen-Lacey et al., 2011c; Morrey et al., 2011; Perrie et al., 2001; Yang et al., 2008). Although Alum, an old and well-known adjuvant for subunit protein vaccines, showed comparable humoural responses to the liposomal systems, it failed to produce cell mediated responses, as expected (Brunel et al., 1999; Davidsen et al., 2005; Rosenkrands et al., 2005).

Overall, in terms of immune responses promoted by the tested liposomal formulations, DOPE:DSTAP, when formed in sucrose and injected under heterologous prime-boost (DDP) immunisation regimen, showed the highest response. DOPE:DSTAP hydrated in PBS showed similar results, with comparable cellular immune responses but lower humoural responses. The higher immune responses of DSTAP-based liposomes can be related to the higher transition temperature of cationic lipid of DSTAP, which makes the bilayer more rigid, compared to the more fluidic DOTAP-based liposomes.

Biodistribution study showed that having a more rigid bilayer caused stronger depot effect at the site of injection, therefore promoting a more sustained drainage into the local draining lymph nodes where dendritic cells (DCs) interact with the antigen and present the antigens to the MHC class II molecules and T cells, which result in induction of the humoural and cell mediated immune responses. Several studies have shown the same trend, showing the effect of bilayer composition on enhancement of immune response (Christensen et al., 2012; Henriksen-Lacey et al., 2011a).

Investigating the impact of incorporating electrolytes in the formulation showed formulations which were hydrated in sucrose induce stronger immune responses compared to the formulations made in PBS. Characterisation studies showed the presence of salt in the liposome formulation can cause aggregation, which may hinder uptake of the liposomal-antigen by DCs infiltrating the site of injection or residing at the local lymph nodes, which leads to the reduction in immune response. In agreement to this result, Henriksen-Lacey et al. (2011b) compared the effect of vesicle size of the liposomes on immune response and for this reason formulated DDA:TDB liposomes in PBS to reach to an extra large vesicle size. The study demonstrated a poor immune response for the formulation when formed in PBS compared to other formulations and suggested that the unexpectedly poor immunogenicity of this formulation may not be solely size induced, but could be as a result of presence of salt. However, another study (Yan and Huang, 2009) has shown presence of small amount of salt in the formulation enhances the development of immune response by the liposomes. Therefore, the ability to exploit electrolytes to enhance immunes responses remains unclear.

Comparing SUV and DRV of DOPE:DSTAP formulated in sucrose showed that, in terms of humoural immune responses, SUV formulations induce significantly stronger

total IgG and IgG1 responses for SUV compared to DRV. However, results demonstrate no significant difference between SUV and DRV formulation in enhancing cellular mediated responses, where amongst all the cytokines tested, only IL-5 production was shown to be significantly higher for SUV, which could be due to the smaller vesicle size of SUV. This is in line with the study (Carstens et al., 2011) that shows smaller liposomal DNA vaccines with low depot effect induce strong humoural immune response, suggesting that liposomal DNA vaccines do not benefit from strong depot effect and other characteristic parameters of the formulation can have an important role in activation of immune system. It has been established through *in vitro* studies that the optimal size for APC uptake is below 500 nm (Foged et al., 2005). Moreover, Singh et al. (2000) have demonstrated an enhanced immunogenicity for particulate-based DNA vaccine with the size of 300 nm over 1 μm size particles.

Furthermore, a recent (Milicic et al., 2012) study compared three different types of liposomes in terms of humoural and cellular immune responses; these studies showed that SUV liposomes of DDA:TDB complexed with OVA induce higher IgG production and consequently stronger humoural immune response compared to MLV and DRV types of the formulation. In contrast, it is shown by Kaur et al. (2012a, b) that DRV liposomal vaccines of DDA:TDB- Ag85B-ESAT-6 provide stronger immunity compared to SUV liposomes. This suggests that the chosen antigen in the formulation may also have an impact.

Finally, to study the impact of different immunisation strategies on immune response, heterologous prime-boost and (DDP) immunisation for DOPE:DSTAP, formed in both hydration media has compared to two homologous vaccination regimens including prime DNA(DD-) and boost (--P) regimens. The results from these studies illustrate

that, compared to the homologous DNA-liposome regimen, heterologous immunisation can stimulate significantly higher antibody and cytokine production in response to HBsAg. Showing a synergistic immune response by the heterologous regimen implies that DDP strategy provides higher immune responses than single i.m injection of liposomal subunit antigen (--P); however, the difference between two strategies is not notably different and potentially a PPP regimen would give higher responses. This indicates that, although numerous studies (Carstens et al., 2011; Liu, 2011; Lu, 2009; O'Hagan et al., 2004; Otten et al., 2005; Stambas et al., 2005; Yang et al., 2008) show promising results for heterologous immunisation, more studies are needed to improve this technology to a level that justifies the cost of this type of vaccination.

Chapter 6:
DDA-based DNA vaccine Formulations: From Characterisation to *in Vitro* and *in Vivo* Studies

6.1. Introduction

To date, there are at least eight liposome-based adjuvant formulations which are approved or going through clinical trial phases (Watson et al., 2012): for example, there are virosomes such as Infexal® V (Herzog et al., 2009) and Epaxal® (Bovier, 2008), which are in the market, and DNA vaccines like Vaxfectin (Sullivan et al., 2010; Veselenak et al., 2012) and JVRS-100 (Dong et al., 2012), which are in Phase II clinical trials. Among the subunit protein vaccines, there is a liposome formulation, which is composed of cationic lipid dimethyl dioctadecyl ammonium (DDA) and the immunomodulating glycolipid trehalose dibehenate (TDB). DDA:TDB (known as Cationic Adjuvant Formulation 01 or CAF01) has recently completed phase I clinical trials in combination with the tuberculosis (TB) vaccine antigen candidate, Ag85B-ESAT-6 (ClinicalTrials.gov, 2012). This formulation has shown to give strong Th1 responses (Henriksen-Lacey et al., 2010b; Henriksen-Lacey et al., 2011c) as required for TB immunisation and its mechanism of action has been extensively investigated; for example, recent studies on DDA:TDB formulations have revealed that immunisation of this formulation produces a strong depot effect at the injection site and induces a high cellular and humoural immune responses against Ag85B-ESAT-6 (Davidsen et al., 2005; Henriksen-Lacey et al., 2010b; Holten-Andersen et al., 2004).

DDA itself has long been recognised to have adjuvant properties (Gall, 1966) and has been shown to enhance both cellular and humoural responses for a range of antigens (Vangala et al., 2007). DDA is a quaternary ammonium lipid with two saturated 18-carbon hydrophobic tails (Figure 6.1A). The adjuvant action of DDA based liposomes has been described to be related to the ability of DDA to protect and deliver antigen to the antigen presenting cells (APCs), and to form the depot effect at the site of injection, causing the antigens to retain for a prolonged time period and hence enhance antigen

uptake and subsequent presentation of antigen material to T cells (Korsholm et al., 2007).

DDA-based liposomes, like other cationic liposomes, are able to convert the antigens into a particulate form and thus enhance their immunogenicity. This is due to the cationic surface charge of DDA and its ability to electrostatically bind anionic antigens (Christensen et al., 2009; Hilgers et al., 1985) and recent studies have confirmed this; by replacing the cationic DDA with the neutral lipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), the biodistribution behaviour of the vaccine adjuvant was changed and dramatically decreased immune responses (Henriksen-Lacey et al., 2010c; Kaur et al., 2011).

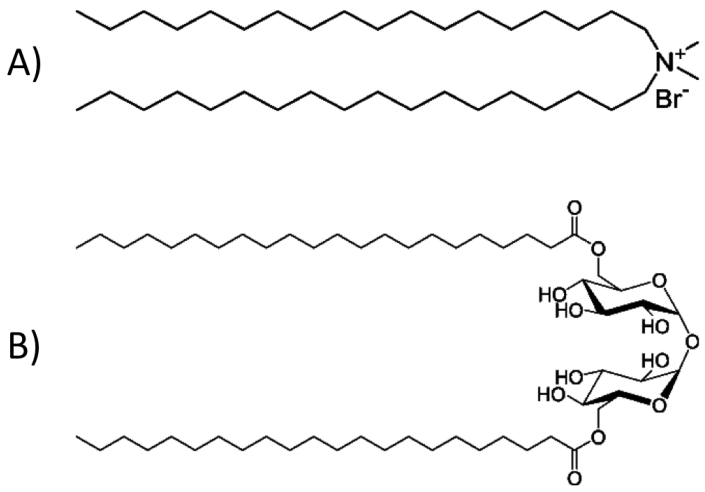

Figure 6.1: Chemical structure of A) the cationic lipid DDA and B) the immunostimulant TDB.

TDB (Figure 6.1B), the second component of DDA:TDB liposomal adjuvant, is a synthetic analogue of trehalose 6,6'-dimycolate (TDM), which is known as cord factor and is an immunostimulatory component in mycobacterial cell wall (Lemaire et al., 1986; Pimm et al., 1979). The advantage of TDB over TDM is that it is less toxic due to

its shorter fatty acid chains, whilst it shows the same immunostimulatory effect as TDM (Olds et al., 1980; Pimm et al., 1979).

As a liposomal adjuvant system, DDA:TDB is able to initiate intracellular activation pathways. Amongst the different classes of signals initiating immune responses, it has been reported that liposomes may enhance signal 1 by providing effective delivery of antigen to secondary lymphoid organs (Perrie et al., 2008; Zinkernagel et al., 1997). Given the high depot effect of DDA:TDB at the injection site, and the consequent slow draining of the incorporated antigen to the local lymph node (Henriksen-Lacey et al., 2010b), suggests that DDA:TDB is able to induce immune responses through signal 1 of the signalling pathways. It has also been shown that induction of immune responses depends upon antigens being available in lymphoid organs, since if an antigen does not reach lymphoid organs it is ignored by immune cells (Zinkernagel et al., 1997). Having antigen in the T cell region of lymph nodes can stimulate Toll-like receptors (TLRs), which activate important mediators of innate and adaptive immunity (Perrie et al., 2008). TLRs transduce signals via the intracellular MyD88 pathways and enhance cellular (Th1) and humoural (Th2) immune responses (Christensen et al., 2009; Milicic et al., 2012).

DDA:TDB was first introduced in 2004 by Holten-Andersen et al. as an efficient adjuvant for TB subunit vaccines (Holten-Andersen et al., 2004), initiating high IFN-γ response considered to be the key cytokine for induction of a Th1 immune response. Since then, DDA:TDB has been extensively studied and optimised with regards to the ratio of components, adjuvant dose and stability of the formulation (Davidsen et al., 2005), resulting in the production of a MLV of DDA:TDB with the weight ratio of 5:1. Differential scanning calorimetry (DSC) studies revealed that during incorporation of

TDB into DDA vesicles, the acyl chains of the TDB might be incorporated into the hydrophobic core of the DDA lipid bilayers, which makes the DDA bilayers more stable (Davidsen et al., 2005).

In a comparative study, the ability of DDA:TDB as a protein subunit vaccine for HBV was studied by incorporating Hepatitis B surface antigen (HBsAg) into the liposome formulation. Similar to DDA:TDB with H1, the HB vaccine produced high levels of Th1 and Th2 responses (Vangala et al., 2007; Vangala et al., 2006). These previous studies have been performed using a homologous immunisation regimen. However, given previous reports (Carstens et al., 2011; Yang et al., 2008) and the results from Chapter 5, which suggest that by applying heterologous prime-boost immunisation, using plasmid DNA and protein subunit of the same antigen, enhances both cellular and humoural immune responses, the purpose of this current study was to consider the use of DDA:TDB in a prime-boost immunisation strategy.

It has been mentioned earlier that DDA as a cationic lipid can be used as a liposomal adjuvant system (Gall, 1966). In addition, there are several cationic lipids such as DOTAP and DC-cholesterol, with immunogenic properties which have been used in liposomal vaccines (Gregoriadis et al., 2002; Henriksen-Lacey et al., 2011a; McNeil et al., 2010; Perrie et al., 2001; Perrie et al., 2002). These studies and those in chapters 3 to 5, have revealed that, to make an efficient liposomal vaccine, an equimolar ratio of helper lipid such as DOPE or cholesterol is needed to be incorporated into the cationic lipid. Hence, in this current study, in addition to DDA:TDB, equimolar ratio of fusogenic lipid DOPE is added to the DDA to make cationic liposomes of DDA:DOPE.

Therefore, the aim of this chapter was to evaluate the immunological efficiency of DDA

based liposomes as a DNA vaccine and in a heterologous prime boost immunisation regimen. To achieve this aim, the overall objectives of the work were to:

- study the characteristics of DDA:TDB and DDA:DOPE as a DNA lipoplex formulation.
- evaluate the *in vitro* transfection efficiency of the formulations.
- investigate the biodistribution of liposomes and their associated DNA and attempt to correlate these to their physicochemical properties and immunological function.
- study the immunological efficiencies of the liposomes in a heterologous prime boost immunisation study.

6.2. Molecular packaging of the lipids

Monolayer studies of DDA:DOPE and DDA:TDB were first investigated to consider potential molecular interactions between the two lipids in the mixtures. Previous studies have shown that monolayer studies can explain the molecular packaging of the lipids and its effect on characteristic properties of the liposomes, as these results could be translated into liposomal systems (Section 3.3, Chapter 3).

Therefore, in this study monolayers of single and mixed lipids were investigated for their interactions within the monolayer in the aqueous sub-phase. Pressure-area (π-A) isotherms are shown in Figure 6.2. Studying the monolayer of TDB was not possible as it is a glycolipid and does not make monolayers on the air/water interface. The collapse pressure and extrapolated (to zero pressure) area per molecule for each isotherm are shown in Table 6.1. Monolayers of single lipids of DOPE and DDA were studied on Langmuir-Blodgett trough individually. Figure 6.2 shows the isotherm of the DOPE

monolayer collapses at 42.3 mN/m at a mean molecular area of 43.4 A^2. The extrapolated area of DOPE monolayer at zero pressure is about 72 A^2/Molecule (Table 6.1). The DDA monolayer seems to have similar molecular packaging arrangements to DOPE as its collapse pressure is ≈44 mN/m. The mean molecular area for DDA monolayer is slightly smaller than DOPE monolayer (≈ 37 A^2), as is its extrapolated area per molecule (64 A^2/Molecule; Table 6.1).

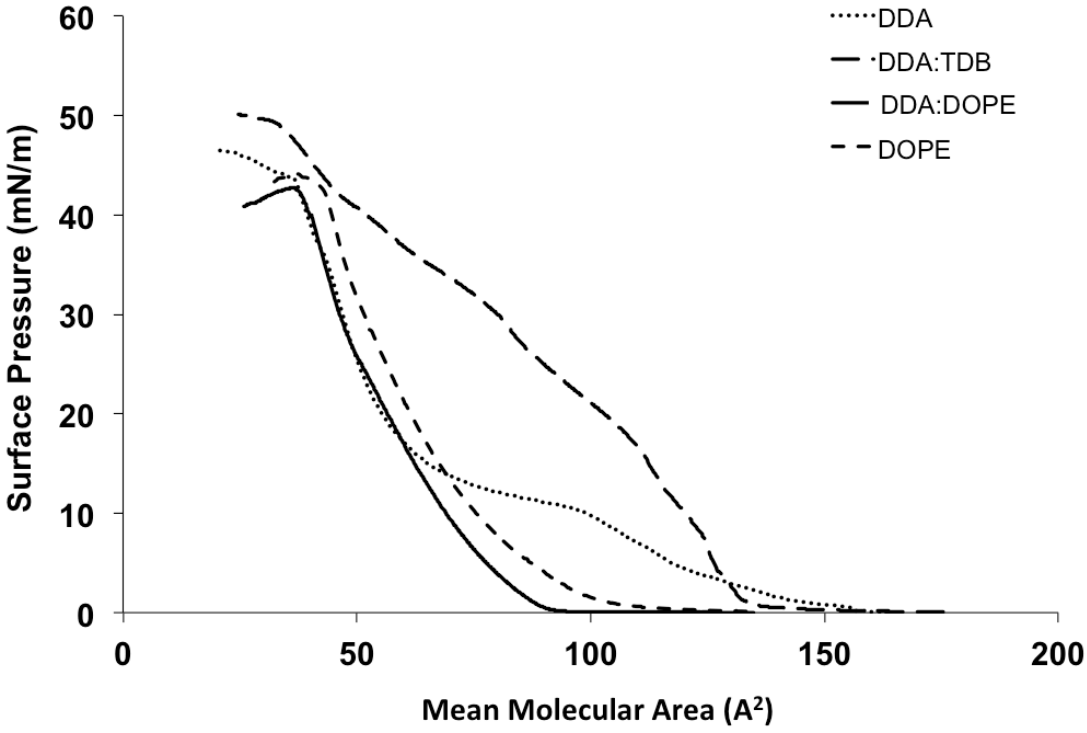

Figure 6.2: Compression isotherm studies of the single and mixture of lipid monolayers of DDA:DOPE and DDA:TDB in deionised water at 20° C. Results are expressed as the means of three experiments. SD has not shown for clarity.

Table 6.1: The experimental extrapolated area and area compressibility of mixed and single monolayers at the air/water interface at 20° C. Results denote mean±SD, n=3 for three independently prepared batches.

Lipid	Extrapolated Area (A^2/Molecule)	Ideal Extrapolated Area (A^2/Molecule)	Deviation from Ideality (%)	Collapse Pressure (mN/m)
DOPE	71.9 ± 6.0	-	-	42.3 ± 0.4
DDA	63.8 ± 4.7	-	-	43.9 ± 0.6
DDA:DOPE	67.4 ± 5.1	67.9	- 0.7	42.8 ± 0.8
DDA:TDB	114.3 ± 7.6	-	-	46.5 ± 1.6

Results show that there is no significant difference between the ideal (67.8 A^2/Molecule; Table 6.1) and experimental areas for the DDA:DOPE mixture (67.4 A^2/Molecule; Table 6.1). Deviation from the ideality can be used to monitor molecular interactions between the molecules in the mixed monolayers such as a condensing effect, as is seen with some phospholipids and cholesterol (Ali et al., 2010).

The mixture of DDA:TDB monolayer (5:1) w/w was also studied to investigate the effect of addition of TDB on DDA monolayer characteristics. As it is shown in Figure 6.2, the DDA monolayer isotherm shows a transition from liquid-expanded to liquid-condensed phase. This transition has disappeared in DDA:TDB monolayer. The results also show that the addition of TDB increases the collapse pressure (Table 6.1), and the extrapolated area at zero pressure for DDA:TDB monolayer compared with the DDA monolayer (114 vs 64 A^2/Molecule respectively), suggesting the overall area per molecule has increased, which maybe indicative of enhanced liposome stability as previously reported (Christensen et al., 2008). Increasing the collapse pressure of DDA monolayer by incorporation of TDB suggests that the trehalose head group of TDB has stronger interactions with water molecules than the DDA quaternary ammonium head groups. Therefore, it can be predicted that incorporation of TDB into the membrane of DDA liposomes will increase the hydration of the membrane and prevent dehydration of the quaternary ammonium head groups. This decreases charge repulsion between the DDA molecules in the bilayer, and reduces aggregation of the liposomes so TDB stabilises the DDA liposomes (Christensen et al., 2008).

6.3. Physicochemical characterisations of the liposomes

Physicochemical characterisations of pDNA lipoplexes of DDA:DOPE and DDA:TDB were studied in terms of their vesicle size, surface charge and antigen loading (Figure

6.3). Similar to the previously studied DOPE:DOTAP and DOPE:DSTAP formulations (Chapter 3), DDA:DOPE and DDA:TDB lipoplexes vesicle size increased with increasing DNA concentration (Figure 6.3A). This is due to the aggregation of the system, which occurs as a result of bridging effect (Ciani et al., 2004). The vesicle size for DDA:DOPE lipoplexes increased from 60 to 600 nm as the DNA concentration increased (Figure 6.3A) and these systems were significantly ($P<0.05$) smaller than DDA:TDB lipoplexes equivalents, which increased in vesicle size from 200 to 800 nm (Figure 6.3A). The difference in size between DDA:TDB and DDA:DOPE may be indicative of differences in molecular packaging of the different lipids particularly given that the DDA:TDB were shown to have more rigid monolayers than DDA:DOPE (Table 6.1 and Figure 6.2). Similarly Zantl et al., (Zantl et al., 1999) also showed that lipoplexes formed from saturated and more rigid bilayers resulted in larger vesicle sizes for the lipoplexes. Furthermore, it is known that different structures exist for the cationic liposome-DNA complex including lamellar and inverted hexagonal structures (Ulrich, 2002) and it is believed that complexation of cationic lipids in the presence of a helper lipid such as DOPE or cholesterol, with DNA would be changed from lamellar to the inverted hexagonal phase, which is preferable for transfection (McNeil and Perrie, 2006) and increases in the fluidity of the bilayer would avoid formation of aggregations (Ulrich, 2002).

Results showed that the zeta potential of any of the studied formulations was constant for DNA content of 0 to 200 μg and was between 60 to 70 mV for both liposomes, but it reduced significantly ($P<0.05$) to ≈50 mV for both DDA:DOPE and DDA:TDB lipoplexes at the highest DNA concentration of 1600 μg, showing that lipid/DNA +/- ratio affects zeta potential of the lipoplexes (Figure 6.3B). DNA association of

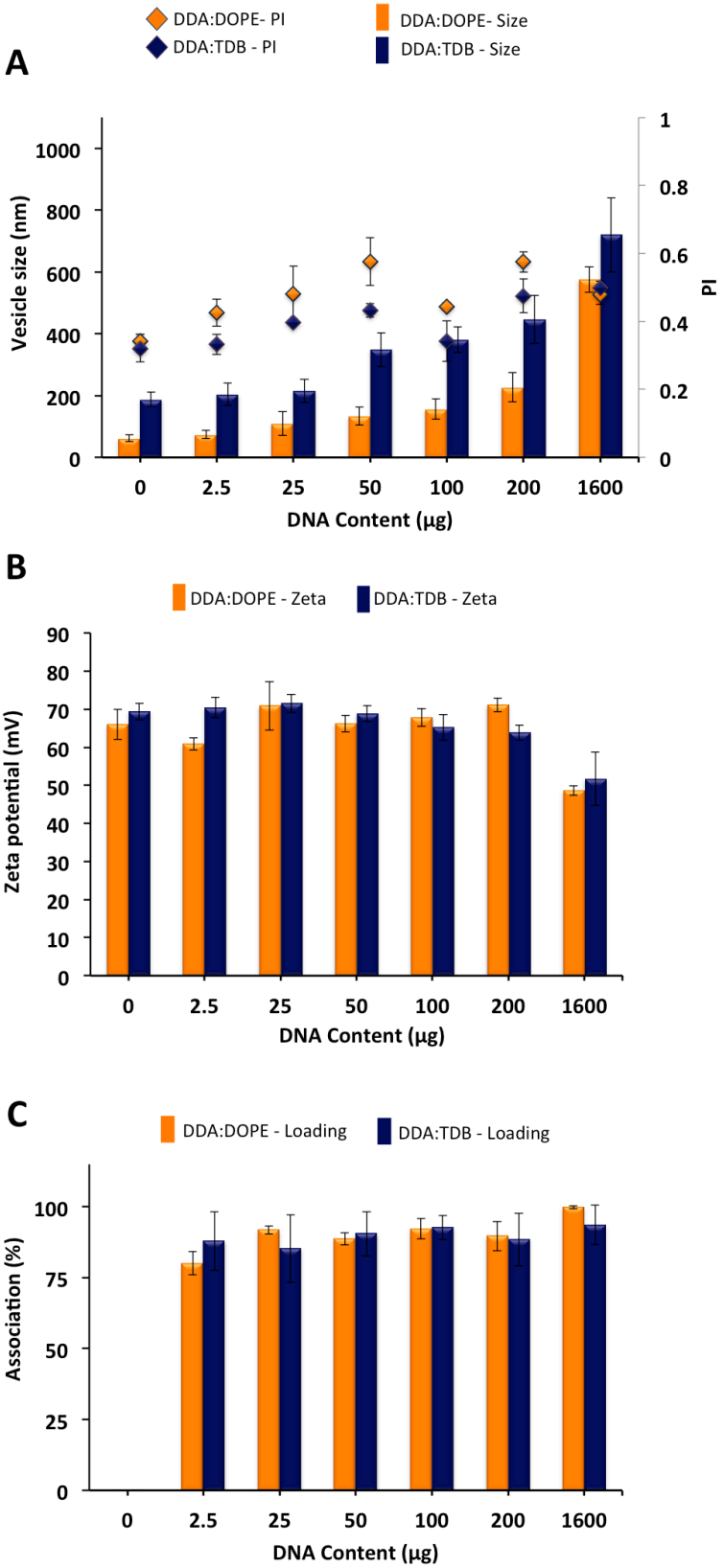

Figure 6.3. A) demonstrates vesicle size and polydispersity index, B) represents zeta potential and C) shows DNA association within SUV lipoplexes of DDA:DOPE and DDA:TDB. Results denote mean ± SD, for three independently prepared batches.

lipoplexes were similar to each other around 90% and was independent from vesicle size, as would be expected for such cationic systems (Figure 6.3C).

6.4. The ability of DDA-based lipoplexes to protect DNA from degradation.

To investigate the DNA protection offered by DDA-based SUV lipoplexes, DDA:DOPE and DDA:TDB complexed with DNA (2μg) were subjected to gel electrophoresis in the presence of SDS at 1.2 % concentration as described in sections 2.7 and 4.2.

Figure 6.4A shows that, on gel electrophoresis of DDA:DOPE (1) and DDA:TDB (2) in the absence of anionic molecules of SDS, DNA remains within the well of the gel, bound to the cationic liposomes. In contrast, following electrophoresis in the presence of SDS, displaced DNA is seen to migrate towards the cathode (Figure 6.4B). Result showed that there is no notable difference in DNA retention between both formulations in either 6.4A or B. Having most of DNA displaced for both DDA:DOPE and DDA:TDB in the presence of SDS suggests that most of the SUV associated DNA is adsorbed on the surface of the lipoplexes and that replacing DOPE with TDB had no notable effect on DNA displacement. This confirms that it is choice of cationic lipid, and not the presence of the helper lipid, which is the key factor for DNA release. This is in line with the results shown in chapter 4 with DOTAP vs DSTAP and in previous studies (Perrie et al., 2004), where replacing DOTAP with DC-choelsterol changed the DNA displacement profiles.

Also similar to section 4.3, to investigate the ability of DDA-based lipoplexes to protect the plasmid DNA from degradation by digestive enzymes, plasmid DNA was incubated

with DNase I as either naked (Figure 6.5A-D, lane 'C') or loaded with cationic liposomes (Figure 6.5A-D, lane 1-2). Figure 6.5 shows naked DNA, which was not exposed to the DNase I (Figure 6.5A and B, lane 'C'), and the digested naked DNA (Figure 6.5C and D, lane 'C'). The band of migrated anionic DNA towards the cathode is shown for undigested naked DNA in the absence (Figure 6.5A, lane 'C') and presence of SDS (Figure 6.5B, lane 'C'). However, when naked DNA exposed to the DNase I, there is no visible band on the agarose gel (Figure 6.5C and D, lance 'C') showing the naked DNA was digested by DNase I.

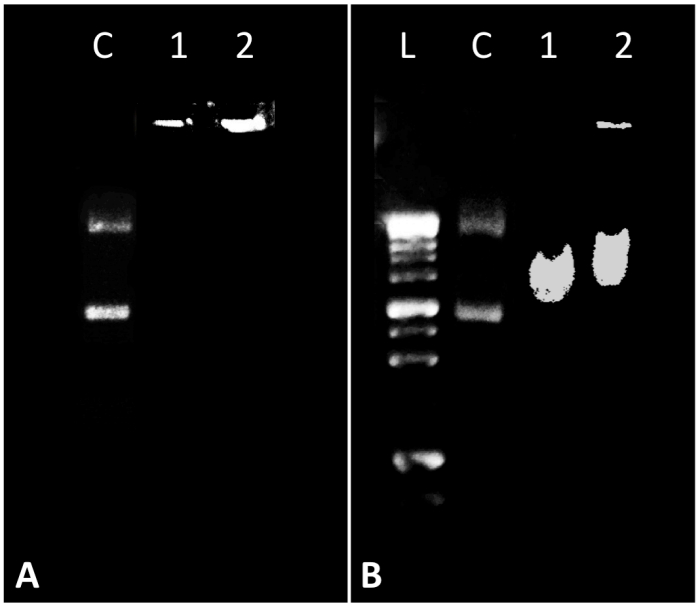

Figure 6.4: A) Gel electrophoresis of SUV lipoplexes of 1)DDA:DOPE, and 2)DDA:TDB. Lane (L) represents the DNA ladder and (C) shows the naked DNA. **B)** As in A, but in the presence of 1.2 % SDS.

For both liposome formulations tested in the absence of SDS (Figure 6.5A, lane 1,2 and Figure 6.5C, lane 1,2), DNA migration is not seen, as the plasmid DNA remains associated with the liposomes and is retained within the wells of the gel. In the presence of the competitive anionic molecules of SDS, the liposomes can be disrupted and the plasmid DNA released (Figure 6.5B, lane1,2). With the liposomes that were exposed to

DNase I (Figure 6.5D, lane 1,2), plasmid DNA can still be seen, demonstrating that both DDA:DOPE and DDA:TDB were able to protect the DNA from degradation contrary to the naked DNA, which is rapidly digested.

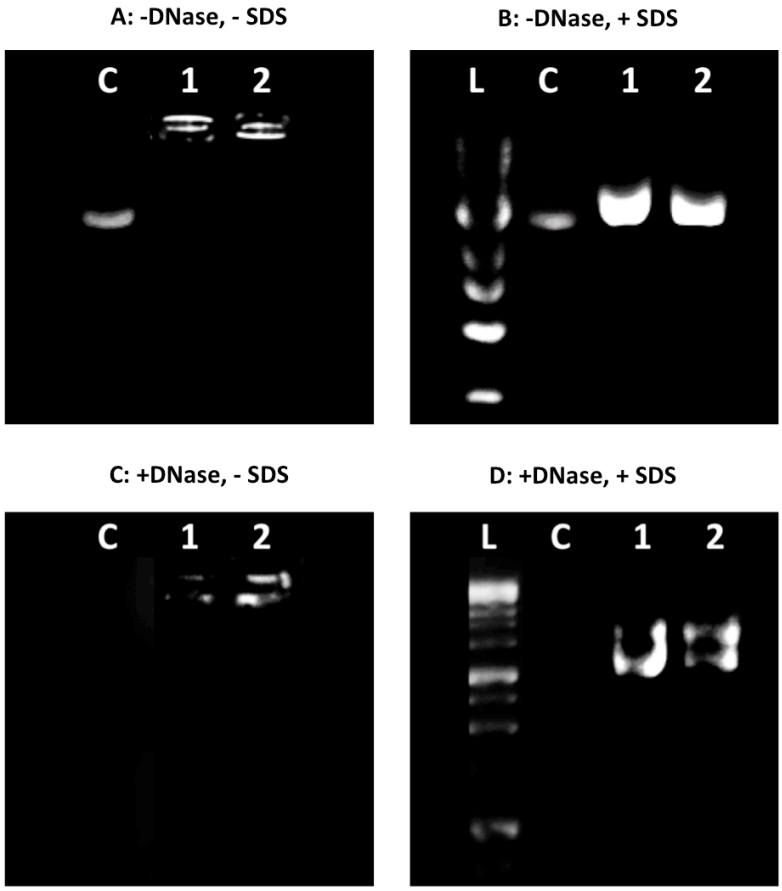

Figure 6.5: Gel electrophoresis of free and liposome entrapped plasmid DNA before and after exposure to digestive enzyme, DNase I. **A)** Gel electrophoresis of 1) SUV DDA:DOPE, 2) SUV DDA:TDB before exposure to DNase I with no SDS, **B)** same as A but in the presence of SDS, **C)** same as A but after exposure to DNase I with no SDS, **D)** Same as C but in the presence of SDS. Lane (L) represents the DNA ladder and lane (C) represents naked DNA.

6.5. *In vitro* DNA release

The release of DNA from DDA:DOPE and DDA:TDB was investigated in PBS, pH 7.4, 37 °C. As it is shown in Figure 6.6, there is no significant difference in the DNA

release rate between the formulations; and similar to the SUV lipoplexes investigated in Chapter 4, there is a burst release after 4 hours, which is 20% of total DNA entrapment (Figure 6.6). After 24 hours the DNA release increased to 30%, and to 40% after 48 hours again with no significant difference between the formulations. From this point, the release rate becomes slower, as after 4 days the release rate for both of the lipoplexes were 45% and finally the total DNA release for DDA:DOPE and DDA:TDB reached to ≈ 55% after 8 days (Figure 6.6).

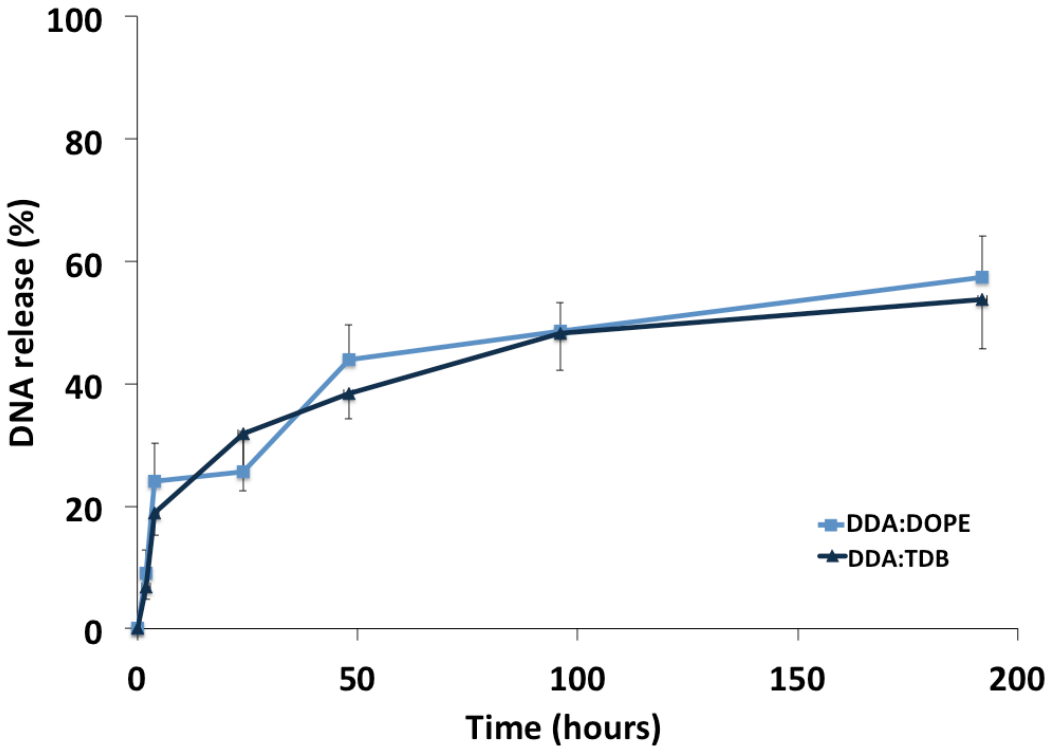

Figure 6.6. DNA release of SUV lipoplexes of DDA:DOPE and DDA:TDB in PBS, pH 7.4 and at 37 °C at time points of 2, 4, 24, 48, 96 and 192 hours. Results represent percentage release initially loaded DNA expressed as mean±SD, for three independently prepared batches.

The similarity in release profiles again demonstrates that DDA has the main role in the DNA release behaviour, as the electrostatic interactions between anionic DNA and cationic DDA is the main interaction involved in complex formation. The presence of

DOPE vs TDB makes no impact on the measured characteristics, with the formulations having similar zeta potentials (Figure 6.3B), DNA association (Figure 6.3C) and similar results for gel electrophoresis studies (Figure 6.4).

6.6. Studying the *in vitro* transfection efficiency of DDA-based lipoplexes

To investigate the transfection efficacy of the two formulations, the ability of the systems to transfect COS-7 cells with plasmid DNA encoding for luciferase was measured and compared to the marketed Lipofectin™ which is made of DOPE:DOTMA (Felgner et al., 1987) as a control. In addition, cell viability was measured using the MTS assay.

Cytotoxicity studies show that cell viability was significantly lower ($P<0.05$) after incubation with the DDA:DOPE formulations compared to cells incubated with plasmid DNA alone (Figure 6.7A). This is in line with a previous study performed by McNeil et al (2010), showing similar results for DDA:DOPE. However, this did not translate into transfection efficacy; the DDA:DOPE formulation was able to promote transfection, at levels similar to Lipofection, however, DDA:TDB, whilst having less impact on cell viability, did not promote transfection (Figure 6.7B).

Previous studies showed that having cationic charge on the surface of the liposome might not be sufficient to initiate high transfection and other parameters might be involved on transfection efficacy of the cationic liposomes (McNeil et al., 2010; Ramezani et al., 2009). These present results also confirm this, although DDA is a cationic lipid, when combined with TDB, the cationic SUV were unable to promote

transfection, despite no measured difference in commonly studied physico-chemical characteristics.

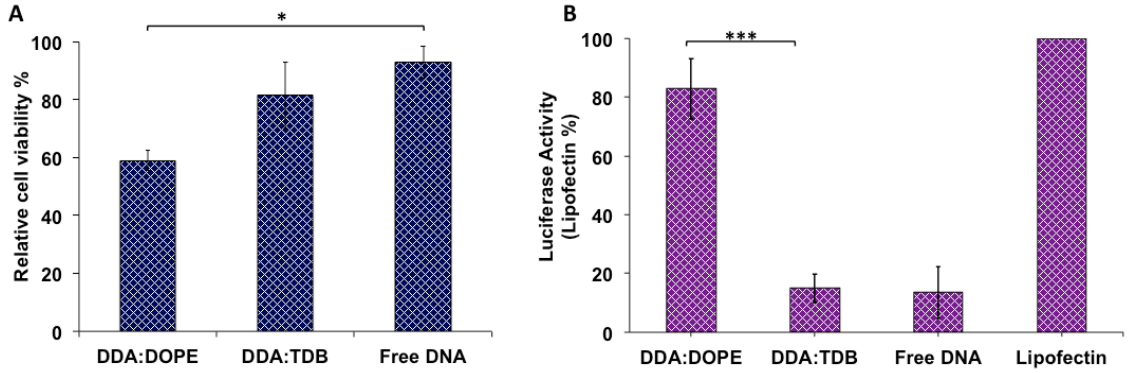

Figure 6.7. A) Relative cell viability of cationic liposomes. B) Comparison of transfection efficiency of cationic liposomes. All results denote mean±SD, for three independently prepared batches.

Clearly, although studies have demonstrated that DDA:TDB is a promising vaccine adjuvant for subunit vaccines and can promote cellular uptake (Henriksen-Lacey et al., 2011b), it is not an ideal transfection reagent with low luciferase activity of 30% (Figure 6.7B). Comparing DDA:TDB and DOPE:DDA suggests the effect of helper lipid DOPE is required to enhance the transfection efficiency of DDA. Felgner et al., in 1987 demonstrated that transfection activity of DOTMA increases when is formulated with DOPE instead of DOPC (Felgner et al., 1987) and it has been proposed that DOPE has the ability of promoting the transition from lamellar phase to an inverted hexagonal phase, as the inverted cone shaped structure of DOPE promotes the conversion of the lamellar lipoplexes phase into a non-lamellar structure. It has also been suggested that after endosomal uptake of the lipoplexes, the presence of DOPE in the formulation causes destabilisation of endosomal membrane leading to entrance of free DNA to the cytosol (Ciani et al., 2004; Farhood et al., 1995; McNeil et al., 2010). This might explain higher transfection efficacy of DOPE:DDA comparing to DDA:TDB.

6.7. Biodistribution studies: Vaccine localisation after intramuscular immunisation.

Previous studies reveal that DDA-based subunit vaccines are able to form a high depot effect at the injection site (Henriksen-Lacey et al., 2010b), which leads to the prolonged and sustained presence of the antigen at the local draining lymph nodes. This is shown to be effective for initiating the immune responses, as more antigens will be exposed to the APCs and as a result TLRs enhance Th1 and Th2 immune responses (Henriksen-Lacey et al., 2010b).

Therefore, to investigate the depot formation abilities of DDA-based DNA vaccines, the concentration of the liposomal carrier and the DNA was measured at the site of injection, the local draining lymph node (PLN), the liver, spleen, kidney and lungs using a dual radiolabelling method (Henriksen-Lacey et al., 2010a). Similar to the results of chapter 4 for DOTAP and DSTAP DNA vaccines, only injection site and PLN showed detectable levels of lipid and DNA (Figure 6.8) and other organs results were negligible (and therefore data not shown).

DNA associated with both the DDA-formulations, gave enhanced retention at the injection site, with approximately 40 % to 60% of the liposome-associated DNA remaining at the injection site after 24 h for DDA:DOPE and DDA:TDB, with no significant difference (Figure 6.8A). Over time the amount of DNA retained at the injection site decreased, and after 8 days levels had fallen to approximately half of those on day 1. However, the levels of DNA retained at the injection site was still significantly ($P<0.05$) higher than naked DNA (Figure 6.8A). In terms of lipid retention, a similar trend is seen for both formulations, with no significant difference between DDA:DOPE and DDA:TDB at each of the time points measured (Figure 6.8A).

The distribution of vaccine components to the draining PLN is shown in Figure 6.8B. Results show that the amount of detectable DNA and lipid for both formulations at the PLN increased over the time. The retained DNA at the PLN on day 1 for the lipoplex formulations was less than 0.003% and this amount increased to about 0.005% by day 8 (Figure 6.8B).

Overall the retention of the vaccine components at the injection site and PLN did not show any significant difference between the two DDA-based liposome formulations (Figure 6.8). This suggests that the presence of DOPE vs TDB has no impact on the biodistribution of the formulations, which could be an outcome of their similar charge and antigen retention. Indeed, previous studies considering DDA:TDB formulations suggest that the vesicle size of these highly cationic vesicles made no difference to their biodistribution and the primary driving factor for clearance rates is the charge of the vesicles (Henriksen-Lacey et al., 2011b). Using pontamine blue as a tracker for monocyte influx (Carstens et al., 2011; Henriksen-Lacey et al., 2010b; Tilney, 1971a), Figure 6.9 shows that the DDA:TDB promoted much higher levels of monocyte recruitment compared to the DDA:DOPE formulation.

Higher monocyte infiltration for DDA:TDB is believed (Henriksen-Lacey et al., 2010b) to be due to the role of immunomodulating molecule of TDB in the activation of the innate immune system in the influx of circulating monocytes to the site of injection. This is in agreement with the previous study (Henriksen-Lacey et al., 2010b) that reported TDB when administered in DDA liposomes activates APCs. The present results (Figure 6.8 and 6.9) show that DDA based liposomes are efficiently able to promote antigen retention at the injection site, as both systems showed similar retention of the lipid and the plasmid DNA at the site of injection and lymph node (Figure 6.8).

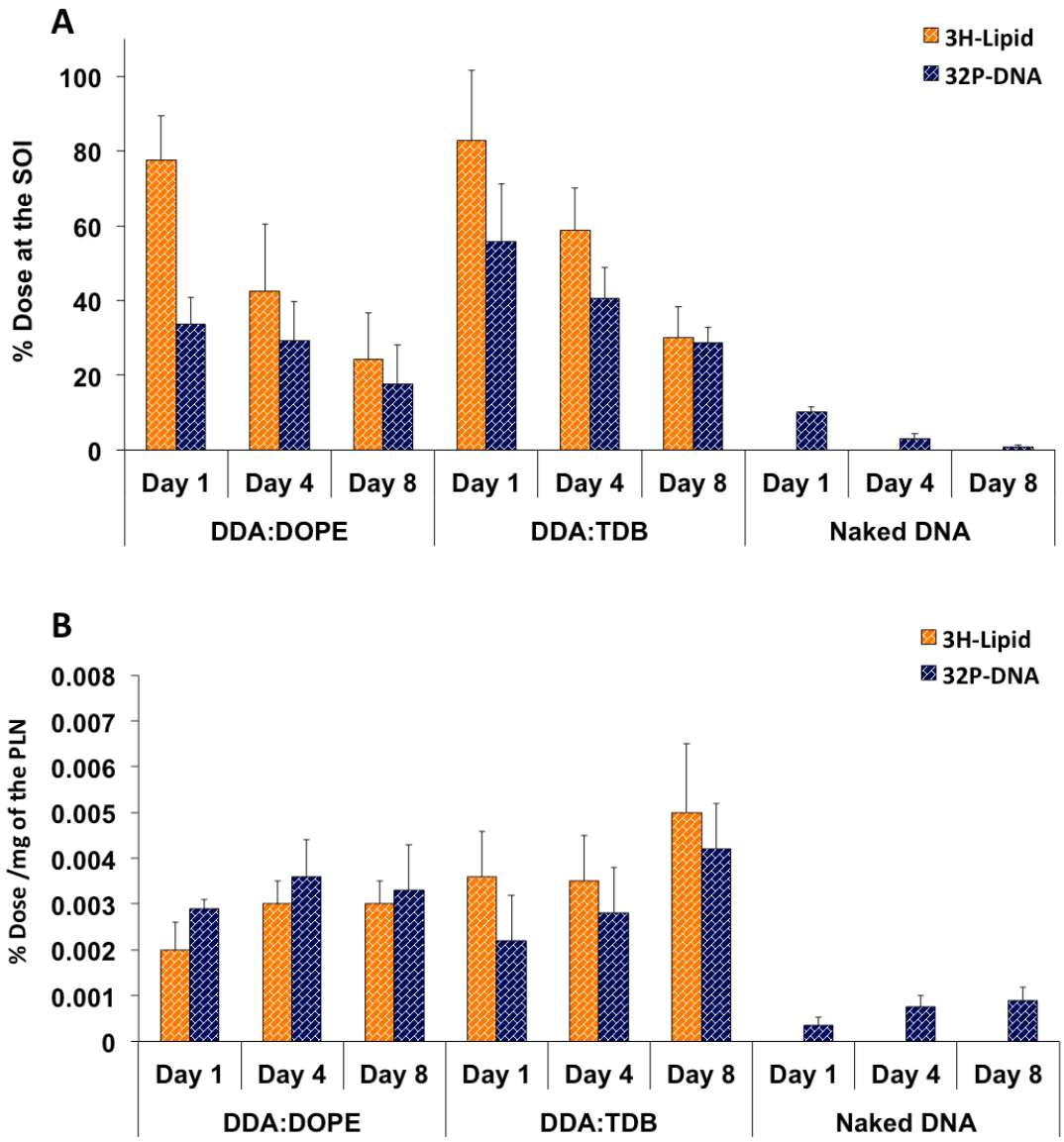

Figure 6.8. pDNA and liposome detection A) at the site of injection and B) draining lymph nodes (PLN) following i.m. injection of DDA:DOPE and DDA:TDB liposomes. pDNA and liposome quantification were determined at days 1,4 and 8 p.i. using radiolabel counting methods. Data represents mean ± SD of 4 mice and is presented % dose at the SOI (B,C) or the % dose per mg PLN harvested (D,E). Naked DNA included for comparison.

Given the similar antigen and liposome retention at the site of injection and the local draining lymph node (Figure 6.8) and the higher monocyte infiltration at the injection

site for DDA:TDB (Figure 6.9) would suggest the necessity of presentation of the antigen along with the liposome to the APCs for higher immune response. Previous studies have also reported this finding (Christensen et al., 2012; Kamath et al., 2009).

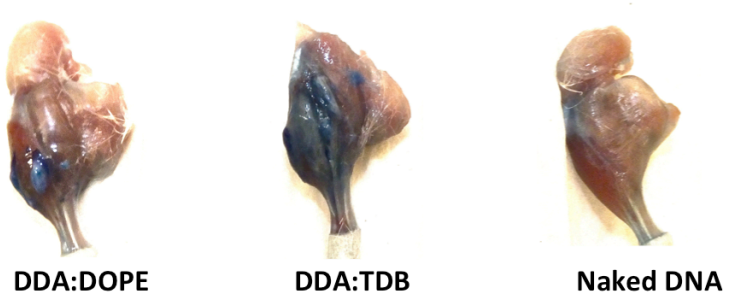

Figure 6.9: Pontamine blue staining on day 8 p.i. of the injection site after i.m injection with DNA lipoplexes of DDA:DOPE, DDA:TDB and naked DNA.

6.8. Immunisation studies

To investigate the potential of these DDA-based lipoplexes as DNA vaccine adjuvants, both the DDA:DOPE and DDA:TDB formulations were considered in a heterologous prime-boost vaccination strategy. Previous research on Hepatitis B vaccines have been performed in two different homologous immunisation methods; DNA vaccines were designed by using plasmid DNA pRc/CMV HBS which encodes the HBsAg protein (Bramwell et al., 2002; Gregoriadis et al., 1997; McNeil et al., 2010; Perrie et al., 2001) or subunit vaccines using HBsAg antigen (Brunel et al., 1999; Vangala et al., 2007; Vangala et al., 2006). DOTAP and DC-Chol were used extensively in DNA vaccine studies and DDA:TDB used in subunit vaccine study against HBV. To investigate the effect of prime-boost immunisation and compare the effect of this heterologous immunisation regimen with previous homologous strategies, in this current study DDA:DOPE and DDA:TDB were formulated as prime DNA lipoplex and boost subunit-liposome complex as explained in section 2.2 and injected as mentioned in 2.10.

The specification and characterisation results of DDA:DOPE and DDA:TDB plasmid DNA and subunit protein complex formulations are shown in details in Table 6.2.

Table 6.2: Characteristics of cationic liposomes od DDA:DOPE and DDA:TDB. Size and zeta potential measured by Malvern Zetasizer Nano-ZS. Results represent mean±SD of triplicate experiments.

Liposome Formulation	Antigen	Vesicle Size (nm)	Polydispersity Index	Zeta Potential (mV)	Loading (%)
SUV DDA:DOPE	DNA	254.2 ± 24.0	0.424 ± 0.02	47.7 ± 1.8	92.6 ± 4.4
	Protein	136.4 ± 14.8	0.415 ± 0.02	61.1± 4.6	76.4 ± 6.1
SUV DDA:TDB	DNA	484.5 ± 65.4	0.324 ± 0.04	49.4 ± 4.3	89.3 ± 5.6
	Protein	266.8 ± 24.2	0.392 ± 0.02	45.5 ± 3.4	63.6 ± 7.3

6.8.1. Antibody production in response to DDA-based vaccines

HBsAg specific antibody titres were determined by antibody ELISA. For total IgG levels, both DDA:DOPE and DDA:TDB were able to induce significantly higher ($P<0.001$) levels of IgG relative to free antigen and alum on days 36 and 49 of the immunisation study, however, there was no significant difference between these liposome formulations (Figure 6.10A).

Figure 6.10B demonstrates the IgG1 and IgG2b responses on day 49; for IgG1 levels, again both DDA:DOPE and DDA:TDB have similar responses; however, their responses are significantly lower ($P<0.05$) than the alum group which has the highest IgG1 responses compared to the liposomes and free DDP. This shows the alum's potency to provide Th2 responses (Brunel et al., 1999; O'Hagan and Valiante, 2003; Rosenkrands et al., 2005; Yang and Hayglass, 1993).

IgG2b antibody isotype immune responses of groups which received formulations show DDA:TDB liposomes induce significantly ($P<0.01$) higher IgG2b production than DOPE:DDA. Interestingly, alum and free subunit antigen did not show any detectable

levels of antibody responses for IgG2b; however, the free DDP group (which is the prime boost immunisation of the plasmid DNA and the subunit protein) shows detectable amounts of IgG2b production (Figure 6.10B) which could be due to the heterologous immunisation as discussed in chapter 5.

As mentioned above, Alum formulations promote a Th2 bias, which shows a high level of humoural immune responses and fail to show cellular responses (Brunel et al., 1999; Davidsen et al., 2005; Diminsky et al., 1996; O'Hagan and Valiante, 2003; Rosenkrands et al., 2005). Conversely, DDA:TDB induces a high level of IgG2b responses (Figure 6.10B), suggesting the ability to induce cellular immune responses. This is in line with previous studies which showed the high levels of Th1 responses induced by DDA:TDB formulations for different antigens (Henriksen-Lacey et al., 2010b; Henriksen-Lacey et al., 2011a; Vangala et al., 2007).

Giving similar levels of total IgG and IgG1 production, both DDA:TDB and DDA:DOPE show the adjuvant effect of cationic lipids and, in particular, DDA, which induces a significantly ($P<0.05$) higher humoural immune response compared to free antigens. This has been reported by several studies (Henriksen-Lacey et al., 2010b; Henriksen-Lacey et al., 2010c; Vangala et al., 2007) with the very first one was shown by Gall et al (Gall, 1966).

This present study also shows that DDA:TDB group induces significantly ($P<0.05$) higher Th1 responses compared to DDA:DOPE group, suggesting that immunomodulating TDB has a crucial role in stimulation of Th1 responses and high immunogenicity of DDA:TDB formulation.

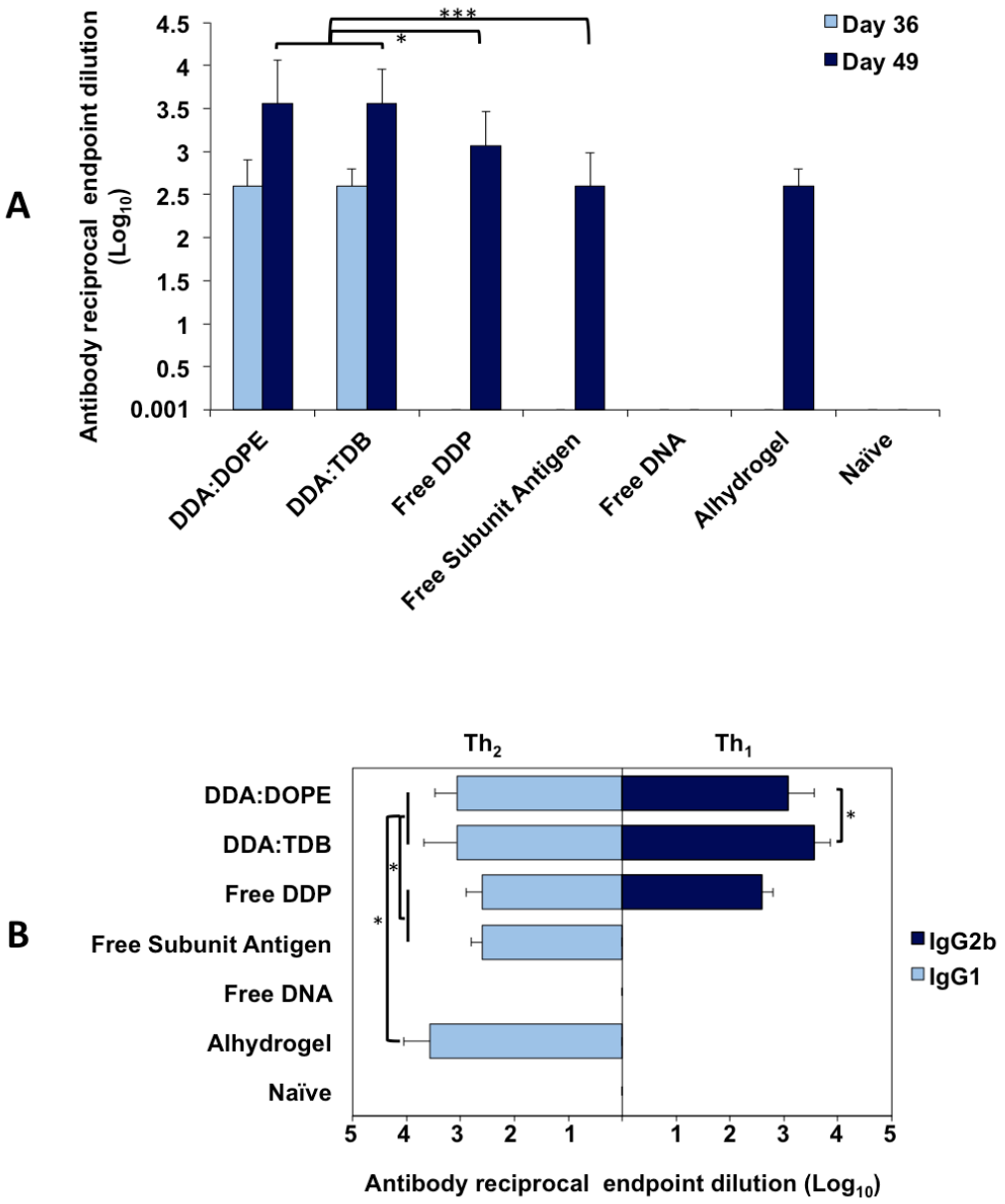

Figure 6.10. HBsAg specific antibody titres. A) Total IgG titre of DDA:DOPE and DDA:TDB, serum taken from day 36 and 49, B) IgG_1(white bars) and IgG_{2b}(black bars) levels for DOPE:DDA, DDA:TDB, serum taken from day 49. Results compared to alhydrogel, free DDP, free subunit antigen, free DNA and naïve group. Results show the mean ± SD reciprocal endpoint dilution (log_{10}) of 5 mice. Significance measured by one-way ANOVA and Tukey's post test, it is shown by *$p<0.05$ and ***$P<0.001$.

6.8.2. Investigating the cytokine production level in response to heterologous vaccination by DDA-based liposomes

The ability of DDA:DOPE and DDA:TDB to elicit cell-mediated as well as humoural immune responses was evaluated by measuring endogenous cytokine levels (IL-2, IL-5, IL-6, IL-10 and IFN-γ) within spleen homogenates to assess the presence of Th1/Th2 response. IL-1ß levels were also studied to see how vesicular vaccine adjuvants promote antigen uptake at the SOI.

To determine the cellular immune responses, IL-2 and IFN-γ levels were determined. Results (Figure 6.11) show that DDA:TDB produced significantly ($P<0.001$) higher levels of IL-2 and IFN-γ compared to DDA:DOPE. Induced levels of IL-2 for DDA:DOPE was 1000 pg/mL compared with 1800 pg/mL for DDA:TDB (Figure 6.11A). Similarly IFN-γ levels induced by DDA:TDB were almost 2 fold higher than DDA:DOPE (4800 Vs 2500 pg/mL) (Figure 6.11E). In both cases, the liposome formulations promoted immune responses significantly higher than those of alum or free DDP (Figure 6.11A,E).

Levels of Th2 indicator cytokines (IL-5, IL-6 and IL-10) were also measured to investigate the ability of the mentioned liposomes in induction of humoural immune responses. As it is demonstrated in Figure 6.11B and D, the induced levels of IL-5 and IL-10 for both liposomal vaccines are similar (80 pg/mL for IL-5 and 100 pg/mL for IL-10) and significantly ($P<0.05$) higher than free DDP levels (Figure 6.11B and D).

Since IL-5 and IL-10 are indicators of humoural immune responses, restimulation of alum group splenocytes promoted high levels of IL-5 and IL-10 comparable to liposomal groups. Data shows induced IL-5 levels for alum group was 60 pg/mL which

was not significantly different from IL-5 levels for DDA:DOPE and DDA:TDB (Figure 6.11B).

Alum cytokine production levels for IL-10 is even more interesting with 150 pg/mL, which was significantly ($P<0.001$) higher than DDA:DOPE and DDA:TDB cytokine production level (Figure 6.11D). For IL-6, DDA:TDB cytokine levels were above 500 pg/mL which is significantly ($P<0.001$) higher than DOPE:DDA with 300 pg/mL (Figure 6.11C). Both liposomes produced notably ($P<0.05$) higher IL-6 than alum and free DDP, however alum levels were also higher than background levels (Figure 6.11C). This shows an intermediate response for IL-6, which has both Th1 and Th2 parameters. IL-6 is known to represent Th2 responses, although this cytokine also promotes differentiation of Th17 cells with production of IL-17 and has been reported to detect both cellular and humoural responses (Diehl and Rincon, 2002; Dienz and Rincon, 2009; Henriksen-Lacey et al., 2011b). This explains the specific trend of IL-6 cytokine levels that have been achieved in this experiment.

Studying the amount of produced IL-1ß at the SOI shows that not only the liposome formulation, but also alum, free DDP, free subunit antigen and free DNA are able to activate dendritic cells to induce the innate immune response; however, DDA:TDB levels of IL-1ß are significantly ($P<0.001$) higher than other immunised groups (Figure 6.11F). This data supports the results provided by pontamine blue staining (Figure 6.9), showing higher stimulation of innate immune system for DDA:TDB.

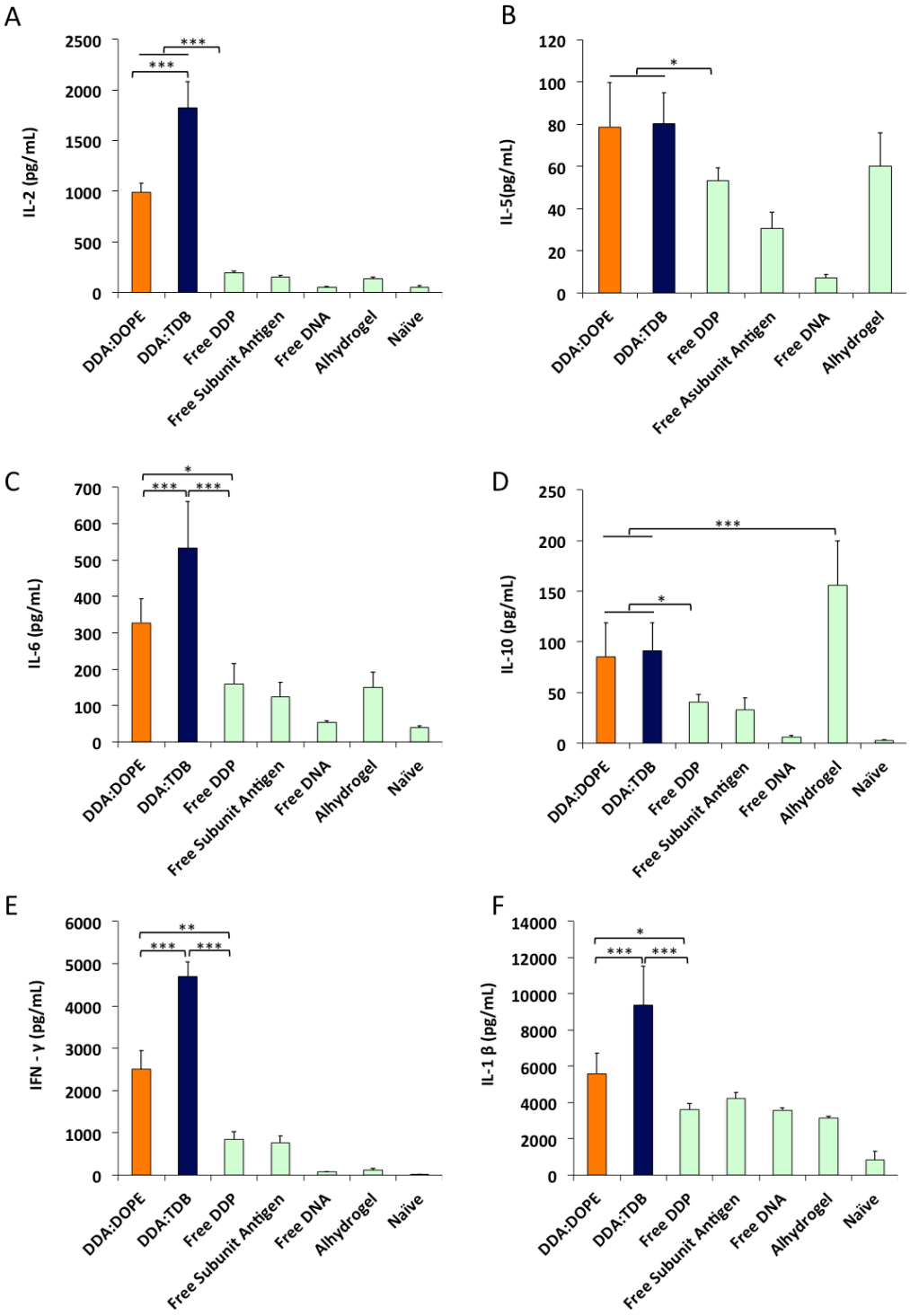

Figure 6.11. IL-2, IL-5, IL-6, IL-10 and IFN-γ cytokine production from splenocytes (A–E) and IL-1β production from excised leg muscle from the SOI (F) derived from mice immunised with DDA:DOPE or DDA:TDB pDNA-lipoplex or subunit protein liposome , compared to free DDP, free subunit antigen, free DNA, alhydrogel and naïve group. Splenocytes and muscle from the SOI were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 48 h in the presence of HBsAg (5 μg/mL). Leg muscle was excised, digested and homogenised. Cytokines were measured from splenocyte or muscle supernatants using sandwich ELISAs.

Overall, these results (Figures 6.11) demonstrate similar Th2 immune responses for both liposomes however the Th1 responses induced by DDA:TDB are higher than those promoted by DDA liposomes. As it has been reported earlier (Davidsen et al., 2005; Henriksen-Lacey et al., 2010b; Holten-Andersen et al., 2004; Vangala et al., 2007) the presence of TDB in the liposomal structure causes more immunogenicity for DDA:TDB. Two key factors have been defined as the main reasons that incorporation of TDB to DDA induce higher immune responses by DDA:TDB rather than DDA:DOPE;

1- TDB is synthetic version of the glycolipid which is in the cell wall of the tuberculosis bacteria and based on this it provides more immunogenicity to the liposome formulations (Olds et al., 1980; Pimm et al., 1979).
2- TDB stabilise the DDA liposomes by inhibiting the fusion between phospholipid vesicles due to the relatively large headgroups of trehalose. This would increase the hydration of the liposomal surface and prevent the dehydration of the quaternary ammonium headgroups so no aggregation would occur. Moreover, due to its large headgroup, trehalose might prevent close contact between opposing liposome and consequently prevent the aggregation of the liposomes (Crowe et al., 1994; Spargo et al., 1991).

6.8.3. Splenocyte proliferation ex vivo in response to HBsAg

Using spleens of immunised mice in an *ex vivo* splenocyte proliferation assay, the ability of DDA:DOPE and DDA:TDB liposomes to deliver the antigen to antigen presenting cells (APCs) was explored. Therefore splenocytes were restimulated with 0.05, 0.5 and 5 μg/mL HBsAg antigen in addition to the positive control stimuli Con A (5 μg/mL). Figure 6.12 shows the proliferative ability of splenocytes of all immunised groups in

response to Con A. It is shown that all the groups showed response to Con A as the ^{3}H-thymidine uptake was shown to be between 30000 to 40000 CPM (Figure 6.12).

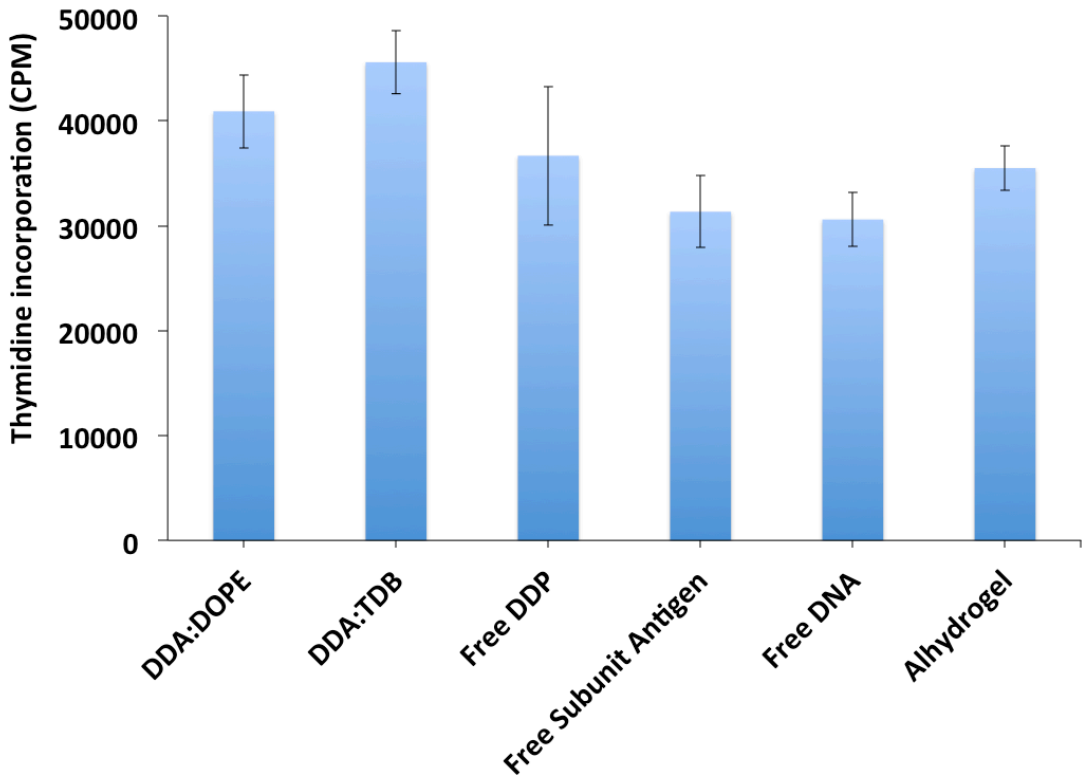

Figure 6.12: Splenocyte proliferation of each immunisation group and naïve group in response to stimulation with 5 µg/mL Con A. Splenocytes were derived on day 49 of the study and restimulated *ex vivo* with Con A as a positive control. After a further 24 hrs cells were harvested and the proportion of ^{3}H-thymidine incorporated in the cells measured using standard scintillation counting.

Figure 6.13 demonstrates that increasing the pooled HBsAg concentration increases the splenocyte proliferation of the formulations. Upon restimulation, with DDA:TDB immunised group showed the highest level of splenocyte proliferation with 4000 CPM at 5 µg/mL of HBsAg.

The level of splenocyte proliferation for DDA:TDB significantly increased ($P<0.05$) from 1500 to nearly 4000 CPM when the HBsAg concentration increased to 0.5 µg/mL of HBsAg (Figure 6.13). Comparing DDA:TDB to DDA:DOPE shows the splenocyte

proliferation levels for DDA:TDB is significantly (P<0.05) higher than DDA:DOPE in 0.5 and 5 µg/mL of HBsAg; however, in lower concentrations of HBsAg, similar results were seen for both formulations. This shows the effect of TDB as an immunomodulating agent in inducing higher levels of cell proliferation, which is in line with a previous study (Vangala et al., 2007) where it is shown that presence of TDB in liposome formulation has a crucial role in increasing the cell proliferation and in total initiating the cell mediated immunity compared to other cationic liposomes with no TDB in their formulation.

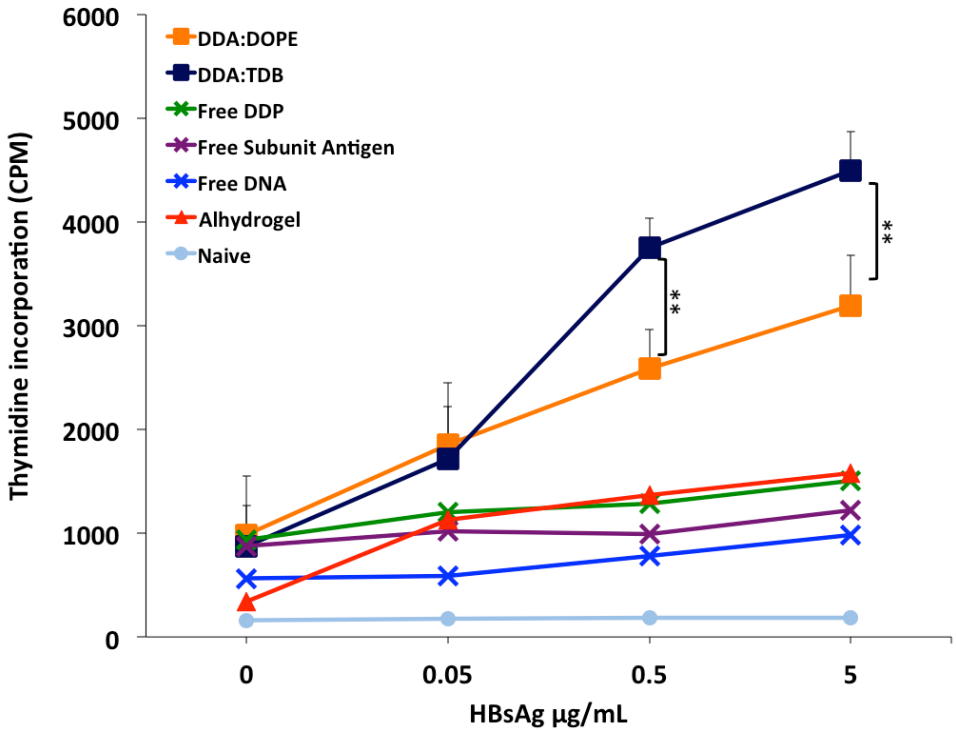

Figure 6.13. Spleen cell proliferation in response to stimulation/re-stimulation with HBsAg antigen. Cell proliferation was measured by incorporation of ^{3}H into cultured splenocytes. Splenocyte proliferation of DOPE:DDA and DDA:TDB injected under heterologous regimen, compared to free DDP, free subunit antigen, free pDNA, alhydrogel and naïve group. ** denotes significantly increased proliferation in compare to naïve controls (n=5 p<0.01).

Alternatively, although DDA:DOPE showed lower cell proliferations compared to DDA:TDB, its level of splenocyte proliferation were significantly higher than alum and free antigens (Figure 6.13), suggesting the ability of cationic liposomes to improve splenocyte proliferation level and delivering the antigen to the APCs.

6.9. Conclusion

The aim of this study was to evaluate the immunogenicity of DDA-based liposomes as DNA vaccines and in a heterologous prime boost immunisation regimen.

From the monolayer studies and characterisation measurements, no notable differences in physicochemical characteristics of DDA:DOPE and DDA:TDB were observed, however their biological activity has been shown to be significantly different. Although DDA is a cationic lipid, lipoplexes of DDA:DOPE and DDA:TDB are not competitive transfection reagents compared to formulations including DOTAP and DSTAP (Section 4.6; Chapter 4). Comparing DDA:DOPE and DDA:TDB shows the presence of TDB does not have specific impact on characteristic properties of the lipoplexes, however absence of DOPE dramatically reduced the *in vitro* transfection of DDA:TDB. This can be due to the effect of DOPE as a helper lipid to change the liposome structure from lamellar to H_{II} inverted hexagonal, which is believed to be more beneficial for *in vitro* transfection efficiency of the lipoplexes (Felgner et al., 1987). The concept of using a fusogenic lipid such as DOPE is to mimic attributes of viruses when fuse with the cell membrane and delivering their genetic material into the cytosol (McNeil and Perrie, 2006). It has been reported that incorporation of DOPE into the liposome formulations enhances the transfection efficiency of the liposomes (Farhood et al., 1995) and this could be the reason of low transfection efficiency of DDA:TDB.

Considering the localisation of the DDA:DOPE and DDA:TDB at the injection site and drainage of the liposome contents to the local draining lymph node showed no significant difference between the two formulations, however pontamine blue staining results demonstrated more monocyte influx for DDA:TDB which is known to be due to the incorporation of immunostimulant molecule of TDB within the liposome structure. These observations are in correlation to what has been observed previously (Henriksen-Lacey et al., 2010b), suggesting the co-stimulatory effect of the adjuvant and the antigen and their presentation to the APCs at the lymph node is essential for stimulation of a high immune response (Christensen et al., 2012; Henriksen-Lacey et al., 2010b; Kamath et al., 2009).

Immunisation studies including exploring antibody and cytokine production of DDA:DOPE and DDA:TDB reveal that both formulations show a similar humoural immune response; however, DDA:TDB liposomal vaccine shows a significantly higher cellular immune response compared to DDA:DOPE. Results show similar total IgG and IgG1 levels for both liposomes as well as similar IL-5 and IL-10 cytokine levels as a result of intramuscular heterologous prime boost immunisation of these formulations, show humoural immune system is triggered similarly by both formulations. In contrast, it has been observed that IgG2b levels produced by DDA:TDB were significantly higher than DDA:DOPE. Furthermore, results of cytokine study showed the same trend for the IL-2 and IFN-γ production levels, suggesting that DDA:TDB induces higher cell mediated immune response rather than DDA:DOPE. The results are in agreement with previous studies (Henriksen-Lacey et al., 2010b; Holten-Andersen et al., 2004; Vangala et al., 2007). They suggest higher cellular immune response of DDA:TDB is related to the incorporation of TDB into the DDA bilayers, which increases the immunogenicity of the liposomes as well as stabilising them.

Chapter 7:
General Discussion

7.1. Monolayer studies: lipid packaging configurations

Lipid monolayer studies have been shown to be able to increase the understanding of bilayer structures, hence it can add knowledge into areas such as the bilayer lipid packaging configurations (Dynarowicz-Latka and Hac-Wydro, 2004), drug-lipid interactions (Sun et al., 2004) and liposome stability (Crowe et al., 1984).

The aim of monolayer studies in this project was to study molecular lipid packaging and interactions, and correlate the results with the physico-chemical properties of the liposomes. Studies demonstrated that saturated lipids have closer packaging arrangements compared to their unsaturated analogues and this influences the bilayer formation as using two saturated lipids, such as DSPE and DSTAP, failed to form liposomes. Characterisation studies also showed liposomes containing saturated and rigid lipids had a tendency to aggregate with DNA molecules, which caused larger vesicle sizes in comparison to liposomes made of unsaturated lipids. Studying the mixture of the unsaturated and bulky DOPE with the saturated and rigid DSTAP in monolayers showed that presence of DOPE reduces the rigidity of DSTAP, which can be translated to the high release profile and high *in vitro* transfection of DOPE:DSTAP lipoplexes.

For liposomes to be able to transfect cells efficiently, they need to be more fluidic in nature so they can destabilise the lipid membrane of the cells, therefore DOPE as a fusogenic lipid has been used extensively to enhance the cell transfection. Monolayer studies conducted within this thesis have shown that incorporation of DOPE with DSTAP reduced the rigidity of DSTAP to a similar level of the DOPE:DOTAP. Considering transfection efficiency of DOPE:DOTAP and DOPE:DSTAP, both

formulations have higher transfection efficiency than a marketed transfection reagent (Lipofectin).

In conclusion, the monolayer studies were shown to be effective in giving insight into the molecular packaging of the lipids, which is helpful for understanding liposomal molecular packaging, and hence the liposomal delivery systems.

7.2. Investigating the effect of electrolyte on liposomal vaccine formulations

It has been reported that presence of small amounts of salt (30 mM) within cationic liposomes could enhance immune responses of the vaccine (Yan and Huang, 2009). This was due to the interference of salt with the electrostatic interactions between the cationic lipid and the antigen, which enhances the antigen release from the liposomes and at the same time activates the antigen presenting cells. Therefore, to investigate the effect of presence of NaCl within the liposomes on their physicochemical properties, *in vitro* transfection efficiency and immune performances, DOPE:DOTAP and DOPE:DSTAP liposomes were hydrated with PBS or dH_2O (Sucrose for *in vivo* studies).

Characterisation studies showed that the presence of electrolytes within the liposomes could lead to increases in the vesicle size of the formulations due to the reduction in electrostatic interactions between systems and the effect of the phosphate group of PBS, which initiates bridging of the polar headgroups of the lipids. Although no influence was observed on DNA association within the lipoplexes as a result of electrolyte existence in the formulations, it decreased the cationic surface charge of the lipoplexes, which was attributed to the neutralisation effect of buffer electrolytes on zeta potential. These results are in line with the previous studies, which shows presence of salts increases the

vesicle size (Ciani et al., 2007; Wasan et al., 1999) and reduces the zeta potential (Li and Hui, 1997).

In vitro transfection studies showed no correlation with characterisation results, as the presence of electrolytes did not show a trend on transfection efficiency. This suggests that the key feature in transfection efficacy could be the lipid composition rather than vesicle size or hydration buffer electrolytes. In contrast, previous studies (Ogris et al., 1998) showed that the impact of salt on transfection efficiency is basically due to the increase in vesicle size of the systems, which is shown to cause higher transfection efficiency (Eastman et al., 1997; McNeil et al., 2010).

Similar to the transfection efficiency, the presence of salt within the liposomes did not make a significant impact on localisation of the liposomes and plasmid DNA at the injection site, showing that not all of the characterisation measurements are translatable to *in vitro* and *in vivo* studies. In contrast, immunisation studies showed that formulations hydrated in sucrose induce higher humoural immunity than the formulations which incorporated electrolytes within their system, whilst the cellular immune system activity remained similar for both kinds of liposomes. This is in agreement with a previous study (Henriksen-Lacey et al., 2011b), which shows the presence of PBS in the formulations caused aggregation and this led to a poor immunity performance by DDA:TDB formulations. It has been described that aggregation of the vesicles at the injection site would slow down and reduce the rate of antigen presentation at the lymph node and consequently would decrease the immune response.

Overall, it has been shown that the main impact of the presence of salt in the liposomal formulation is on its characterisation parameters; particularly increases in the vesicle size of the liposomes, which eventually causes a reduction in immune responses.

7.3. Impact of lipid composition on liposomal vaccines

The importance of cationic charge and antigen (subunit protein or plasmid DNA) adsorption to the lipids for a liposomal vaccine has been established, therefore the role of cationic lipid structure was investigated as unsaturated cationic lipid of DOTAP was compared to the saturated DSTAP in terms of characterisation studies, *in vitro* and *in vivo* studies.

While characterisation studies showed no significant difference in vesicle size, zeta potential and DNA association of the liposomes when DOTAP replaced by DSTAP, *in vitro* studies demonstrated significantly higher transfection efficiency for DSTAP-based lipoplexes. This is in contrast with previous studies, which have reported more rigid bilayers (Kudsiova et al., 2011; Wasungu and Hoekstra, 2006) produce lower transfection in cells. Higher transfection efficiency of DSTAP-based lipoplexes is believed to be due to the fluidic effect of DNA (Lobo et al., 2002) and DOPE on bilayers of DSTAP and the instability of this system compared to DOPE:DOTAP (Regelin et al., 2000). In addition, it has been described that high positive deviation from ideal extrapolated area of the monolayers can lead to increased instability of the bilayer system, and hence facilitating the fusion of the liposome and the cell membrane, and higher transfection efficiency of the lipoplexes (Hac-Wydro and Wydro, 2007; Hac-Wydro et al., 2007).

In vivo studies showed higher localisation of lipid and DNA at the site of injection for DSTAP-based liposomes, which is attributed to the higher transition temperature, and rigidity of DSTAP bilayers. This is in line with previous studies which showed fluidic bilayers will be cleared faster compared to the rigid bilayers (Christensen et al., 2012; Henriksen-Lacey et al., 2011a). A stronger depot effect for DSTAP-based liposomes can cause a higher chance for antigen presentation to APCs, which are located in local lymph nodes leading to higher activity of $CD8^+$ T cells via MHC II molecules and consequently stronger cellular and humoural immune response for DSTAP formulations. Recent studies confirm similar results for DDA:TDB liposomal subunit vaccine (Christensen et al., 2012; Henriksen-Lacey et al., 2011a).

7.4. SUV vs DRV: The effect of liposomal preparation method on their *in vitro* and *in vivo* attributes

The dehydration-rehydration method has been shown to be effective in entrapment of plasmid DNA within liposomes (Kirby and Gregoriadis, 1984), therefore a comparison has been made between SUV and DRV liposomes to see the effect of dehydration-rehydration method on characteristic properties, as well as *in vitro* transfection efficiency and immune performance of the liposomes.

The physiochemical characteristics of DRV liposomes composed of DOPE:DOTAP and DOPE:DSTAP revealed that in contrast to SUV liposomes, vesicle size and zeta potential values did not change significantly in the presence of DNA. This suggests that the plasmid DNA was entrapped within bilayers of the cationic DRV liposomes, presumably bound to the cationic charge of the inner bilayers when produced by the dehydration-rehydration procedure. Gel electrophoresis studies supported these results as only minimal amounts of DNA was displaced by SDS molecules for DRV liposomes.

However, observation of the higher transfection efficiency of SUV liposomes compared to DRV formulations shows having entrapped DNA within the liposomes may not be advantageous. This could be attributed to the inability of the entrapped DNA escaping the vesicles in the time duration of the experimental protocol. *In vivo* studies showed that whilst localisation of both SUV and DRV liposomes at the injections site were similar for both formulations, SUV liposomes induce stronger humoural immunity however cell mediated immune response levels for both formulations are similar. This implies that there was higher APC uptake for SUV liposomal vaccines, which was expected due to higher *in vitro* transfection efficacy of SUV formulations. This could be due to the smaller vesicle size of SUV liposomes compared to DRV formulations. In agreement to this finding, it has previously been reported that induction of immune response by DNA liposomal vaccines is more related to the higher cell transfection and smaller vesicles size of the particles with a optimum size of below 500 nm is desirable for APC uptake of the particles (Carstens et al., 2011).

7.5. How effective are heterologous immunisation strategies in providing desirable levels of immune response?

Investigating the effect of immunisation regimens on immune performance of the liposomal vaccines, a heterologous vaccination schedule including two injections of DNA-liposome vaccines followed by a protein subunit-liposome booster was compared to the homologous immunisation strategies composed of either two DNA-liposome immunisation or a single protein-liposome vaccination. Results of this study showed the occurrence of synergistic response as the activation of immune system in response to the heterologous immunisation was higher than each of homologous regimens. Compared to prime DNA vaccine administration, heterologous vaccination caused a significantly

higher antibody and cytokine production levels. However, the results of prime/boost immunisation were not significantly higher than a single injection of liposome-protein. Therefore considering the cost and number of injections, subunit protein vaccines seem to be more attractive option compared to DNA vaccines or the heterologous vaccination strategies.

7.6. DDA-based liposomal vaccines

Given a high and solid immune activity for DDA:TDB incorporated with subunit antigen of TB; Ag85B-ESAT-6 (Henriksen-Lacey et al., 2010b; Holten-Andersen et al., 2004) and earlier research which showed the cationic liposomes made from DDA have adjuvant properties (Gall, 1966) has led to evaluate DDA-based liposomes as a DNA vaccine in a prime/boost immunisation regimen. To investigate the impact of immunostimulant TDB molecule on immune response and compare it with fusogenic lipid DOPE, which enhances the transfection of the liposomes, DDA:TDB and DDA:DOPE formulations were compared in this study.

While characterisation studies showed larger vesicle size for DDA:TDB compared to DDA:DOPE (which can be as a result of more rigidity and aggregation of DDA:TDB upon its complexation with DNA) zeta potential and DNA entrapment for the formulations did not show significant difference. *In vitro* transfection efficiency of DDA:TDB has been shown to be significantly lower than DDA:DOPE and Lipofectin™. This was suggested to be due to the effect of DOPE, which acts as a fusogenic lipid and enhances the destabilisation of cell membrane by conversion of the DDA:DOPE structure to inverted hexagonal H_{II} structure, which has been shown to be ideal for transfection. Biodistribution studies showed no significant difference in the localisation and movement of the liposomes and their associated plasmid DNA at the

injection site; however, by applying pontamine blue staining technique, results showed significantly higher monocyte infiltration at the site of injection upon administration of DDA:TDB compared to DDA:DOPE. This was attributed to the immunostimulatory effect of TDB molecule, which activated the innate immune response. This was followed by the immunisation studies which showed higher cell mediated immunity of DDA:TDB in comparison to DDA:DOPE while both formulations showed similar levels of humoural immune activity. In agreement to previous studies (Henriksen-Lacey et al., 2010b; Holten-Andersen et al., 2004; Vangala et al., 2007), higher cellular immune responses of DDA:TDB is related to the immunostimulatory effects of TDB molecule.

It is believed that DNA vaccines need high cell transfection to be able to enter to the nucleus of the cell, encode the antigen and start the immune activity. However, low transfection efficiency of DDA:TDB suggests that strong immune activity of this formulation is more attributed to the subunit vaccine injection rather than two DNA vaccine immunisations of this heterologous vaccination, and DNA prime injections are not necessary to induce immune response for this formulation.

7.7. Final conclusions

In summary, investigation of a range of cationic liposome formulations as DNA vaccines showed that:

- Monolayer studies can be useful in giving more insight about the bilayer structure and molecular packaging of the bilayers, which can be translated to the characteristic properties of the liposomes.
- It is known that physicochemical characteristic parameters of cationic liposomes have an impact on their *in vitro* and *in vivo* behaviours and this study showed

lipid composition had the most influence on *in vitro* transfection and *in vivo* biodistribution of the formulations, as well as their immune performances.

- Applying saturated lipids enhances cell transfection and promotes immune responses of the liposomal DNA vaccines.
- Liposomal DNA vaccines induce remarkably stronger cellular and humoural immune responses compared to the free DNA administration.
- Although heterologous prime/boost immunisation produces higher immunity than homologous vaccination, yet more studies need to be performed to improve this technology.

7.8. Future work

Further studies can be conducted in continuation of this work. Monolayer studies could include investigating the effect of presence of DNA in the subphase on monolayer properties of the lipids. This might open new windows to our understanding about lipid-DNA interactions in bilayer systems. Differential scanning calorimetry (DSC) study can be performed on tested lipids and their combination to see the effect of DOPE on lowering the transition temperature of saturated lipid such as DSTAP. Also impact of incorporation of DNA to the bilayer molecular packaging could be investigated using DSC. Microscopic imaging could be conducted showing the size, morphology and antigen entrapment within liposomes and possible aggregations. Transmission electron microscopy (TEM) would be the method of choice due to nano scale size of the vesicles.

In vitro transfection studies could include comparing different periods of transfection for the liposomes, specially to see the difference in optimum transfection time for SUV and DRV liposomes.

To perform the biodistribution of the formulations and localisation of the lipid at different organs, advanced *in vivo* imaging instruments such as Spectrum CT and VECTor/CT can be used to show a real time data about the localisation of the formulation at different time points. This will eliminate need of different groups of animals for each time point.

With regards to the immunisation studies, flow cytometry analysis could be performed to give more knowledge about the induced immune responses and the stage of T cell differentiation based on cytokine production. Moreover, the immunisation studies could be carried on with a challenge study and investigate the effect of the liposomal vaccines to protect the animal form the pathogens. Finally, the ability of studied cationic liposomes in delivery of nucleic acids can be further investigated by using these delivery systems to deliver siRNA to down-regulate the genes which control cancer cells.

References

Agger, E.M., Rosenkrands, I., Hansen, J., Brahimi, K., Vandahl, B.S., Aagaard, C., Werninghaus, K., Kirschning, C., Lang, R., Christensen, D., Theisen, M., Follmann, F., Andersen, P., 2008. Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. PloS one 3, e3116.

Akao, T., Fukumoto, T., Ihara, H., Ito, A., 1996. Conformational change in DNA induced by cationic bilayer membranes. FEBS Lett 391, 215-218.

Al-Dosari, M.S., Gao, X., 2009. Nonviral gene delivery: principle, limitations, and recent progress. The AAPS journal 11, 671-681.

Ali, M.H., Kirby, D.J., Mohammed, A.R., Perrie, Y., 2010. Solubilisation of drugs within liposomal bilayers: alternatives to cholesterol as a membrane stabilising agent. Journal of Pharmacy and Pharmacology, 1646-1655.

Aljaberi, A., Spelios, M., Kearns, M., Selvi, B., Savva, M., 2007. Physicochemical properties affecting lipofection potency of a new series of 1,2-dialkoylamidopropane-based cationic lipids. Colloids and surfaces. B, Biointerfaces 57, 108-117.

Allison, A., Gregoriadis, G., 1974. Liposomes as immunological adjuvants.

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., 2007. Tuberculosis vaccines - an update. Nat Rev Microbiol 5, 484-487.

Andre, F., Booy, R., Bock, H., Clemens, J., Datta, S., John, T., Lee, B., Lolekha, S., Peltola, H., Ruff, T., 2008. Vaccination greatly reduces disease, disability, death and inequity worldwide. Bulletin of the World Health Organization 86, 140-146.

Anwer, K., Kao, G., Rolland, A., Driessen, W.H., Sullivan, S.M., 2004. Peptide-mediated gene transfer of cationic lipid/plasmid DNA complexes to endothelial cells. Journal of drug targeting 12, 215-221.

Arpin, C., Banchereau, J., Liu, Y.J., 1997. Memory B cells are biased towards terminal differentiation: a strategy that may prevent repertoire freezing. The Journal of experimental medicine 186, 931-940.

Artenstein, A.W., 2009. Vaccines : a biography. Springer, New York ; London.

Arvin, A.M., Greenberg, H.B., 2006. New viral vaccines. Virology 344, 240-249.

Audran, R., Peter, K., Dannull, J., Men, Y., Scandella, E., Groettrup, M., Gander, B., Corradin, G., 2003. Encapsulation of peptides in biodegradable microspheres prolongs their MHC class-I presentation by dendritic cells and macrophages in vitro. Vaccine 21, 1250-1255.

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392, 245-252.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. Journal of molecular biology 13, 238-252.

Becker, S.I., Wang, R., Hedstrom, R.C., Aguiar, J.C., Jones, T.R., Hoffman, S.L., Gardner, M.J., 1998. Protection of mice against Plasmodium yoelii sporozoite challenge with P. yoelii merozoite surface protein 1 DNA vaccines. Infection and immunity 66, 3457-3461.

Bedi, D., Musacchio, T., Fagbohun, O.A., Gillespie, J.W., Deinnocentes, P., Bird, R.C., Bookbinder, L., Torchilin, V.P., Petrenko, V.A., 2011. Delivery of siRNA into breast cancer cells via phage fusion protein-targeted liposomes. Nanomedicine : nanotechnology, biology, and medicine 7, 315-323.

Bendelac, A., Bonneville, M., Kearney, J.F., 2001. Autoreactivity by design: innate B and T lymphocytes. Nature reviews. Immunology 1, 177-186.

Bennett, E.P., Hassan, H., Mandel, U., Mirgorodskaya, E., Roepstorff, P., Burchell, J., Taylor-Papadimitriou, J., Hollingsworth, M.A., Merkx, G., van Kessel, A.G., Eiberg, H., Steffensen, R., Clausen, H., 1998. Cloning of a human UDP-N-acetyl-alpha-D-Galactosamine:polypeptide N-acetylgalactosaminyltransferase that complements other GalNAc-transferases in complete O-glycosylation of the MUC1 tandem repeat. J Biol Chem 273, 30472-30481.

Black, M., Trent, A., Tirrell, M., Olive, C., 2010. Advances in the design and delivery of peptide subunit vaccines with a focus on toll-like receptor agonists. Expert review of vaccines 9, 157-173.

Blander, J.M., Medzhitov, R., 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature 440, 808-812.

Bovier, P.A., 2008. Epaxal: a virosomal vaccine to prevent hepatitis A infection. Expert review of vaccines 7, 1141-1150.

Bramwell, V.W., Eyles, J.E., Somavarapu, S., Alpar, H.O., 2002. Liposome/DNA complexes coated with biodegradable PLA improve immune responses to plasmid encoding hepatitis B surface antigen. Immunology 106, 412-418.

Bramwell, V.W., Perrie, Y., 2005a. Particulate delivery systems for vaccines. Critical reviews in therapeutic drug carrier systems 22, 151-214.

Bramwell, V.W., Perrie, Y., 2005b. The rational design of vaccines. Drug discovery today 10, 1527-1534.

Brandt, L., Elhay, M., Rosenkrands, I., Lindblad, E.B., Andersen, P., 2000. ESAT-6 subunit vaccination against Mycobacterium tuberculosis. Infection and immunity 68, 791-795.

Brunel, F., Darbouret, A., 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.

Burger, K.N., Verkleij, A.J., 1990. Membrane fusion. Experientia 46, 631-644.

Caracciolo, G., Pozzi, D., Caminiti, R., Marchini, C., Montani, M., Amici, A., Amenitsch, H., 2007. Transfection efficiency boost by designer multicomponent lipoplexes. Biochimica et biophysica acta 1768, 2280-2292.

Carstens, M.G., Camps, M.G., Henriksen-Lacey, M., Franken, K., Ottenhoff, T.H., Perrie, Y., Bouwstra, J.A., Ossendorp, F., Jiskoot, W., 2011. Effect of vesicle size on tissue localization and immunogenicity of liposomal DNA vaccines. Vaccine 29, 4761-4770.

Cevc, G., 1991. How membrane chain-melting phase-transition temperature is affected by the lipid chain asymmetry and degree of unsaturation: an effective chain-length model. Biochemistry 30, 7186-7193.

Chambers, M.A., Wright, D.C., Brisker, J., Williams, A., Hatch, G., Gavier-Widen, D., Hall, G., Marsh, P.D., Glyn Hewinson, R., 2004. A single dose of killed Mycobacterium bovis BCG in a novel class of adjuvant (Novasome) protects guinea pigs from lethal tuberculosis. Vaccine 22, 1063-1071.

Chen, D., Maa, Y.F., Haynes, J.R., 2002. Needle-free epidermal powder immunization. Expert review of vaccines 1, 265-276.

Chen, W.C., Huang, L., 2005. Non-viral vector as vaccine carrier. Advances in genetics 54, 315-337.

Christensen, D., Agger, E.M., Andreasen, L.V., Kirby, D., Andersen, P., Perrie, Y., 2009. Liposome-based cationic adjuvant formulations (CAF): past, present, and future. Journal of liposome research 19, 2-11.

Christensen, D., Foged, C., Rosenkrands, I., Lundberg, C.V., Andersen, P., Agger, E.M., Nielsen, H.M., 2010. CAF01 liposomes as a mucosal vaccine adjuvant: In vitro and in vivo investigations. International journal of pharmaceutics 390, 19-24.

Christensen, D., Henriksen-Lacey, M., Kamath, A.T., Lindenstrom, T., Korsholm, K.S., Christensen, J.P., Rochat, A.F., Lambert, P.H., Andersen, P., Siegrist, C.A., Perrie, Y., Agger, E.M., 2012. A cationic vaccine adjuvant based on a saturated quaternary ammonium lipid have different in vivo distribution kinetics and display a distinct CD4 T cell-inducing capacity compared to its unsaturated analog. Journal of controlled release : official journal of the Controlled Release Society 160, 468-476.

Christensen, D., Kirby, D., Foged, C., Agger, E.M., Andersen, P., Perrie, Y., Nielsen, H.M., 2008. alpha,alpha'-trehalose 6,6'-dibehenate in non-phospholipid-based liposomes enables direct interaction with trehalose, offering stability during freeze-drying. Biochimica et biophysica acta 1778, 1365-1373.

Churchyard, G.J., Morgan, C., Adams, E., Hural, J., Graham, B.S., Moodie, Z., Grove, D., Gray, G., Bekker, L.G., McElrath, M.J., Tomaras, G.D., Goepfert, P., Kalams, S., Baden, L.R., Lally, M., Dolin, R., Blattner, W., Kalichman, A., Figueroa, J.P., Pape, J., Schechter, M., Defawe, O., De Rosa, S.C., Montefiori, D.C., Nabel, G.J., Corey, L., Keefer, M.C., 2011. A phase IIA randomized clinical trial of a multiclade HIV-1 DNA prime followed by a multiclade rAd5 HIV-1 vaccine boost in healthy adults (HVTN204). PloS one 6, e21225.

Ciani, L., Casini, A., Gabbiani, C., Ristori, S., Messori, L., Martini, G., 2007. DOTAP/DOPE and DC-Chol/DOPE lipoplexes for gene delivery studied by circular dichroism and other biophysical techniques. Biophysical Chemistry 127, 213-220.

Ciani, L., Ristori, S., Salvati, A., Calamai, L., Martini, G., 2004. DOTAP/DOPE and DC-Chol/DOPE lipoplexes for gene delivery: zeta potential measurements and electron spin resonance spectra. Biochimica et biophysica acta 1664, 70-79.

ClinicalTrials.gov, 2012. Trial on the safety of a new liposomal adjuvant system, CAF01, when given with the tuberculosis subunit vaccine Ag85B-ESAT-6 as two injections with two months interval to healthy adult volunteers. clinicaltrials.cov.

Cohen, S.G., 2008. Measles and immunomodulation. The Journal of allergy and clinical immunology 121, 543-544.

Congiu, A., Pozzi, D., Esposito, C., Castellano, C., 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.

Crowe, J.H., Whittam, M.A., Chapman, D., Crowe, L.M., 1984. Interactions of phospholipid monolayers with carbohydrates. Biochimica et biophysica acta 769, 151-159.

Crowe, L.M., Spargo, B.J., Ioneda, T., Beaman, B.L., Crowe, J.H., 1994. Interaction of cord factor (alpha, alpha'-trehalose-6,6'-dimycolate) with phospholipids. Biochimica et biophysica acta 1194, 53-60.

Crowe, L.M., Womersley, C., Crowe, J.H., Reid, D., Appel, L., Rudolph, A., 1986. Prevention of Fusion and Leakage in Freeze-Dried Liposomes by Carbohydrates. Biochimica et biophysica acta 861, 131-140.

Darwis, Y., Kellaway, I.W., 2001. Nebulisation of rehydrated freeze-dried beclomethasone dipropionate liposomes. International journal of pharmaceutics 215, 113-121.

Davidsen, J., Rosenkrands, I., Christensen, D., Vangala, A., Kirby, D., Perrie, Y., Agger, E.M., Andersen, P., 2005. Characterization of cationic liposomes based on dimethyldioctadecylammonium 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 1718, 22-31.

Davis, H.L., Whalen, R.G., Demeneix, B.A., 1993. Direct gene transfer into skeletal muscle in vivo: factors affecting efficiency of transfer and stability of expression. Human gene therapy 4, 151-159.

De Mare, A., Lambeck, A.J., Regts, J., van Dam, G.M., Nijman, H.W., Snippe, H., Wilschut, J., Daemen, T., 2008. Viral vector-based prime-boost immunization regimens: a possible involvement of T-cell competition. Gene therapy 15, 393-403.

De Rosa, S.C., Thomas, E.P., Bui, J., Huang, Y., deCamp, A., Morgan, C., Kalams, S.A., Tomaras, G.D., Akondy, R., Ahmed, R., Lau, C.Y., Graham, B.S., Nabel, G.J., McElrath, M.J.,

2011. HIV-DNA priming alters T cell responses to HIV-adenovirus vaccine even when responses to DNA are undetectable. J Immunol 187, 3391-3401.

Demel, R.A., Dorrepaal, E., Ebskamp, M.J., Smeekens, J.C., de Kruijff, B., 1998. Fructans interact strongly with model membranes. Biochimica et biophysica acta 1375, 36-42.

Deshmukh, T.M., Lole, K.S., Tripathy, A.S., Arankalle, V.A., 2007. Immunogenicity of candidate hepatitis E virus DNA vaccine expressing complete and truncated ORF2 in mice. Vaccine 25, 4350-4360.

Diehl, S., Rincon, M., 2002. The two faces of IL-6 on Th1/Th2 differentiation. Molecular immunology 39, 531-536.

Dienz, O., Rincon, M., 2009. The effects of IL-6 on CD4 T cell responses. Clin Immunol 130, 27-33.

Diminsky, D., Reimann, Z.J., Schirmbeck, R., Barenholz, Y., 1996. Structural and Functional Characterization of Liposomal Recombinant Hepatitis B Vaccine. Journal of liposome research 6, 289-304.

Dong, L., Liu, F., Fairman, J., Hong, D.K., Lewis, D.B., Monath, T., Warner, J.F., Belser, J.A., Patel, J., Hancock, K., Katz, J.M., Lu, X., 2012. Cationic liposome-DNA complexes (CLDC) adjuvant enhances the immunogenicity and cross-protective efficacy of a pre-pandemic influenza A H5N1 vaccine in mice. Vaccine 30, 254-264.

Donnelly, J.J., Ulmer, J.B., Shiver, J.W., Liu, M.A., 1997. DNA vaccines. Annual review of immunology 15, 617-648.

Duclos, P., Hofmann, C.A., 2001. Immunisation safety: a priority of the World Health Organization's Department of Vaccines and Biologicals. Drug safety : an international journal of medical toxicology and drug experience 24, 1105-1112.

Dynarowicz-Latka, P., Hac-Wydro, K., 2004. Interactions between phosphatidylcholines and cholesterol in monolayers at the air/water interface. Colloids and Surfaces B-Biointerfaces 37, 21-25.

Eastman, S.J., Siegel, C., Tousignant, J., Smith, A.E., Cheng, S.H., Scheule, R.K., 1997. Biophysical characterization of cationic lipid: DNA complexes. Biochimica et biophysica acta 1325, 41-62.

Edlich, R.F., Diallo, A.O., Buchanan, L., Martin, M.L., 2003. Hepatitis B virus: a comprehensive strategy for eliminating transmission in the United States. Journal of long-term effects of medical implants 13, 117-125.

Edwards, A.D., Manickasingham, S.P., Sporri, R., Diebold, S.S., Schulz, O., Sher, A., Kaisho, T., Akira, S., Reis e Sousa, C., 2002. Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. J Immunol 169, 3652-3660.

El-Aneed, A., 2004. An overview of current delivery systems in cancer gene therapy. Journal of controlled release : official journal of the Controlled Release Society 94, 1-14.

Elouahabi, A., Ruysschaert, J.M., 2005. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther 11, 336-347.

Fairman, J., Moore, J., Lemieux, M., Van Rompay, K., Geng, Y., Warner, J., Abel, K., 2009. Enhanced in vivo immunogenicity of SIV vaccine candidates with cationic liposome-DNA complexes in a rhesus macaque pilot study. Hum Vaccin 5, 141-150.

Farhood, H., Serbina, N., Huang, L., 1995. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochimica et biophysica acta 1235, 289-295.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proceedings of the National Academy of Sciences of the United States of America 84, 7413-7417.

Fenner, F., 1993. Smallpox: emergence, global spread, and eradication. History and philosophy of the life sciences 15, 397-420.

Fenske, D.B., MacLachlan, I., Cullis, P.R., 2002. Stabilized plasmid-lipid particles: a systemic gene therapy vector. Methods in enzymology 346, 36-71.

Ferdous, A.J., Stembridge, N.Y., Singh, M., 1998. Role of monensin PLGA polymer nanoparticles and liposomes as potentiator of ricin A immunotoxins in vitro. Journal of Controlled Release 50, 71-78.

Filion, M.C., Phillips, N.C., 1997. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. Biochimica et biophysica acta 1329, 345-356.

Fiore, A.E., Wasley, A., Bell, B.P., 2006. Prevention of hepatitis A through active or passive immunization: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control 55, 1-23.

Fitzpatrick, M., 2004. MMR: risk, choice, chance. British medical bulletin 69, 143-153.

Florence, A., Attwood, D., 2006. Physicochemical principles of pharmacy, 4th ed. Pharmaceutical press, London.

Foged, C., Brodin, B., Frokjaer, S., Sundblad, A., 2005. Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. International journal of pharmaceutics 298, 315-322.

Foged, C., Hansen, J., Agger, E.M., 2012. License to kill: Formulation requirements for optimal priming of CD8(+) CTL responses with particulate vaccine delivery systems. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 45, 482-491.

Franklin, B., Brownrigg, W., Farish, 1774. Of the Stilling of Waves by means of Oil. . Philos.Trans.Res.Soc. 64, 445-460.

Frezard, F., 1999. Liposomes: from biophysics to the design of peptide vaccines. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.] 32, 181-189.

Fulford, G., 1968. Pouring holy oil on troubled water. Isis 59, 198-199.

Fumoto, S., Kawakami, S., Ito, Y., Shigeta, K., Yamashita, F., Hashida, M., 2004. Enhanced hepatocyte-selective in vivo gene expression by stabilized galactosylated liposome/plasmid DNA complex using sodium chloride for complex formation. Molecular therapy : the journal of the American Society of Gene Therapy 10, 719-729.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C., Robinson, H.L., 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proceedings of the National Academy of Sciences of the United States of America 90, 11478-11482.

Gaffen, S.L., Liu, K.D., 2004. Overview of interleukin-2 function, production and clinical applications. Cytokine 28, 109-123.

Galanis, E., Vile, R., Russell, S.J., 2001. Delivery systems intended for in vivo gene therapy of cancer: targeting and replication competent viral vectors. Crit Rev Oncol Hematol 38, 177-192.

Gall, D., 1966. The adjuvant activity of aliphatic nitrogenous bases. Immunology 11, 369-386.

Garside, P., Ingulli, E., Merica, R.R., Johnson, J.G., Noelle, R.J., Jenkins, M.K., 1998. Visualization of specific B and T lymphocyte interactions in the lymph node. Science 281, 96-99.

Giarelli, E., 2007. Cancer vaccines: a new frontier in prevention and treatment. Oncology (Williston Park) 21, 11-17; discussion 18.

Gjetting, T., Andresen, T.L., Christensen, C.L., Cramer, F., Poulsen, T.T., Poulsen, H.S., 2011. A simple protocol for preparation of a liposomal vesicle with encapsulated plasmid DNA that mediate high accumulation and reporter gene activity in tumor tissue. Results in Pharma Sciences 1, 49-56.

Gray, D., Dullforce, P., Jainandunsing, S., 1994a. Memory B cell development but not germinal center formation is impaired by in vivo blockade of CD40-CD40 ligand interaction. The Journal of experimental medicine 180, 141-155.

Gray, D., Siepmann, K., Wohlleben, G., 1994b. CD40 ligation in B cell activation, isotype switching and memory development. Seminars in immunology 6, 303-310.

Gregoriadis, Ryman, B.E., 1971. Liposomes as Carriers of Enzymes or Drugs - New Approach to Treatment of Storage Diseases. Biochemical Journal 124, P58.

Gregoriadis, G., 1990. Immunological adjuvants: a role for liposomes. Immunology today 11, 89-97.

Gregoriadis, G., 1994. The immunological adjuvant and vaccine carrier properties of liposomes. Journal of drug targeting 2, 351-356.

Gregoriadis, G., 1998. Genetic vaccines: strategies for optimization. Pharmaceutical research 15, 661-670.

Gregoriadis, G., Bacon, A., Caparros-Wanderley, W., McCormack, B., 2002. A role for liposomes in genetic vaccination. Vaccine 20 Suppl 5, B1-9.

Gregoriadis, G., McCormack, B., Obrenovic, M., Saffie, R., Zadi, B., Perrie, Y., 1999. Vaccine entrapment in liposomes. Methods 19, 156-162.

Gregoriadis, G., McCormack, B., Obrenovich, M., Perrie, Y., 2000. Entrapment of plasmid DNA vaccines into liposomes by dehydration/rehydration. Methods in molecular medicine 29, 305-311.

Gregoriadis, G., Putman, D., Louis, L., Neerunjun, D., 1974. Comparative effect and fate of non-entrapped and liposome-entrapped neuraminidase injected into rats. Biochem J 140, 323-330.

Gregoriadis, G., Saffie, R., de Souza, J.B., 1997. Liposome-mediated DNA vaccination. FEBS Lett 402, 107-110.

Gregoriadis, G., Saffie, R., Hart, S.L., 1996. High yield incorporation of plasmid DNA within liposomes: effect on DNA integrity and transfection efficiency. Journal of drug targeting 3, 469-475.

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

Hac-Wydro, K., Wydro, P., 2007. The influence of fatty acids on model cholesterol/phospholipid membranes. Chem Phys Lipids 150, 66-81.

Hac-Wydro, K., Wydro, P., Dynarowicz-Latka, P., 2004. A study of the interaction between dialkyldimethylammonium bromides and tri-n-octylphosphine oxide (topo) in mixed monolayers at the air/water interface. Journal of Colloid and Interface Science 278, 206-214.

Hac-Wydro, K., Wydro, P., Jagoda, A., Kapusta, J., 2007. The study on the interaction between phytosterols and phospholipids in model membranes. Chemistry and Physics of Lipids 150, 22-34.

Hamdy, S., Haddadi, A., Hung, R.W., Lavasanifar, A., 2011. Targeting dendritic cells with nano-particulate PLGA cancer vaccine formulations. Advanced drug delivery reviews 63, 943-955.

Harper, S.A., Fukuda, K., Cox, N.J., Bridges, C.B., 2003. Using live, attenuated influenza vaccine for prevention and control of influenza: supplemental recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control 52, 1-8.

Hashimoto, C., Hudson, K.L., Anderson, K.V., 1988. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52, 269-279.

Henke, A., 2002. DNA immunization--a new chance in vaccine research? Med Microbiol Immunol 191, 187-190.

Henriksen-Lacey, M., Bramwell, V., Perrie, Y., 2010a. Radiolabelling of Antigen and Liposomes for Vaccine Biodistribution Studies. Pharmaceutics 2, 91-104.

Henriksen-Lacey, M., Bramwell, V.W., Christensen, D., Agger, E.M., Andersen, P., Perrie, Y., 2010b. Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. Journal of controlled release : official journal of the Controlled Release Society 142, 180-186.

Henriksen-Lacey, M., Christensen, D., Bramwell, V.W., Lindenstrom, T., Agger, E.M., Andersen, P., Perrie, Y., 2010c. Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response. Journal of controlled release : official journal of the Controlled Release Society 145, 102-108.

Henriksen-Lacey, M., Christensen, D., Bramwell, V.W., Lindenstrom, T., Agger, E.M., Andersen, P., Perrie, Y., 2011a. Comparison of the depot effect and immunogenicity of liposomes based on dimethyldioctadecylammonium (DDA), 3beta-[N-(N',N'-Dimethylaminoethane)carbomyl] cholesterol (DC-Chol), and 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP): prolonged liposome retention mediates stronger Th1 responses. Mol Pharm 8, 153-161.

Henriksen-Lacey, M., Devitt, A., Perrie, Y., 2011b. The vesicle size of DDA:TDB liposomal adjuvants plays a role in the cell-mediated immune response but has no significant effect on antibody production. Journal of controlled release : official journal of the Controlled Release Society 154, 131-137.

Henriksen-Lacey, M., Korsholm, K.S., Andersen, P., Perrie, Y., Christensen, D., 2011c. Liposomal vaccine delivery systems. Expert opinion on drug delivery 8, 505-519.

Herzog, C., Hartmann, K., Kunzi, V., Kursteiner, O., Mischler, R., Lazar, H., Gluck, R., 2009. Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine. Vaccine 27, 4381-4387.

Hilgers, L.A., Snippe, H., Jansze, M., Willers, J.M., 1985. Combinations of two synthetic adjuvants: synergistic effects of a surfactant and a polyanion on the humoral immune response. Cellular immunology 92, 203-209.

Hilleman, M.R., 1998. Six decades of vaccine development--a personal history. Nature medicine 4, 507-514.

Hilleman, M.R., 1999. Personal historical chronicle of six decades of basic and applied research in virology, immunology, and vaccinology. Immunological reviews 170, 7-27.

Hilleman, M.R., 2000. Vaccines in historic evolution and perspective: a narrative of vaccine discoveries. Vaccine 18, 1436-1447.

Hofland, H.E., Masson, C., Iginla, S., Osetinsky, I., Reddy, J.A., Leamon, C.P., Scherman, D., Bessodes, M., Wils, P., 2002. Folate-targeted gene transfer in vivo. Molecular therapy : the journal of the American Society of Gene Therapy 5, 739-744.

Holten-Andersen, L., Doherty, T.M., Korsholm, K.S., Andersen, P., 2004. Combination of the cationic surfactant dimethyl dioctadecyl ammonium bromide and synthetic mycobacterial cord factor as an efficient adjuvant for tuberculosis subunit vaccines. Infection and immunity 72, 1608-1617.

Hong, D.K., Chang, S., Botham, C.M., Giffon, T.D., Fairman, J., Lewis, D.B., 2010. Cationic lipid/DNA complex-adjuvanted influenza A virus vaccination induces robust cross-protective immunity. Journal of virology 84, 12691-12702.

Hopkins, D.R., 1980. Ramses V: Earliest known victim. World Health Organization 220, 22.

HSCIC, 2012. NHS Immunisation Statistics, England 2011-12, 1 ed. Health and Social Care Information Centre, UK.

Huang, D.B., Wu, J.J., 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. The Journal of infection 49, 179-209.

Huebner, S., Battersby, B.J., Grimm, R., Cevc, G., 1999. Lipid-DNA complex formation: reorganization and rupture of lipid vesicles in the presence of DNA as observed by cryoelectron microscopy. Biophys J 76, 3158-3166.

Israelachvili, J.N., Mitchell, D.J., 1975. A model for the packing of lipids in bilayer membranes. Biochimica et biophysica acta 389, 13-19.

Israelachvili, J.N., Mitchell, D.J., Ninham, B.W., 1977. Theory of self-assembly of lipid bilayers and vesicles. Biochimica et biophysica acta 470, 185-201.

Iwaoka, S., Nakamura, T., Takano, S., Tsuchiya, S., Aramaki, Y., 2006. Cationic liposomes induce apoptosis through p38 MAP kinase-caspase-8-Bid pathway in macrophage-like RAW264.7 cells. Journal of leukocyte biology 79, 184-191.

Janeway, C.A., Jr., 1989. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology 54 Pt 1, 1-13.

Janeway, C.A., Jr., Jones, B., Hayday, A., 1988. Specificity and function of T cells bearing gamma delta receptors. Immunology today 9, 73-76.

Jenner, E., 1798. An inquiry into the causes and effects of the variolae vaccinae : a disease discovered in some of the western counties of england, particularly Gloucestershire, and known by the name cowpox. Sampson Low, London.

Jiao, X., Wang, R.Y., Feng, Z., Alter, H.J., Shih, J.W., 2003. Modulation of cellular immune response against hepatitis C virus nonstructural protein 3 by cationic liposome encapsulated DNA immunization. Hepatology 37, 452-460.

Jones, D.H., Corris, S., McDonald, S., Clegg, J.C., Farrar, G.H., 1997. Poly(DL-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. Vaccine 15, 814-817.

Kamath, A.T., Rochat, A.F., Christensen, D., Agger, E.M., Andersen, P., Lambert, P.H., Siegrist, C.A., 2009. A liposome-based mycobacterial vaccine induces potent adult and neonatal multifunctional T cells through the exquisite targeting of dendritic cells. PloS one 4, e5771.

Karmali, P.P., Chaudhuri, A., 2007. Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. Medicinal research reviews 27, 696-722.

Kaur, R., Bramwell, V.W., Kirby, D.J., Perrie, Y., 2012a. Manipulation of the surface pegylation in combination with reduced vesicle size of cationic liposomal adjuvants modifies their clearance kinetics from the injection site, and the rate and type of T cell response. Journal of controlled release : official journal of the Controlled Release Society 164, 331-337.

Kaur, R., Bramwell, V.W., Kirby, D.J., Perrie, Y., 2012b. Pegylation of DDA:TDB liposomal adjuvants reduces the vaccine depot effect and alters the Th1/Th2 immune responses. Journal of controlled release : official journal of the Controlled Release Society 158, 72-77.

Kaur, R., Chen, J., Dawoodji, A., Cerundolo, V., Garcia-Diaz, Y.R., Wojno, J., Cox, L.R., Besra, G.S., Moghaddam, B., Perrie, Y., 2011. Preparation, characterisation and entrapment of a non-glycosidic threitol ceramide into liposomes for presentation to invariant natural killer T cells. Journal of pharmaceutical sciences 100, 2724-2733.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., Akira, S., 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11, 115-122.

Kawakami, S., Sato, A., Nishikawa, M., Yamashita, F., Hashida, M., 2000. Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. Gene therapy 7, 292-299.

Kelly, R.B., Cozzarelli, N.R., Deutscher, M.P., Lehman, I.R., Kornberg, A., 1970. Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of duplex deoxyribonucleic acid by polymerase at a single strand break. The Journal of biological chemistry 245, 39-45.

Kenney, R.T., Edelman, R., 2003. Survey of human-use adjuvants. Expert review of vaccines 2, 167-188.

Killion, J.J., Fidler, I.J., 1998. Therapy of cancer metastasis by tumoricidal activation of tissue macrophages using liposome-encapsulated immunomodulators. Pharmacology & therapeutics 78, 141-154.

Kirby, C., Gregoriadis, G., 1984. Dehydration-rehydration vesicles: a simple method for high yield drug entrapment in liposomes. Nature biotechnology 2, 979-984.

Kirby, D.J., Rosenkrands, I., Agger, E.M., Andersen, P., Coombes, A.G.A., Perrie, Y., 2008. Liposomes act as stronger sub-unit vaccine adjuvants when compared to microspheres. Journal of drug targeting 16, 543-554.

Koblin, B.A., Casapia, M., Morgan, C., Qin, L., Wang, Z.M., Defawe, O.D., Baden, L., Goepfert, P., Tomaras, G.D., Montefiori, D.C., McElrath, M.J., Saavedra, L., Lau, C.Y., Graham, B.S., 2011. Safety and immunogenicity of an HIV adenoviral vector boost after DNA plasmid vaccine prime by route of administration: a randomized clinical trial. PloS one 6, e24517.

Korsholm, K.S., Agger, E.M., Foged, C., Christensen, D., Dietrich, J., Andersen, C.S., Geisler, C., Andersen, P., 2007. The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. Immunology 121, 216-226.

Kudsiova, L., Ho, J., Fridrich, B., Harvey, R., Keppler, M., Ng, T., Hart, S.L., Tabor, A.B., Hailes, H.C., Lawrence, M.J., 2011. Lipid chain geometry of C14 glycerol-based lipids: effect on lipoplex structure and transfection. Molecular bioSystems 7, 422-436.

Kuroda, T., Nakata, S., Nakamura, T., Ishii, M., Neya, K., Inomoto, O., Ohya, T., Kai, S., Fujii, K., Hayashi, D., 2000. The Marangoni Effect and Its Artistic Application. Forma 15, 203-204.

Lambruschini, C., Relini, A., Ridi, A., Cordone, L., Gliozzi, A., 2000. Trehalose interacts with phospholipid polar heads in Langmuir monolayers. Langmuir 16, 5467-5470.

Langmuir, I., 1917. The constitution and fundamental properties of solids and liquids. II. Liquids.1. Journal of the American Chemical Society 39, 1848-1906.

Langmuir, I., 1920. The mechanism of the surface phenomena of flotation. Transactions of the Faraday Society 15, 62-74.

Lasch, J., Weissig, V., Brandi, M., 2003. Preparation of Liposomes, in: Torchilin, V.P., Weissig, V. (Eds.), Liposomes, 2nd ed. Oxford University Press, Oxford, pp. 3-30.

Lay, M., Callejo, B., Chang, S., Hong, D.K., Lewis, D.B., Carroll, T.D., Matzinger, S., Fritts, L., Miller, C.J., Warner, J.F., Liang, L., Fairman, J., 2009. Cationic lipid/DNA complexes (JVRS-100) combined with influenza vaccine (Fluzone) increases antibody response, cellular immunity, and antigenically drifted protection. Vaccine 27, 3811-3820.

Ledgerwood, J.E., Wei, C.J., Hu, Z., Gordon, I.J., Enama, M.E., Hendel, C.S., McTamney, P.M., Pearce, M.B., Yassine, H.M., Boyington, J.C., Bailer, R., Tumpey, T.M., Koup, R.A., Mascola, J.R., Nabel, G.J., Graham, B.S., 2011. DNA priming and influenza vaccine immunogenicity: two phase 1 open label randomised clinical trials. The Lancet infectious diseases 11, 916-924.

LeibundGut-Landmann, S., Gross, O., Robinson, M.J., Osorio, F., Slack, E.C., Tsoni, S.V., Schweighoffer, E., Tybulewicz, V., Brown, G.D., Ruland, J., Reis e Sousa, C., 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nature immunology 8, 630-638.

Lemaire, G., Tenu, J.P., Petit, J.F., Lederer, E., 1986. Natural and synthetic trehalose diesters as immunomodulators. Medicinal research reviews 6, 243-274.

Levine, M.M., 2010. New generation vaccines, 4th ed. ed. Informa Healthcare ; London : Taylor & Francis [distributor], London.

Li, D., Li, G., Li, P., Zhang, L., Liu, Z., Wang, J., Wang, E., 2010. The enhancement of transfection efficiency of cationic liposomes by didodecyldimethylammonium bromide coated gold nanoparticles. Biomaterials 31, 1850-1857.

Li, L., Saade, F., Petrovsky, N., 2012. The future of human DNA vaccines. Journal of biotechnology 162, 171-182.

Li, L.H., Hui, S.W., 1997. The effect of lipid molecular packing stress on cationic liposome-induced rabbit erythrocyte fusion. Biochimica Et Biophysica Acta-Biomembranes 1323, 105-116.

Li, Y., Carroll, D.S., Gardner, S.N., Walsh, M.C., Vitalis, E.A., Damon, I.K., 2007. On the origin of smallpox: correlating variola phylogenics with historical smallpox records. Proceedings of the National Academy of Sciences of the United States of America 104, 15787-15792.

Liu, M.A., 2003. DNA vaccines: a review. Journal of internal medicine 253, 402-410.

Liu, M.A., 2011. DNA vaccines: an historical perspective and view to the future. Immunological reviews 239, 62-84.

Liu, M.A., Ulmer, J.B., 2005. Human clinical trials of plasmid DNA vaccines. Advances in genetics 55, 25-40.

Lobo, B.A., Rogers, S.A., Choosakoonkriang, S., Smith, J.G., Koe, G., Middaugh, C.R., 2002. Differential scanning calorimetric studies of the thermal stability of plasmid DNA complexed with cationic lipids and polymers. Journal of pharmaceutical sciences 91, 454-466.

Lodmell, D.L., Ray, N.B., Parnell, M.J., Ewalt, L.C., Hanlon, C.A., Shaddock, J.H., Sanderlin, D.S., Rupprecht, C.E., 1998. DNA immunization protects nonhuman primates against rabies virus. Nature medicine 4, 949-952.

Lonez, C., Vandenbranden, M., Ruysschaert, J.-M., 2008. Cationic liposomal lipids: From gene carriers to cell signaling. Progress in Lipid Research 47, 340-347.

Lu, S., 2009. Heterologous prime-boost vaccination. Current opinion in immunology 21, 346-351.

Lu, S., Wang, S., Grimes-Serrano, J.M., 2008. Current progress of DNA vaccine studies in humans. Expert review of vaccines 7, 175-191.

Lul, V.W., Haung, L., 2003. Nonviral approaches for cancer gene therapy, in: Rolland, A., Sulivan, S.M. (Eds.), Pharmaceutical gene delivery systems, 1st ed. Marcel Dekker, Inc., New York, Basel, pp. 279-319.

Lyklema, 2000. Langmuir monolayers, Fundamentals of Interface and Colloid Science

, 1 ed. Academic Press, London, pp. 3.15-13.19.

Ma, B.C., Zhang, S.B., Jiang, H.M., Zhao, B.D., Lv, H.T., 2007. Lipoplex morphologies and their influences on transfection efficiency in gene delivery. Journal of Controlled Release 123, 184-194.

Mackett, M., Williamson, J.D., 1995. Human vaccines and vaccination. Bios, Oxford.

Madigan, M.T., Martinko, J.M., Parker, J., Brock, T.D.B.o.m., 2000. Brock biology of microorganisms, 9th ed. / Michael T. Madigan, John M. Martinko, Jack Parker. ed. Prentice Hall ; London : Prentice-Hall International, Upper Saddle River, N.J.

Mae, M., Andaloussi, S.E., Lehto, T., Langel, U., 2009. Chemically modified cell-penetrating peptides for the delivery of nucleic acids. Expert opinion on drug delivery 6, 1195-1205.

Majeti, B.K., Singh, R.S., Yadav, S.K., Bathula, S.R., Ramakrishna, S., Diwan, P.V., Madhavendra, S.S., Chaudhuri, A., 2004. Enhanced intravenous transgene expression in mouse lung using cyclic-head cationic lipids. Chem Biol 11, 427-437.

Malone, R.W., Felgner, P.L., Verma, I.M., 1989. Cationic liposome-mediated RNA transfection. Proceedings of the National Academy of Sciences of the United States of America 86, 6077-6081.

Manickan, E., Yu, Z., Rouse, R.J., Wire, W.S., Rouse, B.T., 1995. Induction of protective immunity against herpes simplex virus with DNA encoding the immediate early protein ICP 27. Viral immunology 8, 53-61.

Marshall, E., 1999. Gene therapy death prompts review of adenovirus vector. Science 286, 2244-2245.

Maurer, D.M., Harrington, B., Lane, J.M., 2003. Smallpox vaccine: contraindications, administration, and adverse reactions. American family physician 68, 889-896.

Maurer, N., Fenske, D.B., Cullis, P.R., 2001. Developments in liposomal drug delivery systems. Expert opinion on biological therapy 1, 923-947.

Mazumder, S., Maji, M., Das, A., Ali, N., 2011. Potency, efficacy and durability of DNA/DNA, DNA/protein and protein/protein based vaccination using gp63 against Leishmania donovani in BALB/c mice. PloS one 6, e14644.

McCullough, K.C., Summerfield, A., 2005. Basic concepts of immune response and defense development. ILAR journal / National Research Council, Institute of Laboratory Animal Resources 46, 230-240.

McNeil, S.E., Perrie, Y., 2006. Gene delivery using cationic liposomes. Expert Opinion on Therapeutic Patents 16, 1371-1382.

McNeil, S.E., Perrie, Y., 2007. Effect of the liposomal morphological phase and liposome-DNA complex size on in vitro DNA transfection. Journal of Pharmacy and Pharmacology 59, A33-A34.

McNeil, S.E., Vangala, A., Bramwell, V.W., Hanson, P.J., Perrie, Y., 2010. Lipoplexes formulation and optimisation: in vitro transfection studies reveal no correlation with in vivo vaccination studies. Current drug delivery 7, 175-187.

Medzhitov, R., 2007. Recognition of microorganisms and activation of the immune response. Nature 449, 819-826.

Milicic, A., Kaur, R., Reyes-Sandoval, A., Tang, C.K., Honeycutt, J., Perrie, Y., Hill, A.V., 2012. Small cationic DDA:TDB liposomes as protein vaccine adjuvants obviate the need for TLR agonists in inducing cellular and humoral responses. PloS one 7, e34255.

Mischler, R., Metcalfe, I.C., 2002. Inflexal V a trivalent virosome subunit influenza vaccine: production. Vaccine 20 Suppl 5, B17-23.

Moghaddam, B., Ali, M.H., Wilkhu, J., Kirby, D.J., Mohammed, A.R., Zheng, Q., Perrie, Y., 2011. The application of monolayer studies in the understanding of liposomal formulations. International journal of pharmaceutics 417, 235-244.

Morrey, J.D., Motter, N.E., Chang, S., Fairman, J., 2011. Breaking B and T cell tolerance using cationic lipid--DNA complexes (CLDC) as a vaccine adjuvant with hepatitis B virus (HBV) surface antigen in transgenic mice expressing HBV. Antiviral research 90, 227-230.

Moser, B., 2003. Chemokines: role in immune cell traffic. European cytokine network 14, 204-210.

Moser, C., Metcalfe, I.C., Viret, J.F., 2003. Virosomal adjuvanted antigen delivery systems. Expert review of vaccines 2, 189-196.

Muller-Goymann, C.C., 2004. Physicochemical characterization of colloidal drug delivery systems such as reverse micelles, vesicles, liquid crystals and nanoparticles for topical administration. Eur J Pharm Biopharm 58, 343-356.

Mumper, R.J., Wang, J., Claspell, J.M., Rolland, A.P., 1995. Novel polymeric condensing carriers for gene delivery. Proc Control Release Soc, 178-179.

Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., Paul, W.E., 1999. The IL-4 receptor: signaling mechanisms and biologic functions. Annual review of immunology 17, 701-738.

Nichol, K.L., Mendelman, P.M., Mallon, K.P., Jackson, L.A., Gorse, G.J., Belshe, R.B., Glezen, W.P., Wittes, J., 1999. Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: a randomized controlled trial. JAMA : the journal of the American Medical Association 282, 137-144.

Nicolle, J., 1961. Louis Pasteur : a master of scientific enquiry. Hutchinson.

Nordly, P., Rose, F., Christensen, D., Nielsen, H.M., Andersen, P., Agger, E.M., Foged, C., 2011. Immunity by formulation design: induction of high CD8+ T-cell responses by poly(I:C) incorporated into the CAF01 adjuvant via a double emulsion method. Journal of controlled release : official journal of the Controlled Release Society 150, 307-317.

Norrby, E., 2007. Yellow fever and Max Theiler: the only Nobel Prize for a virus vaccine. The Journal of experimental medicine 204, 2779-2784.

O'Hagan, D.T., De Gregorio, E., 2009. The path to a successful vaccine adjuvant--'the long and winding road'. Drug discovery today 14, 541-551.

O'Hagan, D.T., MacKichan, M.L., Singh, M., 2001. Recent developments in adjuvants for vaccines against infectious diseases. Biomolecular engineering 18, 69-85.

O'Hagan, D.T., Rahman, D., McGee, J.P., Jeffery, H., Davies, M.C., Williams, P., Davis, S.S., Challacombe, S.J., 1991. Biodegradable microparticles as controlled release antigen delivery systems. Immunology 73, 239-242.

O'Hagan, D.T., Singh, M., Dong, C., Ugozzoli, M., Berger, K., Glazer, E., Selby, M., Wininger, M., Ng, P., Crawford, K., Paliard, X., Coates, S., Houghton, M., 2004. Cationic microparticles are a potent delivery system for a HCV DNA vaccine. Vaccine 23, 672-680.

O'Hagan, D.T., Valiante, N.M., 2003. Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov 2, 727-735.

Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., Wagner, E., 1998. The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. Gene therapy 5, 1425-1433.

Olds, G.R., Chedid, L., Lederer, E., Mahmoud, A.A., 1980. Induction of resistance to Schistosoma mansoni by natural cord factor and synthetic lower homologues. The Journal of infectious diseases 141, 473-478.

Opferman, J.T., Ober, B.T., Ashton-Rickardt, P.G., 1999. Linear differentiation of cytotoxic effectors into memory T lymphocytes. Science 283, 1745-1748.

Otri, A.M., Singh, A.D., Dua, H.S., 2008. Abu Bakr Razi. The British journal of ophthalmology 92, 1324.

Otten, G.R., Schaefer, M., Doe, B., Liu, H., Srivastava, I., Megede, J., Kazzaz, J., Lian, Y., Singh, M., Ugozzoli, M., Montefiori, D., Lewis, M., Driver, D.A., Dubensky, T., Polo, J.M., Donnelly, J., O'Hagan, D.T., Barnett, S., Ulmer, J.B., 2005. Enhanced potency of plasmid DNA microparticle human immunodeficiency virus vaccines in rhesus macaques by using a priming-boosting regimen with recombinant proteins. Journal of virology 79, 8189-8200.

Ouali, M., Ruysschaert, J.M., Lonez, C., Vandenbranden, M., 2007. Cationic lipids involved in gene transfer mobilize intracellular calcium. Molecular membrane biology 24, 225-232.

Park, S.H., Yang, S.H., Lee, C.G., Youn, J.W., Chang, J., Sung, Y.C., 2003. Efficient induction of T helper 1 CD4+ T-cell responses to hepatitis C virus core and E2 by a DNA prime-adenovirus boost. Vaccine 21, 4555-4564.

Patil, S.D., Rhodes, D.G., Burgess, D.J., 2005. DNA-based therapeutics and DNA delivery systems: a comprehensive review. The AAPS journal 7, E61-77.

Pearce, J.M., 2002. Louis Pasteur and rabies: a brief note. Journal of neurology, neurosurgery, and psychiatry 73, 82.

Perrie, Y., 2006. Vaccines: an overview and update. Pharmaceutical journal 276, 209-213.

Perrie, Y., Barralet, J.E., McNeil, S., Vangala, A., 2004. Surfactant vesicle-mediated delivery of DNA vaccines via the subcutaneous route. International journal of pharmaceutics 284, 31-41.

Perrie, Y., Frederik, P.M., Gregoriadis, G., 2001. Liposome-mediated DNA vaccination: the effect of vesicle composition. Vaccine 19, 3301-3310.

Perrie, Y., Gregoriadis, G., 2000. Liposome-entrapped plasmid DNA: characterisation studies. Biochimica et biophysica acta 1475, 125-132.

Perrie, Y., Kirby, D., Bramwell, V.W., Mohammed, A.R., 2007. Recent developments in particulate-based vaccines. Recent Pat Drug Deliv Formul 1, 117-129.

Perrie, Y., McNeil, S., Vangala, A., 2003. Liposome-mediated DNA immunisation via the subcutaneous route. Journal of drug targeting 11, 555-563.

Perrie, Y., Mohammed, A.R., Kirby, D.J., McNeil, S.E., Bramwell, V.W., 2008. Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. International journal of pharmaceutics 364, 272-280.

Perrie, Y., Obrenovic, M., McCarthy, D., Gregoriadis, G., 2002. Liposome (Lipodine)-mediated DNA vaccination by the oral route. Journal of liposome research 12, 185-197.

Pierre, P., Turley, S.J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R.M., Mellman, I., 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. Nature 388, 787-792.

Pimm, M.V., Baldwin, R.W., Polonsky, J., Lederer, E., 1979. Immunotherapy of an ascitic rat hepatoma with cord factor (trehalose-6, 6'-dimycolate) and synthetic analogues. International journal of cancer. Journal international du cancer 24, 780-785.

Plotkin, S.A., 2005. Vaccines: past, present and future. Nature medicine 11, S5-11.

Pockels, A., 1891. Surface Tension. Nature 43, 437-439.

Ramezani, M., Khoshhamdam, M., Dehshahri, A., Malaekeh-Nikouei, B., 2009. The influence of size, lipid composition and bilayer fluidity of cationic liposomes on the transfection efficiency of nanolipoplexes. Colloids and surfaces. B, Biointerfaces 72, 1-5.

Rao, M., Alving, C.R., 2000. Delivery of lipids and liposomal proteins to the cytoplasm and Golgi of antigen-presenting cells. mangala.rao@na.amedd.army.mil. Advanced drug delivery reviews 41, 171-188.

Rayleigh, L., 1890. Measurements of the amount of oil necessary in order to check the motions of camphor upon water. Proc. R. Soc. London 47, 364-367.

Rayleigh, L., 1899. Investigations in capillarity: the size of drops. - The liberation of gas from supersaturated solutions. - Colliding jets. - The tension of contaminated water-surfaces. A curious observation. Phil.Mag 48, 321-337.

Regelin, A.E., Fankhaenel, S., Gurtesch, L., Prinz, C., von Kiedrowski, G., Massing, U., 2000. Biophysical and lipofection studies of DOTAP analogs. Biochimica et biophysica acta 1464, 151-164.

Reinhardt, R.L., Kang, S.J., Liang, H.E., Locksley, R.M., 2006. T helper cell effector fates--who, how and where? Current opinion in immunology 18, 271-277.

Rhazes, A.B.A.-R.z., 910. A Treatise on the Small-Pox and Measles Sydenham Society, London.

Richards, R.L., Rao, M., Vancott, T.C., Matyas, G.R., Birx, D.L., Alving, C.R., 2004. Liposome-stabilized oil-in-water emulsions as adjuvants: increased emulsion stability promotes induction of cytotoxic T lymphocytes against an HIV envelope antigen. Immunology and cell biology 82, 531-538.

Richmond, J.F., Lu, S., Santoro, J.C., Weng, J., Hu, S.L., Montefiori, D.C., Robinson, H.L., 1998. Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. Journal of virology 72, 9092-9100.

Riedel, S., 2005. Edward Jenner and the history of smallpox and vaccination. Proc (Bayl Univ Med Cent) 18, 21-25.

Rifkin, I.R., Leadbetter, E.A., Busconi, L., Viglianti, G., Marshak-Rothstein, A., 2005. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. Immunological reviews 204, 27-42.

Rigby, P.W., Dieckmann, M., Rhodes, C., Berg, P., 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. Journal of molecular biology 113, 237-251.

Robinson, H.L., Montefiori, D.C., Johnson, R.P., Manson, K.H., Kalish, M.L., Lifson, J.D., Rizvi, T.A., Lu, S., Hu, S.L., Mazzara, G.P., Panicali, D.L., Herndon, J.G., Glickman, R., Candido, M.A., Lydy, S.L., Wyand, M.S., McClure, H.M., 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. Nature medicine 5, 526-534.

Rongen, H.A., Bult, A., van Bennekom, W.P., 1997. Liposomes and immunoassays. Journal of immunological methods 204, 105-133.

Rosenkrands, I., Agger, E.M., Olsen, A.W., Korsholm, K.S., Andersen, C.S., Jensen, K.T., Andersen, P., 2005. Cationic liposomes containing mycobacterial lipids: a new powerful Th1 adjuvant system. Infection and immunity 73, 5817-5826.

Sallusto, F., Cella, M., Danieli, C., Lanzavecchia, A., 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. The Journal of experimental medicine 182, 389-400.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., Lanzavecchia, A., 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401, 708-712.

Savva, M., Aljaberi, A., Feig, J., Stolz, D.B., 2005. Correlation of the physicochemical properties of symmetric 1,3-dialkoylamidopropane-based cationic lipids containing single primary and tertiary amine polar head groups with in vitro transfection activity. Colloids and surfaces. B, Biointerfaces 43, 43-56.

Schijns, V.E., 2000. Immunological concepts of vaccine adjuvant activity. Current opinion in immunology 12, 456-463.

Schirmbeck, R., Melber, K., Kuhrober, A., Janowicz, Z.A., Reimann, J., 1994a. Immunization with soluble hepatitis B virus surface protein elicits murine H-2 class I-restricted CD8+ cytotoxic T lymphocyte responses in vivo. J Immunol 152, 1110-1119.

Schirmbeck, R., Melber, K., Mertens, T., Reimann, J., 1994b. Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: implication for the pathogenesis of HBV-induced hepatitis. Journal of virology 68, 1418-1425.

Schneider, J., Gilbert, S.C., Blanchard, T.J., Hanke, T., Robson, K.J., Hannan, C.M., Becker, M., Sinden, R., Smith, G.L., Hill, A.V., 1998. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. Nature medicine 4, 397-402.

Schoenen, H., Bodendorfer, B., Hitchens, K., Manzanero, S., Werninghaus, K., Nimmerjahn, F., Agger, E.M., Stenger, S., Andersen, P., Ruland, J., Brown, G.D., Wells, C., Lang, R., 2010. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol 184, 2756-2760.

Schubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W., Bennink, J.R., 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 404, 770-774.

Sedegah, M., Hedstrom, R., Hobart, P., Hoffman, S.L., 1994. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. Proceedings of the National Academy of Sciences of the United States of America 91, 9866-9870.

Seder, R.A., Darrah, P.A., Roederer, M., 2008. T-cell quality in memory and protection: implications for vaccine design. Nature reviews. Immunology 8, 247-258.

Seder, R.A., Hill, A.V., 2000. Vaccines against intracellular infections requiring cellular immunity. Nature 406, 793-798.

Sharp, F.A., Ruane, D., Claass, B., Creagh, E., Harris, J., Malyala, P., Singh, M., O'Hagan, D.T., Petrilli, V., Tschopp, J., O'Neill, L.A., Lavelle, E.C., 2009. Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proceedings of the National Academy of Sciences of the United States of America 106, 870-875.

Singh, M., Briones, M., Ott, G., O'Hagan, D., 2000. Cationic microparticles: A potent delivery system for DNA vaccines. Proceedings of the National Academy of Sciences of the United States of America 97, 811-816.

Singh, M., O'Hagan, D.T., 2002. Recent advances in vaccine adjuvants. Pharmaceutical research 19, 715-728.

Spack, E.G., Sorgi, F.L., 2001. Developing non-viral DNA delivery systems for cancer and infectious disease. Drug discovery today 6, 186-197.

Spargo, B.J., Crowe, L.M., Ioneda, T., Beaman, B.L., Crowe, J.H., 1991. Cord factor (alpha,alpha-trehalose 6,6'-dimycolate) inhibits fusion between phospholipid vesicles. Proceedings of the National Academy of Sciences of the United States of America 88, 737-740.

Spier, R.E., 1996. International meeting on the nucleic acid vaccines for the prevention of infectious disease and regulating nucleic acid (DNA) vaccines. Natcher Conference Center NIH, Bethesda, MD 5-8 February, 1996. Vaccine 14, 1285-1288.

Stambas, J., Brown, S.A., Gutierrez, A., Sealy, R., Yue, W., Jones, B., Lockey, T.D., Zirkel, A., Freiden, P., Brown, B., Surman, S., Coleclough, C., Slobod, K.S., Doherty, P.C., Hurwitz, J.L., 2005. Long lived multi-isotype anti-HIV antibody responses following a prime-double boost immunization strategy. Vaccine 23, 2454-2464.

Stetson, D.B., Voehringer, D., Grogan, J.L., Xu, M., Reinhardt, R.L., Scheu, S., Kelly, B.L., Locksley, R.M., 2004. Th2 cells: orchestrating barrier immunity. Advances in immunology 83, 163-189.

Stewart, A.J., Devlin, P.M., 2006. The history of the smallpox vaccine. The Journal of infection 52, 329-334.

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

Storni, T., Kundig, T.M., Senti, G., Johansen, P., 2005. Immunity in response to particulate antigen-delivery systems. Advanced drug delivery reviews 57, 333-355.

Sullivan, S.M., Doukas, J., Hartikka, J., Smith, L., Rolland, A., 2010. Vaxfectin: a versatile adjuvant for plasmid DNA- and protein-based vaccines. Expert opinion on drug delivery 7, 1433-1446.

Sun, L., Xu, M., Hou, X.L., Wu, L.X., 2004. In-situ observation of the aggregated morphology and interaction of dialkyldimethylammonium bromide with DNA at air/water interface by Brewster angle microscopy. Materials Letters 58, 1466-1470.

Tanaka, T., Legat, A., Adam, E., Steuve, J., Gatot, J.S., Vandenbranden, M., Ulianov, L., Lonez, C., Ruysschaert, J.M., Muraille, E., Tuynder, M., Goldman, M., Jacquet, A., 2008. DiC14-amidine cationic liposomes stimulate myeloid dendritic cells through Toll-like receptor 4. European journal of immunology 38, 1351-1357.

Tang, D.C., DeVit, M., Johnston, S.A., 1992. Genetic immunization is a simple method for eliciting an immune response. Nature 356, 152-154.

Tarahovsky, Y.S., Koynova, R., MacDonald, R.C., 2004. DNA release from lipoplexes by anionic lipids: correlation with lipid mesomorphism, interfacial curvature, and membrane fusion. Biophys J 87, 1054-1064.

Tascon, R.E., Colston, M.J., Ragno, S., Stavropoulos, E., Gregory, D., Lowrie, D.B., 1996. Vaccination against tuberculosis by DNA injection. Nature medicine 2, 888-892.

Taylor, K., Nguyen, A., Stephenne, J., 2009. The need for new vaccines. Vaccine 27 Suppl 6, G3-8.

Tilney, N.L., 1971a. Patterns of lymphatic drainage in the adult laboratory rat. J Anat 109, 369-383.

Tilney, N.L., 1971b. Patterns of lymphtic drainage in the adult laboratory rat. Journal of Anatomy 109, 369-383.

Tirabassi, R.S., Ace, C.I., Levchenko, T., Torchilin, V.P., Selin, L.K., Nie, S., Guberski, D.L., Yang, K., 2011. A mucosal vaccination approach for herpes simplex virus type 2. Vaccine 29, 1090-1098.

Toda, S., Ishii, N., Okada, E., Kusakabe, K.I., Arai, H., Hamajima, K., Gorai, I., Nishioka, K., Okuda, K., 1997. HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-gamma antibody. Immunology 92, 111-117.

Trinchieri, G., 1997. Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-gamma). Current opinion in immunology 9, 17-23.

Tristram-Nagle, S., Nagle, J.F., 2004. Lipid bilayers: thermodynamics, structure, fluctuations, and interactions. Chem Phys Lipids 127, 3-14.

Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., et al., 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259, 1745-1749.

Ulrich, A.S., 2002. Biophysical aspects of using liposomes as delivery vehicles. Bioscience reports 22, 129-150.

Uyechi-O'Brien, L., Szoka, J.F., 2003. Mechanism for cationic lipids in gene transfer, in: Rolland, A., Sulivan, S.M. (Eds.), Pharmaceutical gene delivery systems, 1st ed. Marcel Dekker, Inc., New York, Basel, pp. 79-108.

Vaine, M., Wang, S., Hackett, A., Arthos, J., Lu, S., 2010. Antibody responses elicited through homologous or heterologous prime-boost DNA and protein vaccinations differ in functional activity and avidity. Vaccine 28, 2999-3007.

Vangala, A., Bramwell, V.W., McNeil, S., Christensen, D., Agger, E.M., Perrie, Y., 2007. Comparison of vesicle based antigen delivery systems for delivery of hepatitis B surface antigen. Journal of controlled release : official journal of the Controlled Release Society 119, 102-110.

Vangala, A., Kirby, D., Rosenkrands, I., Agger, E.M., Andersen, P., Perrie, Y., 2006. A comparative study of cationic liposome and niosome-based adjuvant systems for protein subunit vaccines: characterisation, environmental scanning electron microscopy and immunisation studies in mice. The Journal of pharmacy and pharmacology 58, 787-799.

Vassaux, G., Nitcheu, J., Jezzard, S., Lemoine, N.R., 2006. Bacterial gene therapy strategies. J Pathol 208, 290-298.

Veselenak, R.L., Shlapobersky, M., Pyles, R.B., Wei, Q., Sullivan, S.M., Bourne, N., 2012. A Vaxfectin((R))-adjuvanted HSV-2 plasmid DNA vaccine is effective for prophylactic and therapeutic use in the guinea pig model of genital herpes. Vaccine 30, 7046-7051.

Wahren, B., 1996. Gene vaccines. Immunotechnology : an international journal of immunological engineering 2, 77-83.

Wang, C., Zhuang, Y., Zhang, Y., Luo, Z., Gao, N., Li, P., Pan, H., Cai, L., Ma, Y., 2012a. Toll-like receptor 3 agonist complexed with cationic liposome augments vaccine-elicited antitumor immunity by enhancing TLR3-IRF3 signaling and type I interferons in dendritic cells. Vaccine 30, 4790-4799.

Wang, S., Parker, C., Taaffe, J., Solorzano, A., Garcia-Sastre, A., Lu, S., 2008. Heterologous HA DNA vaccine prime--inactivated influenza vaccine boost is more effective than using DNA or inactivated vaccine alone in eliciting antibody responses against H1 or H3 serotype influenza viruses. Vaccine 26, 3626-3633.

Wang, T., Upponi, J.R., Torchilin, V.P., 2012b. Design of multifunctional non-viral gene vectors to overcome physiological barriers: dilemmas and strategies. International journal of pharmaceutics 427, 3-20.

Wareing, M.D., Tannock, G.A., 2001. Live attenuated vaccines against influenza; an historical review. Vaccine 19, 3320-3330.

Wasan, E.K., Harvie, P., Edwards, K., Karlsson, G., Bally, M.B., 1999. A multi-step lipid mixing assay to model structural changes in cationic lipoplexes used for in vitro transfection. Biochimica Et Biophysica Acta-Biomembranes 1461, 27-46.

Wasungu, L., Hoekstra, D., 2006. Cationic lipids, lipoplexes and intracellular delivery of genes. Journal of controlled release : official journal of the Controlled Release Society 116, 255-264.

Watson, D.S., Endsley, A.N., Huang, L., 2012. Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine 30, 2256-2272.

Weaver, C.T., Hatton, R.D., Mangan, P.R., Harrington, L.E., 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annual review of immunology 25, 821-852.

Weisman, S., Hirsch-Lerner, D., Barenholz, Y., Talmon, Y., 2004. Nanostructure of cationic lipid-oligonucleotide complexes. Biophys J 87, 609-614.

Werninghaus, K., Babiak, A., Gross, O., Holscher, C., Dietrich, H., Agger, E.M., Mages, J., Mocsai, A., Schoenen, H., Finger, K., Nimmerjahn, F., Brown, G.D., Kirschning, C., Heit, A., Andersen, P., Wagner, H., Ruland, J., Lang, R., 2009. Adjuvanticity of a synthetic cord factor analogue for subunit Mycobacterium tuberculosis vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. The Journal of experimental medicine 206, 89-97.

Whitehead, K.A., Langer, R., Anderson, D.G., 2009. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov 8, 129-138.

Wierzbicki, A., Kiszka, I., Kaneko, H., Kmieciak, D., Wasik, T.J., Gzyl, J., Kaneko, Y., Kozbor, D., 2002. Immunization strategies to augment oral vaccination with DNA and viral vectors expressing HIV envelope glycoprotein. Vaccine 20, 1295-1307.

Wilson-Welder, J.H., Torres, M.P., Kipper, M.J., Mallapragada, S.K., Wannemuehler, M.J., Narasimhan, B., 2009. Vaccine adjuvants: current challenges and future approaches. Journal of pharmaceutical sciences 98, 1278-1316.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P.L., 1990. Direct gene transfer into mouse muscle in vivo. Science 247, 1465-1468.

Wong, J.P., Zabielski, M.A., Schmaltz, F.L., Brownlee, G.G., Bussey, L.A., Marshall, K., Borralho, T., Nagata, L.P., 2001. DNA vaccination against respiratory influenza virus infection. Vaccine 19, 2461-2467.

Writer, M., Hurley, C.A., Sarkar, S., Copeman, D.M., Wong, J.B., Odlyha, M., Jayne Lawrence, M., Tabor, A.B., McAnulty, R.J., Ayazi Shamlou, P., Hailes, H.C., Hart, S.L., 2006. Analysis and optimization of the cationic lipid component of a lipid/peptide vector formulation for enhanced transfection in vitro and in vivo. Journal of liposome research 16, 373-389.

Wrobel, I., Collins, D., 1995. Fusion of cationic liposomes with mammalian cells occurs after endocytosis. Biochimica et biophysica acta 1235, 296-304.

Xu, Y., Szoka, F.C., Jr., 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. Biochemistry 35, 5616-5623.

Yan, W., Chen, W., Huang, L., 2007. Mechanism of adjuvant activity of cationic liposome: phosphorylation of a MAP kinase, ERK and induction of chemokines. Molecular immunology 44, 3672-3681.

Yan, W., Huang, L., 2009. The effects of salt on the physicochemical properties and immunogenicity of protein based vaccine formulated in cationic liposome. International journal of pharmaceutics 368, 56-62.

Yang, K., Whalen, B.J., Tirabassi, R.S., Selin, L.K., Levchenko, T.S., Torchilin, V.P., Kislauskis, E.H., Guberski, D.L., 2008. A DNA vaccine prime followed by a liposome-encapsulated protein boost confers enhanced mucosal immune responses and protection. J Immunol 180, 6159-6167.

Yang, X., Hayglass, K.T., 1993. Allergen-dependent induction of interleukin-4 synthesis in vivo. Immunology 78, 74-79.

Yasuda, T., Dancey, G.F., Kinsky, S.C., 1977. Immunogenicity of liposomal model membranes in mice: dependence on phospholipid composition. Proceedings of the National Academy of Sciences of the United States of America 74, 1234-1236.

Zabner, J., 1997. Cationic lipids used in gene transfer. Advanced drug delivery reviews 27, 17-28.

Zamecnik, P.C., Rapaport, E., Baril, E.F., 1982. Priming of DNA synthesis by diadenosine 5',5"'-P1,P4-tetraphosphate with a double-stranded octadecamer as a template and DNA polymerase alpha. Proceedings of the National Academy of Sciences of the United States of America 79, 1791-1794.

Zantl, R., Baicu, L., Artzner, F., Sprenger, I., Rapp, G., Rädler, J.O., 1999. Thermotropic phase behavior of cationic lipid-DNA complexes compared to binary lipid mixtures. The Journal of Physical Chemistry B 103, 10300-10310.

Zhdanov, R.I., Podobed, O.V., Vlassov, V.V., 2002. Cationic lipid-DNA complexes-lipoplexes-for gene transfer and therapy. Bioelectrochemistry 58, 53-64.

Zhou, F., Huang, L., 1994a. Liposome-mediated cytoplasmic delivery of proteins: an effective means of accessing the MHC class I-restricted antigen presentation pathway. ImmunoMethods 4, 229-235.

Zhou, X., Huang, L., 1994b. DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. Biochimica et biophysica acta 1189, 195-203.

Zinkernagel, R.M., 2002. On differences between immunity and immunological memory. Current opinion in immunology 14, 523-536.

Zinkernagel, R.M., Ehl, S., Aichele, P., Oehen, S., Kundig, T., Hengartner, H., 1997. Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity. Immunological reviews 156, 199-209.

Zuhorn, I.S., Engberts, J.B.F.N., Hoekstra, D., 2007. Gene delivery by cationic lipid vectors: overcoming cellular barriers. European Biophysics Journal with Biophysics Letters 36, 349-362.

Zuhorn, I.S., Oberle, V., Visser, W.H., Engberts, J.B., Bakowsky, U., Polushkin, E., Hoekstra, D., 2002. Phase behavior of cationic amphiphiles and their mixtures with helper lipid influences lipoplex shape, DNA translocation, and transfection efficiency. Biophysical journal 83, 2096-2108.

Appendices

1- PicoGreen® dsDNA quantification assay

Quant-iT™ PicoGreen® dsDNA reagent is an ultrasensitive fluorescent nucleic acid stain which has been used for quantitating double-stranded DNA (dsDNA) in solution. It helps researchers to selectively detect as little as 25 pg/mL of dsDNA with a standard spectroflurometer and fluorescein excitation and emission wavelengths. The assay is linear over their 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 Dynamic range and sensitivity of the Quant-iT™ PicoGreen® dsDNA assay

Figure.2 Fluorescence enhancement of the PicoGreen® Quantitiation Reagent upon biding dsDNA, ssDNA and RNA.

Figure.3 Fluorescence excitation and emission spectra of PicoGreen® dsDNA Quantitation reagent bound to DNA (adapted from Singer et al., 1997)

2. Calibration curves used for DNA association calculations

Figure. 4 Low range calibration curve for DNA using PicoGreen assay. Results denote mean ± SD, from 3 independent patches.

Figure.5 Low range calibration curve for DNA using PicoGreen assay. Results denote mean ± SD, from 3 independent patches.

3. Calibration curve for calculation the ^{3}H and ^{32}P in biodistribution study.