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Summary

Liposomes offer an ideal platform for the delivery of subunit vaccines, due to their versatility and flexibility, which allows for antigen as well as immunostimulatory lipids and TLR agonists to become associated with these bilayered vesicles. Liposomes have the ability to protect vaccine antigen, as well as enhance delivery to antigen presenting cells, whilst the importance of cationic surface charge for delivery of TB subunit vaccines and formation of an 'antigen depot' may play a key role in boosting cell-mediated immunity and Th1 immune responses. The rational design of vaccine adjuvants requires the thorough investigation into the physicochemical characteristics that dictate the function of a liposomal adjuvant. Within this thesis, physicochemical characteristics were investigated in order to show any effects on the biodistribution profiles and the ensuing immune responses of these formulations.

Initially the role of liposome charge within the formulation was investigated and subsequently their efficacy as vaccine adjuvants in combination with their biodistribution was measured to allow the role of formulation in vaccine function to be considered. These results showed that cationic surface charge, in combination with high loading of H56 vaccine antigen through electrostatic binding, was crucial in the promotion of the 'depot-effect' at the injection site which increases the initiation of Th1 cell-mediated immune responses that are required to offer protection against tuberculosis. To further investigate this, different methods of liposome production were also investigated where antigen incorporation within the vesicles as well as surface adsorption were adopted. Using the dehydration-rehydration (DRV) method (where liposomes are freeze-dried in the presence of antigen to promote antigen encapsulation) and the double emulsion (DE) method, a range of liposomes entrapping antigen were formulated. Variation in the liposome preparation method can lead to antigen entrapment within the delivery system which has been shown to be greater for DRV-formulated liposomes compared to their DE-counterparts. This resulted in no significant effect on the vaccine biodistribution profile, as well as not significantly altering the efficacy of cationic liposomal adjuvants. To further enhance the efficacy of these systems, the addition of TLR agonists either at the vesicle surface as well as within the delivery system has been displayed through variation in the preparation method.

Anionic liposomal adjuvants have been formulated, which displayed rapid drainage from the injection site to the draining lymph nodes and displayed a reduction in measured Th1 immune responses. However, variation in the preparation method can alter the immune response profile for anionic liposomal adjuvants with a bias in immune response to Th2 responses being noted. Through the use of high shear mixing and stepwise incorporation, the efficient loading of TLR agonist within liposomes has been shown. However, interestingly the conjugation between lipid and non-electrostatically bound TLR agonist, followed by insertion into the bilayer of DDA/TDB resulted in localised agonist retention at the injection site and further stimulation of the Th1 immune response at the SOI, spleen and draining lymphatics as well as enhanced antibody titres.

Keywords: Tuberculosis, biodistribution, liposome, vaccine adjuvant, immune response

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Own Publications

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Hussain, M.J., Wilkinson, A., Bramwell, V., Christensen, D., Perrie, Y. (2013) "Th1 immune responses can be modulated by varying dimethyldioctadecylammonium and distearoyl-sn-glycero-3-phosphocholine content in liposomal adjuvants" (Manuscript accepted for Journal of Pharmacy and Pharmacology).

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Conference Abstracts

Wilkinson, A., Henriksen-Lacey, M., Mohammed, A.R., Perrie, Y (2011), "The use and characterisation of a novel anionic liposome system for use in vaccine delivery and formulation" *UKI-CRS Conference, Belfast, 13th April 2011.*

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Wilkinson, A., Korsholm, K.S., Christensen, D., Mohammed, A.R., Perrie, Y. (2013) "Investigating the role of antigen entrapment within liposomal adjuvants on the generated immune response" *UK PharmSci Conference, Edinburgh, September 2013.*

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Abbreviations

APC	Antigen Presenting Cell
BCA	Bicinchonic acid protein assay
BCG	Bacille-Calmette Guerin
BLN	Blank lymph node
CAF	Cationic adjuvant formulation
CAF01	DDA/TDB
CAF04	DDA/MMG
CAF05	DDA/TDB/PolyI:C
CAF09	DDA/MMG/PolyI:C
Chol	Cholesterol
CLR	C-lectin type receptor
ConA	ConcanavalinA
CMI	Cell-mediated immune
CpG	Cytosine-phosphate-guanine
Cpp	critical packing parameter
DC	Dendritic cell
DDA	Dimethyldioctadecylammonium bromide
DE	Double emulsion
DIAD	Diisopropyl azodicarboxylate
DLS	Dynamic light scattering
DRV	Dehydration-rehydration vesicle
DSPC	1,2 – distearoyl-glycero-phosphatidylcholine
DSPE	1,2 – distearoylphosphatidylethanolamine
DSPS	1,2 – distearoylphosphatidylserine
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
H56	Ag85B-ESAT6-Rv2660c
H1	Ag85B-ESAT6
H4	Ag85B-TB10.4

HSM	High shear mixing
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
i.m.	Intramuscular
IRM	Immune response modifier
IR	Infra-red
LH	Lipid-film hydration
LPS	Lipopolysaccharides
MHC	Major histocompatibility complex
MPL	Monophosphoryl Lipid A
MLV	Multilamellar vesicle
MMG	Monomycoloyl glycerol
M.tb	Mycobacterium tuberculosis
NFκB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
Non-TLR	non Toll-like receptor
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PDI	Polydispersity
p.i	Post-injection
pI	Isoelectric point
PLN	Popliteal lymph node
PMA	Phorbol 12- myristate 13-acetate
PolyI:C	Polyinosinic:polycytidylic acid
rbf	Round bottomed flask
SA	Succinic anhydride
s.c.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SOI	Site of injection
SSI	Statens Serum Institute
SUV	Small unilamellar vesicle
TB	Tuberculosis
TDB	Trehalose 6,6’ – Dibehenate
Th	T-helper
TLC	Thin layer chromatography
TLR	Toll-like receptor
TPP	Triphenylphosphine
Treg	Regulatory T-cell
ZP	Zeta potential

Chapter 1: Introduction

1.1. Historical Background

1.1.1. Vaccination

The groundwork of modern vaccination was put down in the 18th century by Edward Jenner. He discovered that patients with smallpox could be protected with the mild related form of the disease, cowpox. This ultimately led to the development of an effective vaccine against smallpox, and laid the foundation for the treatment and protection against other contagious diseases (Plotkin, 2005). The idea behind vaccination can be traced back as early as 430 BC, when survivors of smallpox were used to nurse sufferers of this disease, however it was not until the 10th century AD and over the following few centuries that efforts were made in China to transfer smallpox infection to susceptible individuals, with the aim of combating this disease in a process termed inoculation (Gross and Sepkowitz, 1998).

The basic theory behind vaccination is the attainment of immunity against an infectious agent without the prior need for an initial infection against the specific disease. The initial innate immune response is unable to counteract the initial major disease symptoms. However, the memory cells involved in the adaptive immune response 'remember' the first encounter with the disease pathogen. This results in the rapid onset of protective reactions against a specific disease (Bramwell and Perrie, 2005b, 2006, Perrie et al., 2007). For a vaccine to be deemed effective it must pass a certain number of aspects in that it is able to generate increased immune responses, as well as offer an enhanced safety profile and provide a safe form of health care. There are a range of vaccine types currently available or in clinical trials which will be described in more detail in Section 1.2. Subunit vaccines offer several advantages over live vaccines, however they tend to lack efficacy therefore adjuvants are often employed to improve immune responses.

1.1.2. Liposomes

Liposomes were originally described in the mid 1960s by Bangham and colleagues who observed that smears of lipid (egg lecithin) were able to react with water to form intricate vesicular structures, and following analysis by electron microscopy the spontaneous formation of vesicles was observed (Bangham *et al.*, 1965). Following their discovery, in the initial years of liposome research these vesicles were used as models for artificial membranes (due to their similar membrane bilayer structure) in order to simulate cellular systems for the investigation of transport functions and

permeation properties (Bangham et al., 1967, Papahadjopoulos and Watkins, 1967). Furthermore, initial pioneering work on liposomes was undertaken by Gregoriadis and colleagues in the 1970s and 80s who described their ability to act as delivery systems for drugs (Gregoriadis and Ryman, 1971, Allison and Gregoriadis, 1974, Gregoriadis, 1988). Simultaneously, liposomes were shown to have the ability to change the *in vivo* biodistribution of drugs (Poste and Papahadjopoulos, 1976, Poste et al., 1976), as well as enhance the drug loading and subsequent controlled release in order to increase bioavailability, and also increase entrapment efficiency (Szoka and Papahadjopoulos, 1978).

Many of the advantages of liposomes as drug delivery systems can be applied in their use as vehicles for the delivery of vaccine antigens, through adsorption at the liposome surface or entrapment within (Gregoriadis and Ryman, 1971, Allison and Gregoriadis, 1974, Gregoriadis et al., 1987, Gregoriadis, 1989, 1994, Gregoriadis et al., 1999). Therefore, the delivery of vaccine antigens within liposome delivery systems will be primarily addressed during this thesis.

1.2. Current vaccine strategies against Tuberculosis (TB)

1.2.1. Types of Vaccines

There are three main sub-categories of vaccines: live, inactivated and sub-unit. The adjuvant capability of live vaccines is based on the sustained ability of the immune system to detect signals of hazardous microbes. The application of live vaccines has been shown to reduce the effects of disease; however, there are certain disadvantages such as virus reversion, perseverance and production issues. As described in more detail in Section 1.2, an example of a live vaccine is the TB vaccine, Bacille-Calmette Guerin (BCG). This vaccine is fairly cost-effective and economical and provides high efficacy against TB for infants. However BCG had markedly reduced efficacy in adults, due to the inability to protect against the pulmonary form of this disease in adults (Delogu and Fadda, 2009).

In terms of vaccine subtypes, subunit vaccines have the best safety profile (Perrie *et al.*, 2008). This vaccine subset provide several key advantages in that they contain pure recombinant antigens which have the ability to decrease disease-associated side effects and generate an increased safety profile (Black *et al.*, 2010). Although there are several drawbacks to these vaccines including reduced potency, requirement of more doses, the induction of unneeded host reactions and the limited nature of the produced level of immunity only resulting in humoural responses. However, administration of subunit vaccines alone lack the inherent immunogenicity themselves to produce

an effective immune response. Therefore, these vaccines require the combined presence of an adjuvant delivery system to increase and lengthen the levels of protective immunity (Bramwell and Perrie, 2005a, O'Hagan and De Gregorio, 2009, Reed et al., 2009).

Even though there have been many successes and breakthroughs in the fields of vaccination and disease prevention, there remains a requirement for the development of vaccines against malaria and human immunodeficiency virus (HIV) and new effective TB vaccines, amongst others. Therefore ongoing research into vaccine formulation and technology is crucial to provide safe effective vaccines against infectious diseases (Martin, 2005, Delogu and Fadda, 2009, Reed et al., 2009).

1.2.2. Success criteria for vaccines

Vaccination is one of the primary methods that can be used in order to control the spread of disease and infection (especially tuberculosis, HIV, and malaria) by the introduction of antigenic material to stimulate the immune system of an individual. The main objective of a vaccine is to 'promote long-term immunological protection against the establishment of a specific infection' (Perrie *et al.*, 2007). There are some general criteria that a vaccine must satisfy, or a vaccine would significantly benefit from. A good vaccine must be able to elicit an appropriate immune response required for vaccine efficacy in order to control and eradicate a specific infectious disease. The vaccine must also be safe to administer, cost-effective and be administered in a stable and reproducible formulation (Perrie *et al.*, 2007). If these criteria are satisfied this could have dramatic implications in the development of a successful vaccine. For a vaccine delivery system to be effective it requires the ability to 'prime' the immune system so that it can respond quickly and efficiently to limit infection to a specific pathogen in the vaccinated host organism (Altin and Parish, 2006).

1.2.3. Tuberculosis

As mentioned, tuberculosis (TB) remains a major global healthcare concern. TB is an airborne disease and is one of the commonest serious infectious diseases worldwide with about 8.8 million new cases diagnosed worldwide, with 1.4 million people dying in 2010 (WHO, 2011). This infection is out of control in developing and poverty-stricken regions such as Africa, where HIV-infection is also a serious problem. There is a clear synergistic relationship between *M. tuberculosis* and the human immunodeficiency virus (HIV), as individuals with active tuberculosis (TB) are more likely to become

immunodeficient to the HIV virus, thus causing increased mortality (Toossi et al., 2001, Corbett et al., 2003). Control strategies and antibiotics against TB increasingly rely on at least three drugs for long periods of time; however, resistance to these drugs and difficulties with patient compliance has limited the control of this disease (Martin, 2005).

The only current available vaccine against this disease is the nearly century-old Bacille-Calmette-Guerin (BCG) vaccine, which is based on an attenuated form of the *Mycobacterium bovis* strain. This vaccine is able to protect against childhood forms of TB, but it struggles to protect against pulmonary TB in adults (Delogu and Fadda, 2009, Dheda et al., 2010). Although greater than 90 % of individuals infected with TB are able to effectively contain infection in the latent or subclinical stage, this huge number of latently infected individuals leads to a high number of active TB cases later in life (Ottenhoff and Kaufmann, 2012).

This vaccine is unable to confer protection against TB in adults due to the existence of many strains of TB in the world today, including multidrug-resistant-TB (MDR-TB) and extremely drug resistant-TB (XDR-TB) strains. All of these strains have genetic differences which have resulted in this vaccine being unable to protect against all forms of this disease. Also, these strains have many antigenic and immunological differences (Delogu and Fadda, 2009) that lead to their potential reduction in efficacy against TB. Therefore it is of critical importance to develop an improved vaccine formulation against all forms of this disease. This vaccine must be capable of eliciting an appropriate immune response in individuals at high risk of developing TB. Therefore this situation 'emphasises the need for a vaccine that can reduce, or even prevent transmission of TB' (Ottenhoff *et al.*, 2010). The first new-generation TB vaccines are currently involved in clinical trials. These vaccines are summarised in Table 1.1.

The lung is the point of entry of the *M. tuberculosis* (*M. tb*) bacterium as it enters the body through the airways whilst this infection is firmly established in macrophages, local dendritic cells and epithelial cells of the distal alveolar compartment before being recognised by CD4+ and CD8+ T-cells in order to generate levels of protective immunity (Martin, 2005, Reece and Kaufmann, 2008). *M. tb* organisms are able to be transmitted through aerosols originating from the lungs of individuals with active TB and have the unique ability to establish infection while also delaying the onset of adaptive immune responses by 2-3 weeks (Wolf *et al.*, 2008). This strategy allows *M. tb* to establish infection resulting in the formation of two populations of these microorganisms; dormant (or latent) organisms which can be retained in a low metabolic state and resuscitate later in life, or active metabolically active bacterium which stimulate the local immune response but also lead to

pathological hallmarks of TB including the formation of necrotic lesions (Reece and Kaufmann, 2012).

The host immunity to mycobacterial infections is dependent on IFN-γ and IL-12, therefore showing the importance of a Th1-mediated immunity against *M. tuberculosis.* The most effective vaccination strategies against TB involve the stimulation of CD4 and CD8 T-cell responses in order to produce cytokines associated with the Th1 (cell mediated immune or CMI) response, such as IFN-γ and IL-2.

Table 1.1. Current TB vaccine candidates in clinical trials

Vaccine Strategy	Vaccine Candidate	Vaccine Description	Clinical trials?
Pre-exposure prime vaccination using recombinant BCG	VPM 1002	rBCG-expressing listeriolysin and urease deletion	Phase IIa
	rBCG30	rBCG-expressing Ag85B	Phase I
	Aeras-422	rBCG-expressing perfringolysin and Ag85A, B, Rv3407	Phase I terminated due to side effects
Pre-exposure booster vaccination using viral vectors	Aeras-485	Modified vaccinia Ankara-expressing Ag85A	Phase IIb ongoing
	Aeras-402	Ad35-expressing Ag85A, B and TB10.4	Phase IIb ongoing
	AdAg85A	Ad5-expressing Ag85A	Phase I
Pre-exposure booster vaccination using fusion protein in adjuvants	Hybrid1 in combination with IC31	Fusion of Ag85B and ESAT6 in IC31 adjuvant	Phase I/IIa
	Hybrid56 in combination with IC31	Fusion of Ag85B, ESAT6 and Rv2660c in IC31 adjuvant	Phase I
	Hybrid1 in combination with CAF01	Fusion of Ag85B and ESAT6 in CAF01 adjuvant	Phase I
	Aeras-404	Fusion of Ag85B and TB10.4 in IC31 adjuvant	Phase I
Therapeutic vaccination using whole bacteria	RUTI	Detoxified *M. Tb* in liposomes	Phase IIa

1.2.4. Mycobacterial antigens and their potential as vaccine candidates

Whereas mycobacterial whole cell vaccines are highly immunogenic, vaccines based on sub-unit antigens rely on effective adjuvants or delivery systems in order to promote their immune response. However, vaccines based on these simplified sub-units have an increased profile when it comes to safety and quality control (Perrie et al., 2007, Perrie et al., 2008). During the past several years, the combination between antigen and adjuvant has been used extensively in order to provoke specific

immune responses against mycobacteria in a tuberculosis vaccine model (Davidsen *et al.*, 2005). Subunit vaccines for TB, in the majority, are based on recombinant proteins in combination with proper adjuvants. The aim of TB subunit vaccines is often considered for them to be to used as 'booster vaccines' in able to initiate strong, long-lived immune responses in already primed individuals (Ottenhoff and Kaufmann, 2012) who have been immunised with BCG- or *M. tb* priming vaccines.

Over the past decade, a number of TB vaccine research groups have screened more than 250 mycobacterial antigens for their potential as vaccine candidates, diagnostic tools or biomarkers (Christensen *et al.*, 2009). The most recent approach towards the discovery of antigens has focussed on the identification of antigens that are preferentially recognized in hosts latently infected with mycobacteria with the purpose of using such antigens in future vaccines (Aagaard *et al.*, 2011). To date, a number of antigen candidates have been identified for the purpose of identifying a subunit-based TB vaccine; two of these are preventive vaccines, and the other is a post-exposure vaccine candidate (Figure 1.1). This is important as up to one third (1/3) of the population is estimated to be infected with TB, either in the active form or the latent form. So an effective vaccine must be capable of producing a sufficient immune response to both forms of this disease.

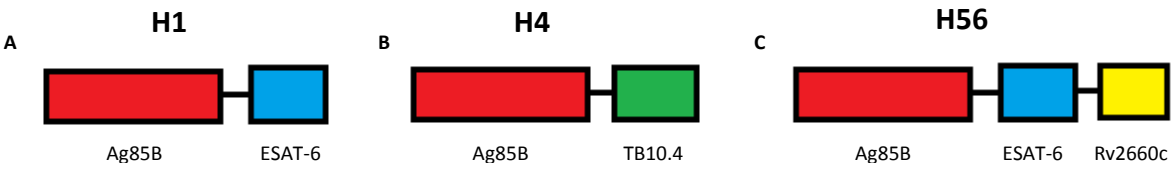


Figure 1-1. Pre- and post-exposure TB-vaccine candidates [A: H1 (Ag85B-ESAT6); B: H4 (Ag85B-TB10.4); C: H56 (Ag85B-ESAT6-Rv2660c) which have been used in combination with liposomes in a TB vaccine model.

The first of these vaccine candidates is termed Hybrid1 (H1) which is a fusion of the two proteins Ag85B and ESAT6 (Figure 1.1). Both of these proteins have been shown to have immunogenicity by themselves (as vaccine antigens), but when in combination (as Hybrid 1) these have a greater effect on the immune response (Weinrich Olsen *et al.*, 2001).

The second of these candidates is referred to as Hyvac (H4), which is a fusion of the two proteins Ag85B and TB10.4, which is also expressed by BCG (Elvang *et al.*, 2009). Whereas the post-exposure vaccine candidate, termed H56, consists of the Ag85B-ESAT6 fusion protein attached to the latency antigen Rv2660c (Figure 1.1). The strategy for this post-exposure vaccine candidate is to selectively target the mycobacteria in the dormant stage of infection so therefore this antigen candidate can be preferentially expressed as the bacteria adapt to long-term persistence in the immune host,

particularly against late chronic infection (Aagaard *et al.*, 2011). The Rv2660c gene had been selected for use in this subunit vaccine combination due to its strong upregulation under starvation conditions.

Both H1 and H56 vaccine antigens have been included in cationic liposome formulations based on DDA in combination with TDB. In 2005, Davidsen et al (Davidsen *et al.*, 2005) showed that by associating this promising TB-fusion protein antigen, Ag85B-ESAT6 (Weinrich Olsen *et al.*, 2001) with DDA/TDB liposomes this could initiate both a strong Th1-immune response and an antibody response. This antigen is highly anionic (negatively charged) and has a low isoelectric point (pI) value of around 4.0. Therefore high adsorption of this fusion protein antigen is able to occur to cationic DDA/TDB liposomes, due to the opposite charge of these components and this interaction is likely to be electrostatic in nature. This subunit protein antigen has been used in subsequent studies in order to investigate the importance of various physicochemical characteristics of liposomal adjuvants, with strong Th1 immune responses also being initiated (Henriksen-Lacey et al., 2011b, Kaur et al., 2012). This formulation, in combination with H1, is currently in phase 1 clinical trials in humans.

In further studies by Christensen et al (Christensen et al., 2007a, Christensen et al., 2009) freeze-dried DDA/TDB liposomes have been shown to retain their ability to generate antigen-specific immune responses, and this was shown after the immunisation of mice with Ag58B-ESAT6 with this trehalose-stabilised adjuvant. Aagaard and colleagues (2011) have investigated the H56 antigen in combination with DDA/TDB. In these studies, this combination has been shown to generate a significantly better immune response than the BCG vaccine against tuberculosis (TB). This H56 vaccine is able to promote a T-cell response against all protein components that is characterised by a very high proportion of polyfunctional $CD4^+$ T-cells. This vaccine candidate has also been shown to be almost ten times more efficient in reducing the bacterial load than the H1 fusion protein (Aagaard *et al.*, 2011) and this has been shown in two separate mouse models. These promising results have led to the recent advancement of the H56 vaccine into a number of clinical trials.

Subunit vaccines can also be delivered through viral vector delivery systems with several systems currently involved in phase IIb clinical trials. These include the modified vaccine virus Ankara (MVA) expressing the subunit antigen Ag85A, which is highly immunogenic in both naive and BCG-primed individuals (McShane et al., 2004, Pathan et al., 2007). Also, replication-deficient adeneoviral systems (Ad5 or Ad35) containing the subunit antigens Ag85A, B and TB10.4 induce highly strong CD4+ and CD8+ T-cell responses as characterised by IFNγ production, therefore showing high immunogenicity in humans (Radosevic *et al.*, 2007).

1.2.5. Live "Priming" Vaccines

Another avenue of research into TB vaccines is to replace the Bacille-Calmette-Guerin (BCG) vaccine with recombinant BCG or genetically attenuated *M. tb* strains. This can be achieved through the overexpression of antigens (such as Ag85A, B, TB10.4 or RD1) that BCG already expresses by itself, but not in high enough quantities throughout infection. An example being the recombinant BCG30 vaccine (rBCG30) which overexpresses Ag85B in BCG and offers improved protection against TB (Tullius *et al.*, 2008).

The second group of live TB vaccines are genetically attenuated *M. tb* derivatives, which can be attained through deletion of essential virulence genes that are involved in the metabolism of essential nutrients for these organisms. Examples of these vaccines include ΔRD1 ΔpanCD and ΔPhoP Δfad *M. tb* strains which lead to the induction of protective immunity in normal and immunocompromised mice and boast improved safety (Martin et al., 2006, Sambandamurthy et al., 2006).

A further vaccine in Phase IIa trials is termed RUTI, which is produced from *Mycobacterium tuberculosis* fragmented into tiny particles and detoxified in order to eliminate the toxic substances produced by the bacillus, and delivered in liposomes. This resulted in the induction of T-cell responses and shortens the treatment of latent TB infection (Cardona, 2006).

1.3. Components of the immune system

1.3.1 Adaptive Immunity

It is important to understand how the human immune system responds to combat a specific infection, and how it responds to vaccination. As shown in Figure 1.2, the human immune system consists of three types of cells (Dendritic cells, T-lymphocytes and B-lymphocytes) that work in conjunction in order to provoke a specific immune response (Sprent and Webb, 1987).

Dendritic cells, such as macrophages, are involved in taking up foreign antigens (derived from pathogens) by endocytosis or phagocytosis. Upon uptake, antigen can be broken down by lysosomes before they then be presented at the cell surface of T-cells, with Class II MHCs (Major Histocompatability Complexes) in association with CD4 glycoproteins (Nordly *et al.*, 2009). These cells can then migrate from the site of infection to the regional lymph nodes. At this site, the antigen

can then be presented to naïve T-lymphocytes, which causes the secretion of various interleukins depending on whether the cellular or humoural arm of the immune response is activated. These signals (interleukins) result in the activation of B-lymphocytes; which in turn causes the formation of plasma cells and subsequently the secretion of antibodies specific to the antigen presented during the initial stage (infection). This leads to an antigen-specific immune response being activated in response to the specific antigen used during vaccination (Perrie *et al.*, 2007).

Figure 1-2. The three cell types that act together to induce an effective immune response [(A) Uptake of foreign antigen by dendritic cells such as macrophages before (B) presentation of antigen at the surface of T-cells at the lymph nodes which causes interleukin secretion which (C) activates B-lymphocytes and hence leading to antibody secretion (Perrie *et al.*, 2007).

When an individual encounters a specific antigen for the first time, the immune response is generally slow to react because the immune system does not know how to deal with this pathogen, which leaves the human body susceptible to infection. During this initial encounter with this specific antigen the body can develop an acquired immunity. Therefore, a number of T- and B-lymphocytes can survive and differentiate into highly reactive memory and plasma cells accordingly, so upon re-infection the body can respond in a quicker fashion in order to provide a stronger immune response (Figure 1.3). The process of vaccination (with antigen) negates the need for a specific infection in an individual in order to cause appropriate immunity to that pathogen, so a quicker immune response can be activated when that certain individual encounters infection (for the first time) without that individual developing symptoms of that disease (Figure 1.3).

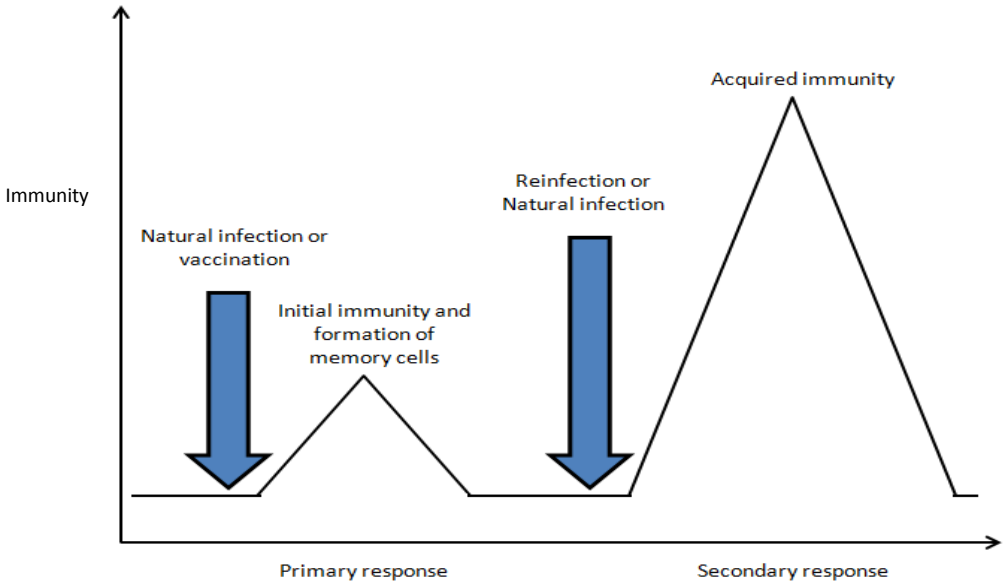


Figure 1-3. The development of an immune response after natural infection or vaccination.

CD4+ T-cells can be subdivided into a number of different subsets depending on the cytokines they produce and the specific function they perform (Figure 1.4). The early events in an immune response can stimulate cytokine production therefore directing the development of these T-cell subsets to a distinct lineage, which is strongly dictated by the type of microorganism or antigen which is invading the host and also depends strongly on antigen dose and vaccine administration route (Mosmann et al., 1986, O'Garra, 1998). Th1 cells can be characterised by their production of interferon-gamma (IFN-γ) which is primarily responsible for the eradication of intracellular pathogens, such as *M. tb*, whereas Th2 cells can be characterised by their production of interleukins 4, 5 and 10 which are responsible for B-cell growth and differentiation. A further distinct lineage of Th cells can be characterised by the production of interleukin-17 (IL-17) and have, therefore, been termed Th17 cells (Harrington *et al.*, 2005). These cells are important in mediating autoimmune pathology, which was previously thought to be a role for Th1 cells. These three separate Th cell lineages can be regulated depending on the cytokine milieu with IL-12 and IL-4 previously being shown to regulate Th1 and Th2 differentiation respectively, with the differentiation of Th17 cells (Bettelli *et al.*, 2006) from naive CD4+ T-cells being shown to be due to IL-6, IL-23 and tumour growth factor-beta (TGF-β). A further subset of T-cells, termed regulatory T-cells (T_{reg}), have been shown to be crucial for promoting 'immunological homeostasis' as well as maintaining the tolerance to self-antigens and also curtailing immune responses against pathogens (Cooke, 2006). T_{reg} cells, therefore, shut down the immune response after the successful elimination of invading organisms such as *Mycobacterium tuberculosis*.

These $CD4^+$ T-cells (often referred to as helper cells) express the CD4 receptor and, therefore, aid in immune responses, whereas a further sub-population of T-cells expressing the CD8 receptor are termed $CD8^+$ T-cells (or cytotoxic T-lymphocytes). This further T-cell subpopulation recognise antigen that is presented on the surface of MHC class I molecules, therefore leading to their destruction (Guy, 2007). Antigen when formulated within liposome-TLR agonist adjuvants further results in the enhanced delivery of antigen and the stimulation of innate immunity through a process called 'cross-priming' in which the antigen can be presented to $CD8^+$ T-cells (Zaks *et al.*, 2006).

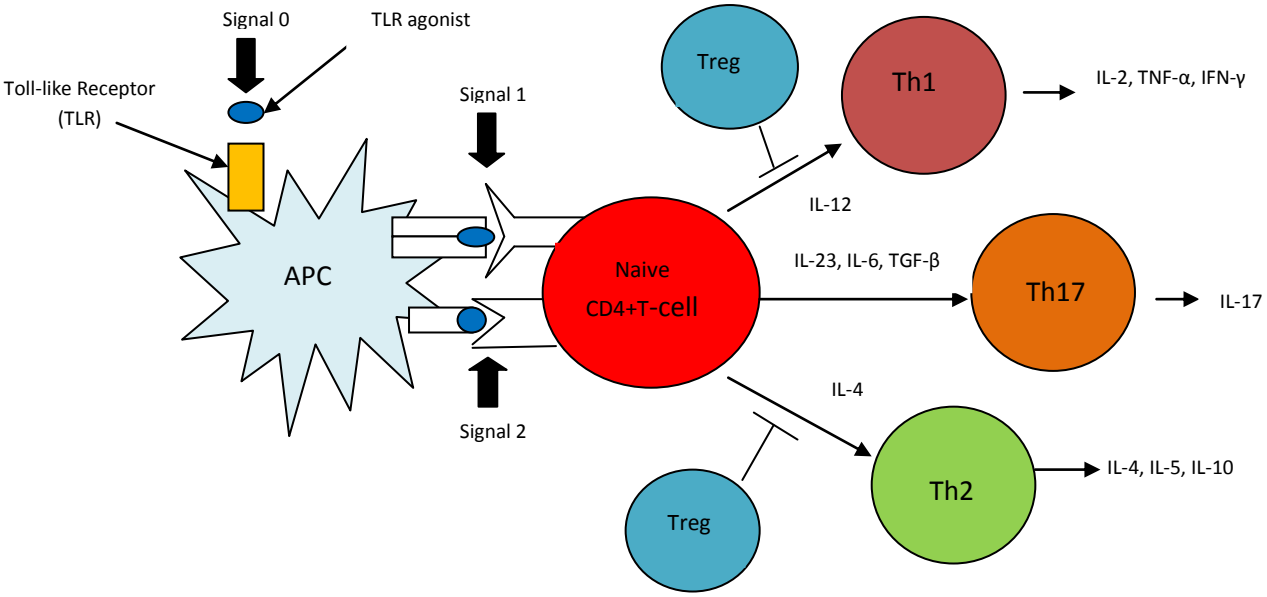


Figure 1-4. Initiation of T-cell responses by pathogen-associated molecular patterns (PAMPs), which are present on TLR agonists (SIGNAL 0). The antigen is presented at the surface of APCs (macrophages) and is taken up at the surface of T-cells (SIGNAL 1). This will provide the simultaneous delivery of co-stimulatory molecules (SIGNAL 2) to the APC. This has the ability to activate several distinct lineages of T-cells; Th1 and Th2, Th17 and Treg.

1.3.2 Innate Immunity

The innate immune system plays an important role as the first line of defence against pathogens. The main components of this system are phagocytes including macrophages, neutrophils and dendritic cells. Phagocyctes can kill these pathogens by engulfing and digesting them. The main function of these phagocytes is the discrimination between self and other pathogens, which is helped via the family of Toll-Like Receptors (TLRs) (Akira et al., 2003, Akira and Takeda, 2004). The stimulation of TLRs results in the activation of dendritic cells leading to the production of cytokines (interleukins), indicating a role for these receptors in adaptive immunity as well as innate immunity. The innate immune system is known to rely on receptors encoded by the germline in the detection of pathogenic or microbial stimuli that takes place during the processes involving cellular stress. All

receptors of the TLR family are characterised by containing an extracellular amino-terminal (-NH_2) domain which comprises a number of leucine rich repeats, as well as a globular cytoplasmic domain termed the Toll/interleukin 1 receptor (or TIR) domain. These TLRs can either recognize their specific ligands on the cell surface (E.g. TLRs 1, 2, 4, 5, 6, 10 and 11) or intracellularly (TLRs 3, 7, 8,and 9); with TLRs located at the cell surface being able to recognise microbial cell wall components such as lipopolysaccharides (LPS). In contrast, TLRs can also be localised to intracellular membranes (phagosomes and endosomes) where they can recognise viral or microbial nucleic acids (ligands) such as dsRNA and ssRNA (Figure 1.5).

In response to the activation of Toll-Like Receptors (TLRs) by their specific molecules or agonists, the activation of the downstream signal transduction pathway is largely dependent on a number of signalling and adaptor proteins. Upon activation of the majority of TLRs (with the exception of TLR3 and TLR4), the initial interaction event is with the Toll interleukin1 receptor (TIR) -containing adaptor molecule (Figure 1.5), myeloid differentiation factor (Myd88) (Akira et al., 2001). This has been shown by the fact that Myd88-deficient mice (Myd88-/-) are unable to produce inflammatory cytokines, including IL12 and TNF-α, which are required in the production of a downstream immune response (Akira and Hoshino, 2003). These Myd88-deficient (KO) mice are more susceptible to infection by various mycobacterial infections, such as *M. tb* (Scanga et al., 2004). The activation of Myd88 in turn leads to the induction of a downstream signalling pathway including the association with members of the IL-1 receptor-associated kinase-1 (IRAK) family, such as IRAK-1 and IRAK-4 (Figure 1.5). This will lead to activation of the TNF Receptor-associated Factor (TRAF6) and subsequently the association with the transcription factor Nuclear Factor Kappa-B (NF-κB), which is able to translocate into the cytoplasm which causes the increased production of cytokines and chemokines (Suzuki et al., 2002, Akira and Takeda, 2004). This complex TLR signal transduction therefore causes the initiation of various intracellular signalling pathways which results in the activation of the innate immune response (Figure 1.6), as well as modulating the adaptive immune response (Quesniaux *et al.*, 2004).

Figure 1-5. Toll–like receptors and their ligands and an overview of major TLR signalling pathways (Quesniaux *et al.*, 2004).

With regards to signalling via Toll-Like Receptors 3 and 4, upon activation these require the TIR domain-containing adaptor-inducing interferon-β (TRIF) and/or the TRIF/TRIF-related adaptor molecule (Quesniaux et al, 2004). These pathways are crucial in the activation and maturation of antigen presenting cells (APCs) and dendritic cells as well as the subsequent activation of IRF-3 (IFN-regulated factor3). This causes the late activation of the transcription factor, NF-κB, as well as the increased production of IFN- β. Therefore, these key adaptor proteins, along with TLRs, play a key role in the induction of innate immunity. These TLR agonists will be described in more detail in Section 1.6.

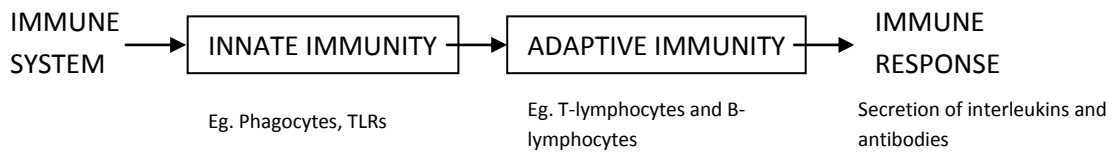


Figure 1-6. The human immune system consists of two separate barriers in order to respond to foreign pathogen or antigen.

1.4. The importance of particulate delivery systems for vaccine delivery

Most of the vaccines which are currently available are based on live organisms, due to their strong potency. In most cases, these vaccines are safe and effective but they also have associated risks and cause adverse symptoms (Perrie *et al.*, 2007).

In terms of an increased safety profile, vaccines can be administered as synthetic peptides or sub-unit proteins (Perrie *et al.*, 2008). Adjuvants are substances or formulations which can non-specifically enhance or modify the immune response to a specific antigen. Many typical adjuvants which are currently in use are particulate-based. These particulates are passively targeted to antigen presenting cells (APCs) and have specific ability to provide persistent antigen due to slow degradation, due to association within the particulate delivery system. Examples of these delivery systems include mineral salts (aluminium-based adjuvants), saponins, liposomes, cationic lipid-based systems, niosomes (non-ionic surfactants), virosomes, immunostimulatory complexes (ISCOMs), polymer-based systems (E.g. PLGA microspheres), emulsions and combination systems.

Formulation of antigens in particulate delivery systems leads to a number of advantages including antigen protection against degradation, uptake by antigen presenting cells (APCs), as well as the co-delivery of antigen and adjuvant (Perrie *et al.*, 2008). This co-delivery of both of these components to the same APC, therefore can direct the specific immune response generated. These APCs (dendritic cells and macrophages) are able to capture pathogens, pathogen-associated molecular patterns (PAMPs) contained on TLR agonists and particulate material by phagocytosis therefore being able to process antigens for presentation as described previously in Section 1.3 (Figure 1.4).

1.4.1. Mechanisms of adjuvant action by particulate delivery systems

Adjuvants (coming from the latin word *adjuvare*, meaning to help or aid) can be described as particles or molecules which can non-specifically enhance the immune response. Adjuvants, when used as substances in combination with antigen are able to produce more robust immune responses than antigen alone. Schijns et al (Schijns, 2000) have displayed the most common grouping for the classification of adjuvants, which is based on the five concepts of immunogenicity shown by Storni et al (2005) and in Figure 1.7 (Henriksen-Lacey *et al.*, 2011c).

A

1. the geographical concept of immune reactivity;
2. the theory of depot effect (emphasising the importance of antigen localisation);
3. the paradigm that adjuvants act as Signal 0, which precedes the induction of the epitope Signal 1 and co-stimulatory Signal 2;
4. the role of Signal 2 molecules as natural adjuvants in the activation of naıve T-helper cells which co-subsequently co-ordinate T-cell dependent immune responses;
5. the hypothesis that immunity is activated by exogenous and eventually endogenous danger signals.

B

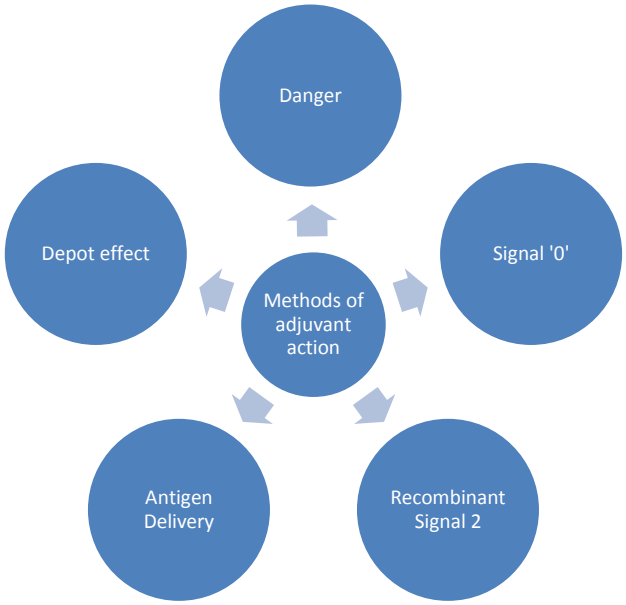


Figure 1-7. Concepts of immunogenicity (A) and methods of adjuvant action (B) by liposomes and particulate-based delivery systems, as explained by Storni et al, 2005.

The process of immunisation has been shown to partly depend upon antigen reaching various lymphoid organs, and this takes place so that antigen-loaded APCs can interact with T-cells in order to provide signal 2 (Zinkernagel *et al.*, 1997). This mode of adjuvant action has been demonstrated to enhance the movement of antigen from the site of injection (SOI) towards the draining lymphatics (nearest to where the immunisation was administered). This enables sufficient amount of antigen to be delivered to the lymphoid tissue (Nordly *et al.*, 2009) in order to mount an extensive immune response. The use of various particulate delivery systems can increase the mode of adjuvant action, as these systems are increasingly recognised by APCs. These adjuvant delivery systems can also function by causing the up-regulation of co-stimulatory molecules on the surface of APCs. Therefore, this will lead to the delivery of signal 1 and signal 2 at the same time, thus causing the production of

inflammatory cytokines and the generation of an enhanced immune response [Figure 1.4; (Schijns, 2000)]. These adjuvants and particulate delivery systems can, therefore, induce additional characteristics to an antigen in order to generate a sufficient immune response based on these concepts of immunogenicity ((Figure 1.7; (Storni *et al.*, 2005)).

The uptake of liposomes, as well as the associated antigen (either surface adsorbed or entrapped), by dendritic cells leads to the processing of pathogen-derived antigen and selection of T-cell clones *in vivo* (Steinman, 2008b, a). This processing step results in the breakdown of protein antigen into smaller peptide fragments (Schijns and Lavelle, 2011). This allows the efficient formation of peptide-MHC complexes at the cell surface, before these dendritic cells migrate to the lymphoid tissue and immune organs in order to join an immune cell network of dendritic cells (Steinman, 2007) by continuously probing the local environment. These dendritic cells (DCs) are located at the most commonly-used injection sites (i.m and s.c) and can scan the local area and follow chemotactic stimuli in order to be on alert to the presence of extracellular material and injected antigen (Schijns and Lavelle, 2011).

Particulate vaccines (associated with antigen) have a significant advantage over soluble (free) antigen due to their increased uptake by antigen-presenting cells (APCs) and their increased ability to 'mimic' pathogenic material. The uptake of particulate-based vaccine delivery systems will ideally follow the same route as the pathogen against which you are vaccinating; initially via phagocytosis to create an early-organelle termed the phagosome which enable and are important in the cross-presentation of antigen (Ackerman *et al.*, 2003). Further intracellular vesicles are involved in the processing of these particulate systems, with phagosome maturation leading to the fusion with the endosome which activates the formation of a phagolysosome. This sequential processing of antigen-loaded particulate delivery systems leads to the acquisition of increasing degradative conditions, which leads to the generation and subsequent presentation of antigen.

The interaction of antigen-bearing dendritic cells (containing liposomes) leads to the interaction of the peptide-MHC complex with T-cells (T-helper cells) at the lymphoid tissue which is referred to as 'signal 1'. When antigen is delivered to the lymphoid tissue this will lead to a selected T-cell response, including an increase in the clonal expansion of a T-cell population (Hawiger *et al.*, 2001), which allows these T-cells to acquire their specific function. These T-cells have the ability to acquire memory (production of memory T-cells), so that upon reinfection with pathogen-derived antigen, these immune cells can produce an effective immune response. The complete activation of T-cells

requires the induction of a second signal event (termed signal 2), which is provided by the release and recognition of costimulatory molecules (such as CD80/86) on APC.

Another important group of molecules involved in the activation of the immune response are chemokines. These chemokines act as stimuli for activation of inflammatory cells, such as macrophages and monocytes, with these chemoattractive molecules being involved in the regulation of immune cell traffic and the interaction of these antigen presenting cells (APCs) with T-lymphocyes in secondary lymphoid organs and tissues (Sallusto et al., 1999a, Sallusto et al., 1999b, Moser, 2003). Therefore, the release of chemokines leads to the attraction of dendritic cells which constantly monitor their environment for any changes in the levels of these stimuli (Yan *et al.*, 2007). A recent study by Yan et al (2007), has investigated the premise of the use of liposomes of varying charge and composition for the induction of chemokine activation in the activation of the immune response. These studies have shown that positively-charged liposomes, including DOTAP, leads to increased production and levels of the CCL-2 chemokine (Yan *et al.*, 2007).

1.4.2. The use of liposomes as drug delivery systems and vaccine adjuvants

Liposomes are closed spherical vesicles consisting of an aqueous inner core surrounded by one or more concentrically arranged bilayer membranes (Perrie and Rades, 2010). In general, phospholipids are used to formulate these liposomes. Lipids from this group can be derived from natural and synthetic sources. Phospholipids are amphiphilic in nature, in the fact that they have a polar (hydrophilic) head group and a hydrophobic tail. The polar head group that is present in these lipids confers the surface charge of the liposomes (for example, phosphoserine and phosphatidylcholine are anionic and neutral in charge respectively). The phospholipid tail is also very important in the formation, as well as the packing and assembly of these liposomes (Figure 1.8). Liposomes are an ideal platform for drug delivery systems and vaccine adjuvants due to their versatility and flexibility in formulation, therefore allowing the incorporation of a large range of drugs and immunogenic molecules. A list of commercially available liposomal-based drugs available on the market is summarised in Table 1.2.

Liposomes were the first delivery system to be described as being able to act as immunological adjuvants (Allison and Gregoriadis, 1974). These vesicles have been extensively tested and utilised as effective delivery systems for a wide variety of disease antigens (Gregoriadis et al., 1987, Christensen et al., 2007b). The ability of liposomes to act as adjuvants for protein-based vaccines (using subunit

antigens) can occur due to the incorporation (Perrie and Gregoriadis, 2000) or association (May et al., 2000, McNeil et al., 2010) of protein antigen into the liposome membrane or bilayer. These liposomes can function as adjuvants due to their specific ability to fuse with the cell membrane of APCs in order to deliver vaccines.

Liposomes are an ideal vaccine delivery system due to their particulate nature, versatility and flexibility in formulation and it is these parameters that can be used to promote a range of immune responses (Foged *et al.*, 2004). This is due to the ability of liposomes as delivery systems to render soluble antigen into a particulate form, thereby leading to the lengthening of their *in vivo* retention (Henriksen-Lacey *et al.*, 2011c). The surface charge of liposomes has also been shown to have a positive effect on the immune response, with cationic liposomes having an advantage over their neutral and anionic counterparts (Henriksen-Lacey et al., 2011c). The importance of charge, antigen isoelectric point (pI) and buffer pH for liposomes offers an important platform for antigen adsorption due to electrostatic interaction between oppositely charged liposome and antigen components.

Figure 1-8. Liposome constructs: when dispersed in an aqueous phase, lipids can form bilayer vesicles which can be prepared with varying physicochemical characteristics, including size (Perrie *et al.*, 2013).

Table 1.2. Liposome-based drugs currently on the market

Liposome Formulation	Drug Name	Company	Target disease	Delivery Route
Liposomal Amphotericin B	Abelcet	Enzon Pharmaceutical	Fungal infection	i.v
Liposomal Amphotericin B	AmBisome	Gilead Sciences	Fungal/protozoal infection	i.v
Liposomal Cytorabine	DepoCyt	Skye Pharma/Enzon Pharmaceutical	Malignant lymphomatous meningitis	Spinal injection
Liposomal Daunorubicin	DaunoXome	Galen	HIV-related Kaposi's Sarcoma	i.v
Liposomal Doxorubicin	Myocet	Zeneus Pharma	Metastatic breast cancer	i.v
Liposomal IRIV Vaccine	Epaxal	Berna Biotech	Hepatitis A	i.m
Liposomal IRIV Vaccine	Inflexal V	Crucell	Influenza	i.m or s.c.
Liposomal Morphine	DepoDur	Pacira Pharmaceuticals/Skye Pharma	Post-surgical analgesia	Epidural
Liposomal Verteporfin	Visudyne	Novartis	Macular degeneration	i.v
Liposome PEG-Doxorubicin	Doxil	Schering Plough, Ortho Biotech	HIV-related Kaposi's Sarcoma, Breast & Ovarian Cancers	i.v

Liposomes have been shown to partly act through a mechanism of action termed the ‘depot effect’ and they act through this mechanism due to their retention at the site of injection, which leads to the sustained release of antigen to the immune cells at this site. This release of antigen can then drain to the lymph nodes in order to activate APCs and initiate T-cells to activate a Th1 cell-mediated immune response. This ‘depot effect’ has been shown to be the mechanism of action for cationic liposomes (Vangasseri et al., 2006, Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2010c, 2011a). The liposome composition, such as the lipid head-group structure and membrane fluidity, as well as the cationic surface charge have been shown to play an important role in the retention of vaccine at the site of injection in order to initiate a vaccine-specific immune response. This has been shown using various cationic lipids, including dimethyldioctadecylammonium (DDA) bromide, 1,2-dioleoyl-3-trimetylammonium propane (DOTAP) and 3β-[N-(N-N-dimetylaminoethane)carbomyl] cholesterol (DC-Chol) in combination with the immunostimulatory glycolipid trehalose 6,6’-dibehenate (TDB).

1.4.3. The promise of cationic adjuvant formulations

The immunological properties of cationic lipids has been described since 1966 when Gall and colleagues (Gall, 1966) described the lipid, dimethyldioctadecylammonium (DDA) bromide to have these properties. The adjuvant properties of cationic lipids, including DDA, has been shown to be due to the presence of quaternary ammonium ion head groups and long alkyl chains, which enables the formation of liposomes upon exposure to an aqueous buffer environment.

DDA is a cationic surfactant which has the ability to promote a weak Th1 immune response (Weinrich Olsen *et al.*, 2001). DDA contains a quaternary ammonium group and two methyl groups which make up the polar head group. This lipid also has two carbon-alkyl chains (18 carbons in length) which make up the hydrophobic tail region of the lipid. Although liposomes based on solely DDA alone are effective adjuvants for the generation of humoural and cell-mediated immune responses, these liposomes are physically unstable, which has limited their development as adjuvant delivery systems (Davidsen *et al.*, 2005).

This limitation of these liposomes can be substantially improved by the addition of immunostimulatory molecules within these cationic delivery systems, such as trehalose 6,6' – dibehenate (TDB). This inclusion of TDB into DDA-based liposomes leads to an increasingly stable (Christensen et al., 2007a, Christensen et al., 2009) and efficient adjuvant delivery system, which has the advantage of greatly improving the Th1-mediated cell immune response and the antibody response (Davidsen *et al.*, 2005). The enhanced stability of this cationic adjuvant formulation (CAF01) is caused by the interaction of the glucose headgroups of TDB with the surrounding aqueous buffer which is used to hydrate the liposomes (Christensen et al., 2007a, Christensen et al., 2008).

These cationic-based liposomes have a selective advantage over other particulate delivery systems, such as niosomes (Vangala *et al.*, 2006) and PLGA microspheres (Kirby *et al.*, 2008a) which shows the importance of cationic liposomes compared to other delivery systems. The positive head group of DDA confers the immunogenic properties of this formulation, with these liposomes able to target the negatively-charged cell membrane of APCs which leads to the enhanced uptake of protein antigen (Korsholm *et al.*, 2007). The incorporation of TDB into DDA-based liposomes confers an increased immune response against various sub-unit vaccine antigens due to the binding of TDB to a C-type Lectin Mincle Receptor (Ishikawa et al., 2009, Werninghaus et al., 2009, Schoenen et al., 2010) which can activate the TLR-independent FcR-Syk-Card9 pathway in APCs in order to cause a sufficient innate immune response, characterised by protective Th1- and Th17-immunity.

Due to the successful passage of cationic DDA/TDB liposomes into clinical trials (NCT 00922363), in combination with the tuberculosis vaccine antigen, Ag85B-ESAT6 (H1), this adjuvant delivery system has been combined with antigens against a variety of other disease models. This subunit vaccine approach builds on the concept of boosting the immune response to these selected antigens in the form of a recombinant antigen, such as Ag85B-ESAT6 (Holten-Andersen *et al.*, 2004). These subunit vaccines offer great advantages such as safety and quality control when compared to vaccines based on live or attenuated microbes, but require to be delivered within effective delivery systems in order to exert their effect on the immune response. Research studies by Vangala et al (Vangala et al., 2006, Vangala et al., 2007) investigated the potential and efficacy of this liposome delivery system in combination with the hepatitis B surface antigen (HbsAg) or malarial antigens (MSP1 and GLURP). These studies showed that these adjuvants had the ability to increase antigen-specific splenocyte proliferation and T-cell responses characterised by high production of interferon-gamma (IFN-γ) in order to generate an efficient cell-mediated immune response.

The trivalent influenza vaccine (Vaxigrip; Sanofi Pasteur MSD), when administered with DDA/TDB, is able to generate an efficient cell-mediated immune response (characterised by the production of interferon-γ (IFN- γ), when delivered by the intranasal (i.n.) route, rather than without adjuvant alone. During this research using an *in vitro* study with the Calu-3 cell culture model has shown that CAF01 is able to increase the permeability through the ciliated mucus layer and the epithelium (Christensen *et al.*, 2010). More recently, CAF01 has been developed and optimised as a dry powder vaccine formulation which can be administered via spray drying (Vehring, 2008, Ingvarsson et al., 2013). The development of a dried powder vaccine formulation is advantageous due to enhanced storage stability and the ability upon rehydration for original particle characteristics to be maintained. These studies have shown that upon immunisation with the spray-dried CAF01 with associated H1 TB antigen (in combination with the stabiliser, trehalose) results in the preservation *in vivo* of the adjuvant activity of this vaccine.

1.5. Further particulate delivery systems for use in vaccine formulation

1.5.1. Aluminium-based adjuvants

To date the most common and successful adjuvants in vaccine development are aluminium-based mineral salts (alum) which have a proven track record in terms of their safety profile and use in vaccines, however these adjuvants are only capable of producing humoural immune responses (Brewer, 2006, Christensen et al., 2007b). Aluminium compounds were originally identified as adjuvants around 80-90 years ago (Glenny et al, 1926), and remain one of the major adjuvants in vaccine design. It is widely accepted that physical association between both aluminium and antigen components is required for these vaccines to retain their adjuvanticity (Brewer, 2006).

The continued development of aluminium-based compounds for use in vaccines has been restricted by these adjuvants lacking the ability to promote a cell-mediated immune (CMI) response. These adjuvants have the ability to stimulate Th2 immune response and hence the production of IL-4 and IL-5 cytokines, as well as B-cell production of antibodies. Therefore, these aluminium-based adjuvants are unable to be used in vaccines against diseases such as TB, malaria and HIV which primarily require a Th1-immune response (Seder and Hill, 2000). Until recently, the immunostimulatory activity of alum-adjuvants was attributed due to its ability to retain antigen at the injection site (Glenny *et al.*, 1926). However in the recent literature, several studies have challenged this theory, reasoning that Alum is likely to elicit Th2 responses independent of a depot formation at the injection site (Romero Mendez et al., 2007, Noe et al., 2010, Hutchison et al., 2012).

1.5.2. Niosomes and virosomes

Non-ionic surfactant vesicles (or niosomes) are potentially a more stable delivery vesicles compared to liposomes, with advantages including their relative chemical stability, low toxicity (Brewer and Alexander, 1992) and reduced costs. These factors make niosomes an attractive delivery system for antigenic components. The most commonly investigated niosome vesicles consists of monopalmitoyl glycerol, cholesterol and dicetyl phosphate (Baillie *et al.*, 1985), with these delivery systems combined with antigen being able to provide higher production of cytokines, such as IFN-γ. Virosomes are composed of naturally occurring phospholipids (70 %) and phospholipids originating from the influenza virus (30 %; (Perrie *et al.*, 2007)). These vesicles have been used as systems for the delivery of entrapped antigen against infections such as Hepatitis B and Influenza.

Following oral vaccine administration, liposomes and niosomes can be destabilised due to the presence of intestinal bile salts in the gastrointestinal (GI) tract which leads to subsequent disruption of the membrane causing release of macromolecules or antigen prior to reaching the intended site of action. Incorporation of bile salts into the vesicle bilayer membrane has been shown to protect liposomes (and associated antigen) to digestion by enzymes present in the GI tract (Schubert et al., 1983, Shukla et al., 2008, Wilkhu et al., 2011). Oral vaccines can be targetted to specialised M-cells which are located in the follicle-associated epithelium of intestinal Peyer's patches for uptake of particulate vaccine antigen in order to improve immune responses (Brayden and Baird, 2004, Brayden et al., 2005, Wilkhu et al., 2011).

1.5.3. Microparticles

Microparticles also have the potential to be used as delivery vesicles with microspheres consisting of polylactides, polyglycolides and their copolymers (PLGA) being used in the field of drug and vaccine delivery for the delivery of promising sub-unit vaccines such as Ag85B-ESAT6 (Kirby et al., 2008a, b). Although the microsphere formulations induced a cell-mediated immune response, this was reduced in comparison to the same DDA/TDB formulation prepared as a liposome adjuvant system (Kirby *et al.*, 2008b).

1.5.4. Immune-stimulating complexes (ISCOMs)

ISCOMs are particulate delivery systems, closely related to liposomes, which consist of phospholipids and cholesterol as well as a built-in immuostimulatory saponin vaccine adjuvant (Morein et al., 1984, Nordly et al., 2009). Therefore this combination within a delivery system is enough to reduce the cytotoxic effect associated with the saponin adjuvant. The incorporation of antigen, including tumour and viral antigens, within these spherical hollow assemblies is powerful and can induce both cellular and humoural immune responses (Cox and Coulter, 1997, Cox et al., 1998). The mechanism of antigen association within these systems is by insertion into the membrane bilayer or entrapment within the structure itself.

1.5.5. Oil in Water Emulsions

The successfully licensed oil in water emulsion (o/w) termed MF59 was licensed in the late 1990's in the influenza vaccine (Novartis, Italy) Fluad® (O'Hagan and De Gregorio, 2009). MF59 is composed of 5 % squalene (oil) combined with two surfactants, sorbitan trioleate and polyoxyethylenesorbitan monooleate. This adjuvant has been shown to have an enhanced safety profile in humans. Mosca and colleagues have noted that MF59 induces cell recruitment to the injection site, renders soluble antigen to a particulate form and enhances the trafficking of antigen to the draining lymph nodes (Mosca *et al.*, 2008). This also leads to increased antigen uptake by dendritic cells (Dupuis *et al.*, 2001).

1.5.6. Further particulate systems

Another promising liposome formulation contains the TLR4 ligand MPL and QS21 (a derivative of saponin), and is termed AS01B (GlaxoSmithKilne). This liposome contains the TB vaccine antigen Mtb72F (fusion protein), which comprises the Rv1196 and Rv0125 antigens. This vaccine has been shown to enhance the immune response, and can be used to boost the BCG vaccine (Brandt *et al.*, 2004). A modified heat-labile toxin derived from *E. coli* in combination with the Ag85B-ESAT6 fusion protein has been developed for nasal immunisation, called LTK63. This formulation has led to a significant immune response, with marked increases in IgG2 Ag85B-ESAT6 specific antibody titres (Palma *et al.*, 2008).

1.5.7. Currently licensed adjuvants used in vaccine formulation

To date there are only a relatively small number of licensed adjuvant systems, which include a number of aluminium-based systems such as aluminium hydroxide or phosphate (Perrie et al., 2008) which have been used in vaccine formulations for Tetanus and Hepatitis B. The adjuvants have been demonstrated to have a good track record in terms of safety (Clements and Griffiths, 2002).

More recently licensed adjuvant systems (AS) include AS03 and AS04, which have been developed by GlaxoSmithKline (Garcon et al., 2007, Henriksen-Lacey et al., 2011c). The adjuvant system AS04 is composed of aluminium hydroxide in combination with the TLR4 agonist monophosphoryl lipid A

(MPL) and is currently in vaccines for hepatitis B and human papillomavirus (HPV). Whereas, AS03 is similar to the MF59 adjuvant, consisting of a squalene oil-in-water emulsion (w/o) and is included in an influenza vaccine (Pandemrix). However, disease targets such as TB, HIV and malaria require the strong induction of Th1 immune responses; whereas these currently licensed adjuvants lack the ability to produce these effects on the immune response.

1.6. Investigation into the combination of TLR agonists within vaccine formulations

The addition of TLR agonists into liposome-based vaccine formulations results in the increase in immunostimulatory profile of these particulate delivery systems in order to cause a more diverse immune response. This has been of increased interest in the past several years with a number of experimental liposome-based vaccine formulations being tested (Nordly et al., 2011b, Nordly et al., 2011c). Adjuvants are substances and molecules which can directly enhance the immune response and these can be split into two distinct groups, vaccine delivery systems and immunopotentiators (Nordly *et al.*, 2009). The delivery system ensures the efficient delivery of antigen to antigen presenting cells (including macrophages), whereas immunopotentiators (such as TLR agonists) are able to activate the various cells of the immune system, through various intracellular pathways. The flexibility and versatility of liposomes allows these vesicles to be used as delivery systems, in combination with antigen, and also for the inclusion of immunostimulatory molecules into the membrane bilayer. Thus, the inclusion of these molecules into a liposome formulation will be of increased interest in the synergistic presentation of antigen, as well as immunopotentiators to the same antigen presenting cell (APC), and hence broaden the immune profile of the vaccine formulation in question. An alternative approach may also be to physically link antigen with TLR agonist (for example, flagellin) in order to provide synergistic delivery to the same APC (Guy, 2007, Eckl-Dorna and Batista, 2009). Of the candidate TLR agonists, a number have been used in liposome vaccine formulations including Monophosphoryl Lipid A (MPL), unmethylated CpG (cytosine-phosphate-guanine) DNA, polyinosinic-polycytidylic acid [poly(I:C)], di- and triacylated lipopeptides, lipopolysaccharides (LPS) and imidazoquinolines (Figure 1.5). The combination of TLR agonist and adjuvant delivery vesicles (liposomes) is a novel prospect in the field of drug delivery and during the past decade a number of these combinations have been tested, in terms of their ability to deliver vaccine formulations which are known to engage TLR signalling. These combination systems will be reviewed in the following section in relation to the production of an appropriate immune response.

1.6.1. Monophosphoryl Lipid A (MPL)

MPL is a synthetic and slightly modified version of the lipopolysaccharides (lipid A) derived from the cell surface of Gram-negative bacteria. MPL acts through the TLR4 agonist located at the cell membrane which results in the activation of downstream intracellular pathways leading to the transcription of co-stimulatory molecules and cytokines. MPL has been licensed in a number of adjuvant systems by GlaxoSmithKline, in which this TLR4 agonist is combined with a secondary adjuvant such as liposomes (AS01), QS21 (AS02) and aluminium hydroxide (AS04).Therefore the ability of these adjuvants to act through TLRs demonstrates that these adjuvants can act as pathogens therefore leading to the initiation of the immune response.

The inclusion of MPL within the cationic liposome formulation, DDA/TDB, results in a significant decrease in the main phase transition temperature for this delivery system, as well as leading to an increase in surface pressure at the final collapse point (when the monolayer was fully compressed) and reduction in the mean molecular area. These factors demonstrated that this TLR4 agonist was inserting into the bilayer membrane of the liposome delivery system, therefore affecting the packing properties of the membrane (Nordly *et al.*, 2011a). The inclusion of MPL within the delivery system also resulted in an enhanced antigen-specific immune response, as shown by a higher population of CD8+ T-cells. This increased T-cell population led to a significant increase in the production of the cytokine interferon-gamma (IFN-γ), so this liposome formulation can activate a number of signalling pathways in the production of a Th1 immune response (Nordly *et al.*, 2011a).

The development of new sub-unit protein vaccines against diseases such as TB has been an urgent research priority in the past decade. With this in mind, a number of adjuvants were developed including the liposome formulation, DDA/MPL. The cationic surfactant, DDA, alone achieves low levels of immunity (as originally described by Gall et al in 1966) but these adjuvants inherently lack stability in terms of their characteristics (Davidsen *et al.*, 2005). The inclusion of the TLR4 ligand monophosphoryl lipid A (MPL) within this liposome formulation and combined with TB subunit antigen is able to initiate significant levels of immunity characterised by a stable deposition of antigen and a strong Th1 immune response characterised by production of interferon-gamma (Holten-Andersen *et al.*, 2004).

1.6.2. Polyinosinic:polycytidylic acid (polyI:C)

The combination of liposome delivery systems (DDA/TDB) with the CD8-inducing immunopotentiator, polyI:C, has been investigated in a number of research studies in the literature (Schlosser et al., 2008, Nordly et al., 2011c, Milicic et al., 2012). These studies have demonstrated the inclusion of polyI:C within the liposome membrane due to electrostatic binding to cationic lipid head groups, as well as resulting in a monolayer expansion of DDA/TDB. The inclusion of this agonist within the liposome formulation (using OVA as model antigen and C57/BL6 as mouse strain) results in an enhanced immune response (as shown by a CD8+ T-cell response), due to the synergistic delivery of both antigen and immunopotentiator to the same antigen presenting cell, which leads to the activation of further intracellular signalling pathways within the APC causing a more broad immune profile of the liposome formulation. Recent studies within our laboratory have demonstrated the immunostimulatory ability of polyI:C following incorporation within liposome vaccine adjuvants (Milicic *et al.*, 2012). Addition of polyI:C significantly increased the adjuvant capability of DDA/TDB liposomes formed as multilamellar vesicles (MLVs) and antigen-entrapping dehydration rehydration vesicles (DRVs). However, DDA/TDB formed as small unilamellar vesicles (SUVs) offered enhanced adjuvant capacity without the addition of TLR agonists to the system.

1.6.3. Unmethylated CpG DNA

The ability of adjuvants to initiate a CD8+ T-cell response is crucial in the field of vaccine immunology. An example of an agonist is unmethylated CpG-containing oligodeoxynucleotides (CpG ODN) which is able to activate TLR9 located in the cytoplasmic compartment of the cell. This TLR9 agonist contains regions and repeats of nucleotides of DNA with a cytosine next to guanine separated by only one phosphate (which is part of the phosphodiester backbone of DNA). The ability of CpG ODN and its use as a vaccine adjuvant has been shown through the activation of natural killer (NK) T-cells, which in turn leads to the production of type 1 interferons (Suzuki *et al.*, 2004). Therefore, the optimisation of cationic adjuvant formulations by the inclusion of CpG-containing ODN in the formulation is a viable option as a vaccine adjuvant with the possibility of increasing CD8+ T-cell responses (Gursel *et al.*, 2001).

1.6.4. Imidazoquinolines

During the early 1980's, effort was going into the identification of small molecules which were able to inhibit infection by herpes simplex virus-2 (HSV-2) (Bernstein and Harrison, 1989, Miller et al., 1999, Wagner et al., 1999). This led to the identification of a family of low molecular weight molecules including imiquimod, resiquimod and gardiquimod (Figure 1.9) which have been shown to activate dendritic cells (DCs) therefore leading to the secretion and production of cytokines and chemokines (IFN-γ, IL-6, IL-18 and IL-10) (Wagner *et al.*, 1999). These imidazoquinoloine molecules are also able to activate B-cells to proliferate and differentiate into antibody-secreting cells therefore leading to enhanced antibody production (Bishop et al., 2000, Tomai et al., 2000). This family of molecules are able to activate both Toll-like receptor (TLR) 7 and 8 agonists leading to the skewing of immunity towards a Th1 immune response (Jurk *et al.*, 2002). These molecules present in the imidazoquinolines are termed synthetic immune response modifiers (IRM) due to their ability to selectively activate these specific TLRs. These TLRs are located within the endosomal compartment of the cell and are able to recognise guanosine- and uridine-rich single stranded (ss)RNA (Diebold et al., 2004, Heil et al., 2004, Gorden et al., 2006) as well as IRM such as imidazoquinolines. Therefore activation of these TLRs occurs at the endosome, which has been shown to be due to activation of these TLRs (by imiquimod and resiquimod), leading to endosomal acidification and downstream events, leading to the activation of the specific immune response (Tomai *et al.*, 2007). This has been further demonstrated by agents that are able to block acidification of endosomes and hence block the subsequent activation of the immune response (Hart *et al.*, 2005).

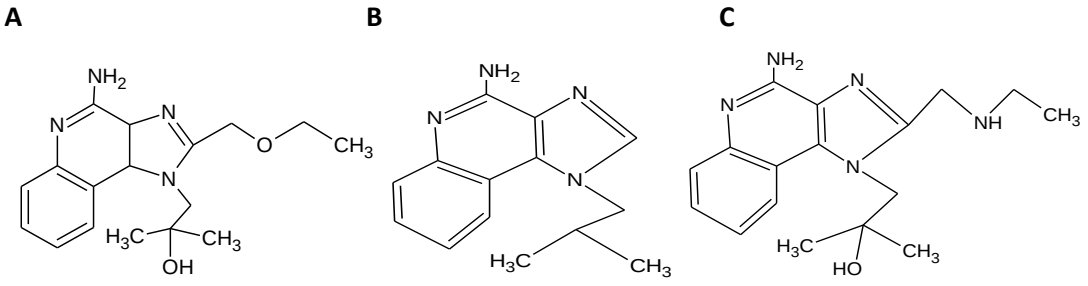


Figure 1-9. Members of the imidazoquinoline family of Toll-like receptor (TLR) agonists [A- Resiquimod; B – Imiquimod; C- Gardiquimod)

Even though these Toll-like receptors are closely related they seem to be activated to a different degree by their natural ssRNA ligands as well as by IRMs in which they stimulate the human forms of TLR7 and TLR8, as well as the mouse TLR7 but not mouse TLR8. This is due to TLR8 in mice being

non-functional as has been shown by its response to ssRNA and immune response modifiers that are usually activated through TLR8 (Hemmi et al., 2002, Jurk et al., 2002, Gorden et al., 2006).

The first member of the imidazoquinoline family to become commercially available was imiquimod in 1997, which has generally been used in the form of a cream (ALDARA™) for treatment against anogenital warts (caused by human papilloma virus infection) and actinic keratosis (Abramovits and Gupta, 2004, Swanson et al., 2010). These clinical studies have shown that topical application of both imiquimod and resiquimod and their synthetic analogues is a safe and effective method in which to locally activate the immune response. This kind of treatment can be considered as a form of endogenous vaccination in which the virus acts as the antigen and the TLR agonists act to boost the antigen-specific immune response (Tomai *et al.*, 2007).

However, in subsequent studies another member of the imidazoquinoline family of molecules, resiquimod (or R848), has been discovered to be a more potent and soluble analogue which is able to produce an increased cytokine response compared to imiquimod (Tomai *et al.*, 1995). Resiquimod is able to exert its immunostimulatory activity via activation of mouse TLR7 and human TLRs 7 and 8. Therefore resiquimod has shown great promise as a vaccine adjuvant and has been included in further vaccine formulations. The adjuvant activity of these TLR agonists has also been studied by vaccination in animal models in which imiquimod has been used in combination with herpes simplex virus (HSV) glycoprotein, which has led to increased levels of response to HSV challenge (Bernstein *et al.*, 1993), both prophylactically and therapeutically. The resiquimod TLR7 agonist has also been used in animal mouse models in combination with viral antigen in order to show enhanced T-cell and antibody responses (Otero et al., 2004, Wille-Reece et al., 2005).

Resiquimod has been investigated as a vaccine adjuvant in initial studies (Vasilakos *et al.*, 2000) using ovalbumin (OVA) as a model protein antigen. This was shown to lead to enhanced Th1 antibody (IgG2) and cytokine (IFN-γ) production, as well as inhibition of Th2 antibody and cytokine production (IgE and IL5). These studies also showed that secondary immunisation of this vaccine (resiquimod, in combination with model antigen OVA) in combination with the alum delivery system leads to a more effective primary memory response, thus showing the importance of a delivery system for retaining both TLR agonist and antigen. In further studies resiquimod has been compared with the TLR9 agonist CpG ODN DNA for their ability to act as vaccine adjuvants. Both of these TLR agonists (in combination with the hepatitis B surface antigen) were experimentally tested in vaccine formulations in mice with CpG ODN giving a superior immune response to its resiquimod alternative. This was hypothesised to be due to resiquimod having a smaller molecular weight (M_w) than CpG ODN, therefore upon vaccination it will more likely be distributed more quickly throughout the body

rather than remain at the vaccination site, whereas CpG ODN is more likely to remain at this site (Weeratna et al., 2005, McCluskie et al., 2006).

Although resiquimod and other analogues (which activate through TLR7 and/or TLR8) have been demonstrated as effective vaccine adjuvants in a number of model systems, several other studies have given mixed success in their ability to act as vaccine adjuvants (Wille-Reece *et al.*, 2005). The short half-life, in combination with small size and high water solubility, may lead to the rapid distribution throughout the body rather than staying at the injection site which is not ideal for the local activation of dendritic cells at the injection site. Therefore, these TLR7/8 agonists may need to be colocalised with other stimuli (antigen) at the injection site in order to offer enhanced immunity (Ahonen *et al.*, 2004). Therefore for optimal vaccine adjuvant formulation it may be required to retain the TLR agonist at the site of injection in order to induce local adjuvant effects without inducing systemic cytokines which may give side effects. This could be achieved by formulating the agonist within a delivery system such as liposomes.

1.6.5. Lipopolysaccharides

The bacterium *Mycobacterium tuberculosis* is able to regulate APCs due to TLR2 signalling. This occurs via lipoproteins which are present in the cell wall of this bacterium, including LpqH, LprA and PhoS1. These lipoproteins are able to regulate the functions of APCs, therefore leading to downstream cytokine production and further innate immune responses (Drage *et al.*, 2009). During these responses TLR2 either associates with TLR1 or TLR6 in order to form a heterodimer, therefore leading to these downstream signalling effects. Both of these heterodimers can bind to different forms of lipid from the mycobacterial cell wall, with TLR1 leading to the recognition of triacylated peptides whilst TLR6 leads to diacyl peptide recognition (Schroder et al., 2004, Buwitt-Beckmann et al., 2005, Kang et al., 2009).

1.6.6. Flagellin

Flagellin is present in the flagellar structure of many bacteria (Gram positive and Gram negative), is recognised by TLR5. Activation of the TLR5 receptor, through bacterial flagellin, leads to the stimulation of proinflammatory cytokines through signalling the adaptor protein Myeloid differentiation primary response gene 88 (Myd88) which activates downstream pathways to activate the immune response (Gewirtz et al., 2001, Hayashi et al., 2001, Smith and Ozinsky, 2002). The immunisation of animals with a recombinant fusion protein containing flagellin has been shown to generate significant antigen specific CD8+ T-cell responses. This occurs through stimulation of antigen presenting cells (APCs), thus resulting in the subsequent maturation of these cells leading to the secretion of proinflammatory cytokines.

1.6.7. Modified TLR agonists through chemical conjugation to lipids

An important characteristic of antigen-loaded vaccine adjuvants is the specific ability to target professional antigen presenting cells (APCs) by the targeting of pattern recognition receptors, such as Toll-like receptors (TLRs). The association of TLR agonist to the liposome bilayer can be achieved by one of two different methods; physical adsorption or chemical conjugation (Demento *et al.*, 2011).

Physical adsorption of pathogen-associated molecular patterns (PAMPs), such as TLR agonists, to the liposome surface can be achieved by electrostatic interactions, hydrogen bonding and weak intermolecular interactions (such as Van der Waals). This occurs due to electrostatic interactions between cationic delivery system and anionic TLR agonist. However these systems may be inherently unstable. Delivery of PAMPs can also occur by entrapment of TLR agonist, polyI:C or CpG, within the delivery system itself (Gursel et al., 2001, Gursel et al., 2002, Nordly et al., 2011c).

However, an interesting approach towards coupling of PAMPs to lipids is through chemical conjugation. This can occur through simple covalent bonding or chemical coupling between adjacent functional groups between lipid and PAMP. Chemical coupling between amine and carboxylate can lead to ‘ester formation’ between these two components (Hermanson, 2008, Demento et al., 2011). This approach of chemical and bioconjugation, although intriguing, may have a drawback in that the

interaction with serum proteins might mask the efficacy of the agonist and its ability to target its specific receptor.

An interesting study by Andrews and colleagues (2011) has shown that immunostimulatory CpG oligodeoxynucleotides (ODN) can be chemically conjugated to lipid and incorporated into liposomes as a means to enhance the delivery of TLR agonist to the TLR9 receptor and, therefore, generate an increased immune response (Andrews *et al.*, 2011).

1.6.8. Synthetic TLR agonists

Encapsulation of TLR agonists within liposome formulations (in combination with protein antigen) has been studied previously as a means of enhancing the immune response (Gursel et al., 2001, Nordly et al., 2011c). Also synthetic TLR7 ligands such as 3M-019 and 3M-052 (synthesised by 3M Pharmaceuticals) have been used and prepared in liposome vaccine formulations (Johnston et al., 2007, Smirnov et al., 2011). Johnston and colleagues (2007) demonstrated that the imidazoquinoline derivative, 3M-019, was able to effectively modulate the immune response to the model vaccine antigen ovalbumin (OVA). This immune response, in terms of IgG2a antibody production, was further increased upon encapsulation of the TLR agonist into the liposome formulation in comparison to TLR agonist or antigen alone thus showing the importance of a delivery system (Johnston *et al.*, 2007). Another synthetic IRM, 3M-052 was designed and synthesised with the aim of forming a depot and sustained release of compound and for incorporation into the bilayer of liposomes. Upon formulation of this agonist within neutral liposomes composed of PC (phosphatidylcholine) this resulted in increased immune responses compared to vaccination using antigen or agonist alone (Smirnov *et al.*, 2011).

Also in other recent studies, some compounds have been specifically developed in order to activate TLRs 7 and 8 (Smits *et al.*, 2008). Activation of TLR7 has been shown to occur due to action by ligands, 3M-001 and 852A. Whereas TLR8-mediated activation occurs through the action of the specific ligand, 3M-002 (or CL075), in order to activate the downstream cellular immune response (Gorden et al., 2006, Dudek et al., 2007, Harrison et al., 2007).

In further studies (Chan *et al.*, 2009) another group of low molecular weight TLR7 activators termed purine-like molecules has been investigated, including 9-benzyl-8-hydroxy-2-(2-methoxyethoxy) adenine (otherwise termed SM360320). This compound has been shown to be a specific TLR7 agonist (Kurimoto *et al.*, 2004). Upon conjugation of this agonist to phosphatidylethanolamine (PE)

this leads to the production of an increased immune response as shown by enhanced antibody production (when compared to agonist alone) upon immunisation (Chan *et al.*, 2009).

1.6.9. The inclusion of non-TLR agonists within liposome formulations

TDB is a synthetic analogue of the mycobacterial 'cord factor', otherwise termed as trehalose dimycolate (TDM) and shows less toxicity *in vivo* whilst retaining immunogenic properties. This is due to the reduced number of mycolate chains which results in a shorter chain length (Matsunaga and Moody, 2009). These properties allow for the successful inclusion of TDB into cationic liposome delivery systems, with an optimal ratio of 11 mol % (8:1 M/M) shown to display the highest titres of antibody (Davidsen *et al.*, 2005) in order to generate the optimal immune response.

A growing number of studies have investigated the role of further mycobacterial-derived lipids in the process of interaction between mycobacteria such as (*M.tb*) and dendritic cells (DCs) at the host-pathogen interface. Initial studies have identified mycobacterial cell envelope components such as phosphatidylinositol mannoside and lipoarabionmannan as having the ability to have an immunopotentiary effect on the immune response (Sprott *et al.*, 2004). Also, further studies (Andersen et al., 2009a, Andersen et al., 2009b) have indicated monomycoloyl glycerol (MMG) as a prominent glycolipid from the mycobacterial cell wall, which has an enhanced effect on the immune response. Formulation of the cationic liposome system, with the addition of MMG (in combination with the TB antigen H1) within the liposome bilayer, has been shown to lead to an increase in antigen-specific immunity. MMG was identified as an important lipid in this process following the extraction of apolar and polar lipid fractions from *M. bovis* BCG (Andersen *et al.*, 2009b). Following isolation and extraction of MMG from the apolar lipid fraction, this lipid was formulated within a cationic DDA-based liposome delivery system. This delivery system, in combination with the Ag85B-ESAT6 fusion protein (Hybrid 1) was found to be highly efficient in the activation of APCs (by MMG) and the production of a Th1-biassed immune response, as characterised by high levels of interferon-gamma (IFN-γ) and IL-2 as well as neglible levels of Th2 cytokines IL-4 and IL-5. This has been studied previously using complex mycobacterial extracts delivered with cationic liposomes which have given enhanced immune responses in combination with TB antigen (Rosenkrands *et al.*, 2005).

A number of research groups have also used synthetic analogues of monomycoloyl glycerol (MMG) to show the effect on the resulting immune response (Andersen et al., 2009a, Bhowruth et al., 2009, Nordly et al., 2011b). A synthetic C32 MMG analogue has been produced by the coupling of C32

mycolic acid with isopropylidene glycerol. This liposome adjuvant system has been shown to give comparable immune responses to both the CAF01 and CAF04 delivery system (Andersen *et al.*, 2009b). A further synthetic MMG analogue, termed MMG-1, has been shown to have a stabilising effect on cationic DDA-based liposomes as well as stimulating the Th1/Th17 immune response of this adjuvant system as characterised by increased production of interferon-gamma (IFN-γ) and IL-2. Also in further studies this liposome vaccine formulation, DDA/MMG (otherwise known as CAF04) has been delivered in combination with the TLR3 agonist polyI:C in order to increase the CD4/CD8 T-cell immune response.

A number of research studies have suggested that several vaccine adjuvants may function in a TLR-independent pathway; molecules including NOD-like receptors, retinoic acid inducible gene (RIG)-like receptors, scavenger receptors, neuronal apoptosis inhibitory proteins (NAIP5) as well as Dectin-1 like receptors have been shown to play a role in activating the immune response (Lahiri *et al.*, 2008).

Double stranded (ds) RNA and its synthetic analogues including polyI:C have been shown to be potent inducers of innate immunity, as well as acting through Toll-Like Receptor 3 (TLR3) and RIG1-like receptors which causes the production of key cytokines (including IFN-γ and IL-12). This enables the cross presentation of antigen to MHCI complexes in order to enhance the CD8+ T-cell response. The nucleotide-binding oligomerisation domain (NOD) receptors are localised in the cytoplasm and are able to recognise components of the bacterial peptidoglycan, such as muramyldipeptide (MDP) and diaminopimelic acid (DAP). These NOD-like proteins are able play key roles in innate immunity against a number of pathogens, including *Chlamydia trachomatis* (Fritz et al., 2006, Shaw et al., 2008).

1.6.10. The role of the inflammasome in innate immunity

The inflammasome is a multi-protein complex that is involved in the promotion of an innate immune response, specifically the activation of caspase-I which promotes the processing and secretion of proinflammatory cytokines (Ogura et al., 2006) including IL-1β. Inflammation is the response of the host to 'danger signals', tissue damage and infectious agents (Stutz *et al.*, 2009). These inflammatory responses are extensively co-ordinated by cells of the innate immune system, with cytokines and chemokines playing a significant role as mediators of the downstream signalling cascades. The inflammasome becomes activated in response to external extracellular stimuli leading to caspase-I

activation, which was initially known as the IL1-converting enzyme due to its ability to proteolytically cleave pro IL-1β into the bioactive cytokine IL-1β. This process occurs due to the recruitment and oligomerisation of pro-caspase-1 upon stimulation of the inflammasome complex.

This proinflammatory cytokine, IL-1β, is important as it activates lymphocytes in the initiation of the immune response. The activation of caspase-I (and hence secretion of cytokines) is a highly regulated event which involves the priming and maturation of this complex. Several other cytokines are processed by the inflammasome including IL-18 and IL-33, which are important in IFN-γ induction and activation of natural killer (NK) T-cells. The activation of the cytokines IL-18 and IL-33 has been shown to be also due to cleavage caused by activation of the caspase-1 enzyme, with the latter of these cytokines inactivated by caspase-1 and actually activated by the calpain enzyme (Cayrol and Girard, 2009, Luthi et al., 2009).

1.7. Enhancing liposomal adjuvants: Investigating the effect of physicochemical characteristics on the function of liposomal adjuvants

Key advantages of these particulate delivery systems lie in the fact that they have the ability to be tailored to achieve desired physicochemical properties through variation of lipid components as well as the method of preparation. This versatility of liposomes allows protein antigen, peptides, nucleic acids and immunomodulatory components including Toll-like receptor (TLR) and non-TLR agonists to be incorporated within the system (Perrie *et al.*, 2013). Therefore liposomes can be used as efficient delivery vehicles for the delivery of vaccines with these vesicles being able to differ in size and lamellarity as well as their membrane fluidity and surface charge (Figure 1.10). The importance of these physicochemical parameters, in terms of enhancing immune responses, will be described below.

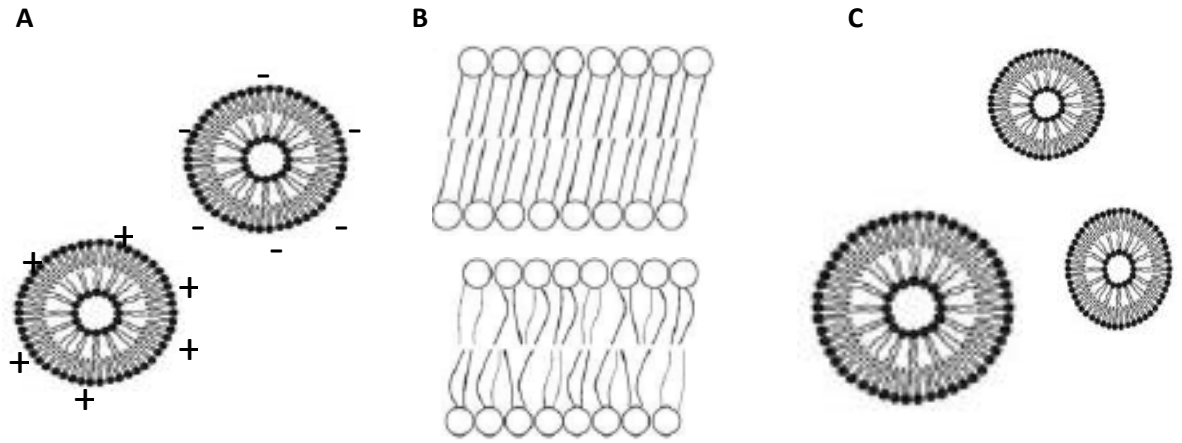


Figure 1-10. Liposome formulation parameters have the ability to influence specific immune responses. These include membrane surface charge (A), bilayer fluidity (B) and vesicle size (C).

1.7.1. The role of liposome surface charge in vaccine delivery and formulation

The role of surface charge of liposome delivery systems in vaccine delivery and formulation has been investigated in a number of research studies. As described previously (Henriksen-Lacey et al., 2010c), cationic liposomes have an advantage over their neutral and anionic counterparts due to their ability to adsorb anionic antigen to their surface and thus enabling the formation of an 'antigen depot effect'. Therefore these particulate vaccine carriers have an increased adjuvant activity and are more likely to deliver antigen to T-cells in the lymphoid tissue. Original studies by Allison and Gregoriadis in 1974 demonstrated the use of diphtheria toxoid in combination with negatively-charged liposomes caused a significantly elevation in the levels of antibody in comparison to positively-charged liposomes (Allison and Gregoriadis, 1974). In addition, other studies (Nakanishi et al., 1999, Foged et al., 2004, Henriksen-Lacey et al., 2010c) have shown that cationic liposomes (in combination with anionic antigen) are more potent inducers of antigen-specific immune responses compared to neutral and anionic liposomes. The use of liposomes of varying charge has also been investigated by Yanasarn et al, in which some negatively-charged liposomes (including the anionic lipid DOPA) are able to generate strong and functional antibody responses, as well as up-regulating gene expression related to the activation and maturation of dendritic cells (Yanasarn *et al.*, 2011). This occurred due to the simple mixing of pre-formed negatively charged liposomes with antigens.

1.7.2. The role of liposome vesicle size and administration route on vaccine delivery and formulation

The vaccine administration route of particulate delivery systems may be importance as particles in the sub-micron range can be naturally phagocytosed by antigen presenting cells (APCs). These particulate vesicles can also be formulated in order to control their characteristics so as to target specific tissues or cells (Mohanan *et al.*, 2010), including the draining lymphatics so as to stimulate and regulate adaptive immune responses (O'Hagan and De Gregorio, 2009). Bachmann and colleagues (2010) have investigated the role of vesicle size on targeting to the lymph nodes by several administration routes. Particulates of less than 200 nm in size have the ability to self-drain to the lymphatics without the need for uptake by APCs before being taken up by lymphoid-organ resident dendritic cells (Joffre *et al.*, 2012), however larger particulates (500-1000 nm) require to be previously taken up by APCs before transport into the LN (Bachmann and Jennings, 2010, Joffre et al., 2012). However drainage of liposomes is also charge-dependent, with neutral DSPC/TDB liposomes (Henriksen-Lacey *et al.*, 2010c) draining rapidly from the injection site due to low retention at this site (which is not size-dependent). However for cationic formulations, drainage of liposomes at early timepoints to the lymph nodes was shown to be size-dependent, thus suggesting increased uptake of larger liposomes by macrophages at the site of injection before subsequent drainage to lymph nodes (Manolova et al., 2008, Henriksen-Lacey et al., 2011b).

A number of various factors have been investigated which may affect the immune response; including the route of vaccine administration, inclusion of Toll-Like Receptor (TLR) ligands, and the amount of antigen and delivery system administered (Mohanan *et al.*, 2010). The route of administration is the site at which an individual or experimental animal are vaccinated. During a recent study (Mohanan *et al.*, 2010), various administration routes were investigated and the ensuing immune response was shown. Each administration route requires a different volume of vaccine, and the antigen drains to the various lymph nodes where an immune response is generated. These routes of vaccine administration, via injection, possess various safety risks with an increased risk of injury from sharp needles (Pruss-Ustun *et al.*, 2005).

Most vaccines are administered via subcutaneous (s.c) or intramuscular (i.m) injection due to relatively cheap cost and ease of administration; however, during recent studies the mucosal, transdermal, intralymphatic and vaginal routes have been a major focus of investigation. These alternative administration routes have been used to increase patient compliance, control and prevent disease at the site of infection and to target the vaccines to local lymphoid tissue. Vaccine administration via the mucosal route is a promising topic in the field of vaccine development, with the use of lipid-based particulate delivery systems being of increased focus (Christensen *et al.*, 2010). The major properties of a vaccine to be delivered via the mucosal route are the ability to penetrate through the mucosal tissue and to be delivered across the epithelium in order to interact with APCs. The trivalent influenza vaccine, when administered with DDA/TDB (CAF01), is able to generate an efficient cell-mediated immune response [characterised with production of interferon-γ (IFN-γ)] when delivered by the intranasal (i.n.) route, rather than without adjuvant alone. A stronger immune response may be expected the closer a vaccine is administered to the lymph node. With this in mind, vaccines administered via the intralymphatic (i.ln) route are able to increase the amount of protein antigen in the lymph node as well as increase cell-mediated and humoural immune responses (Johansen *et al.*, 2010).

1.7.3. The role of liposome preparation method on vaccine delivery and formulation

By varying the preparation method, liposomes (cationic, anionic and neutral) can be formed in a number of ways (Bangham et al., 1965, Gregoriadis et al., 1999). In order to act as efficient delivery systems, liposomes must become associated with antigen in order to induce an effective immune response as these systems are able to protect antigen from degradation (McNeil *et al.*, 2011)

Antigen can either become adsorbed at the liposome surface, associated within the phospholipid bilayer or become entrapped within the liposome itself. In order for antigen to become associated at the liposome surface, both of these components have to be opposite in charge in order to associate via electrostatic interactions. Antigen can also become entrapped within the liposome, and this interaction itself can be devoid of electrostatic interactions, therefore allowing the association of similarly charged liposomal and antigenic components (Henriksen-Lacey *et al.*, 2011c). This entrapment procedure often yields low levels of antigen entrapment, but antigen is protected from degradation by protease enzymes (Gregoriadis, 1990, 1994). Many of the initial studies into liposome-based vaccines compared encapsulated antigens with surface-conjugated antigens with

the main findings showing improved antibody production and immune responses when antigen is exposed on the liposome surface and can attach to B-cell receptors (White *et al.*, 1995).

Various studies have shown that depending on the antigen location and its subsequent physical association with the liposome, the type and strength of the immune system is varied. Fortin and colleagues (1996) have demonstrated that these different locations can influence on the route in which antigen is processed within antigen presenting cells and thus dictate the immune response (Fortin *et al.*, 1996). Antigen may be entrapped within dehydration-rehydration vesicles which gives rise to high antigen entrapment efficiency and subsequent improved immunogenicity (Kirby and Gregoriadis, 1984), however various other studies suggest that the physical location of the antigen has no bearing on the subsequent immune response (Gregoriadis et al., 1987, Kaur et al., 2011).

1.7.4. The role of liposome membrane fluidity in vaccine delivery and formulation

The role of membrane fluidity has been extensively reviewed in the literature. The general trend is for high transition temperature lipids to have an advantage over their low transition temperature counterparts when formulated within liposomal vaccine adjuvants, with Yasuda et al (1977) demonstrating that higher transition lipids gave rise a 4-fold increase in anti-DNP antibody responses (Yasuda *et al.*, 1977). Also, mixed compositions of DSPC (high transition temperature) and DOPC (low transition temperature) led to enhanced antibody responses upon increased titration of DSPC lipid within the formulation (Dancey *et al.*, 1978). More recent studies have compared liposomes formulated using either the high transition lipid DDA or the low transition lipid DODA (dimethyldioleoylammonium bromide) in combination with the immunostimulatory glycolipid TDB. Rigid DDA/TDB liposomes retained a higher percentage of antigen at the injection than their fluid DODA/TDB liposome counterparts, thus leading to significantly higher Th1 responses (100-fold) thus confirming that membrane fluidity plays an important role in the immunogenicity of liposome adjuvants (Christensen *et al.*, 2012).

So to conclude, liposomes consisting of high transition temperature lipids give rise to vesicles of greater rigidity which leads to enhanced antibody and cell-mediated immune (CMI) responses to a number of surface-adsorbed and encapsulated protein antigens (Watson *et al.*, 2012), whereas low transition temperature lipids give rise to more fluid liposomes which lead to a significant reduction in Th1 immune responses.

1.7.5. Enhanced targeting of liposomal vaccines to the draining lymphatics

Lymph nodes are the primary peripheral lymphoid organs that are essential for vaccine-induced immune responses. These draining lymph nodes are strategically positioned in order for the immune response to be initiated after coming into contact with foreign antigens (Roozendaal *et al.*, 2009). The enhanced targeting of liposome-bound antigen is not fully understood in terms of improving the efficiency of cationic liposome-formulated vaccines. Previous studies have shown the role of vesicle size and surface charge as well as membrane fluidity on the retention of liposome at the vaccination site and their subsequent draining to the popliteal lymph nodes (PLN). These studies suggested that as cationic liposomes led to vesicle aggregation at the vaccination site, however neutral DSPC/TDB liposomes are less well retained at this site therefore are more likely to drain to the LN (Henriksen-Lacey *et al.*, 2010c).

Liposome drainage to the lymph nodes has also been investigated by Kaur et al (2011) and Zhuang et al (2011) by incorporating varying concentrations of polyethylene glycol (PEG) within the formulation. These studies have demonstrated that incorporating PEG into the formulation led to the blockage of the 'depot effect' due to 'masking' of the cationic surface charge and, therefore, subsequently led to increased liposome drainage to the LN (Kaur *et al.*, 2011). However, this did not correlate with antigen retention at this site, with no significant differences noted between formulations and a subsequent reduction in immune responses observed. However in terms of design for the manufacture of adjuvants, the promotion of a strong liposome depot also leads to the potentiation of strong Th1 immune responses (Perrie *et al.*, 2013).

Zhuang and colleagues demonstrated that PEGylation is important in the modification of liposome vaccine formulations. Incorporation of PEG could shield the surface charge of nanoparticles and reduce non-specific absorption by the extracellular matrix, thereby effectively accelerating the drainage of nanoparticles into the lymphatic system (Zhuang *et al.*, 2011). Further studies have been carried out including the PEGylation of cationic liposomal adjuvants (Kaur *et al.*, 2012). These studies indicated that size reduction of liposomes (to around 150 nm) and PEGylation of these liposome adjuvants led to enhanced drainage to the popliteal lymph nodes (PLN) and also led to earlier antibody responses. Carstens and colleagues (2011) also noted an enhanced lymphatic drainage of liposomal DNA vaccine carriers following subcutaneous injection, however this did not correlate with enhanced immune responses (Carstens *et al.*, 2011).

The draining lymph nodes are functionally partitioned into various compartments which contain areas that are high in cell content for B- and T-cells respectively (Roozendaal et al., 2009, Gonzalez et

al., 2010) . Therefore these cellular interactions in distinct compartments of the LN are able to direct both the cell-mediated immune response and humoural immunity. B-cells are able to circulate throughout secondary lymphoid organs, such as the spleen, in an effort to become more susceptible to encounter with antigen. These B-cells then are able to concentrate and become activated in the follicular region of the lymph nodes due to chemotactic migration in response to CXCL13, where they accumulate at the T-cell/B-cell border in order to interact with T-cells.

In general, the lymphatic draining of cationic liposome vaccine formulations is inefficient, which has been previously demonstrated in the literature. This is due to their increased retention at the vaccination site due to non-specific electrostatic binding between the delivery system and extracellular matrix proteins (Foged *et al.*, 2004). However, these vaccine formulations have still been shown to be immunopotent and capable of increasing levels of immunity as characterised by high production of interferon-gamma (IFN-γ) which is required for effective Th1 immunity. Therefore, these cationic liposome vaccine formulations are immunostimulatory but future challenges in research will be to augment enhanced lymphatic targeting of these systems in order to induce better immunoprotection by vaccination.

1.8. Aims and Objectives

A liposomal adjuvant currently shown to offer strong potential as an adjuvant for sub-unit vaccines including those for TB is composed of the cationic lipid DDA and the immunostimulatory glycolipid TDB (CAF01). Through previous studies, one contributing mechanism of action of this vaccine formulation is termed the 'depot effect'. However, further information on the downstream immune responses elicited by this adjuvant remain unclear. With this in mind, the aim of this of this work was to further investigate these cationic liposome formulations in order to better understand the controlling link of formulation and function. To achieve this aim, the main objectives of these studies were to:

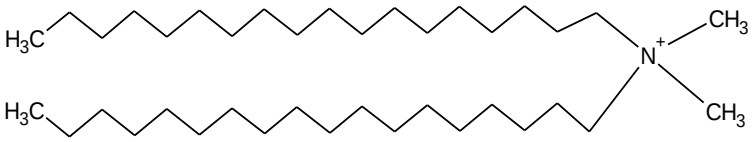
1. Formulate and characterise a high transition temperature anionic formulation.
2. Develop and formulate an optimised liposome formulation, which maintains nanoscale properties.

3. Investigate the role of surface charge in liposome vaccine formulation, stability, biodistribution and vaccine efficacy.
4. Investigate the role of liposome preparation method in formulation, stability, biodistribution and vaccine efficacy.
5. Identify TLR and non-TLR ligands we should consider in the context of liposome vaccine formulations.
6. Optimise liposome vaccine formulations through inclusion of surface-bound or entrapped TLR agonists or insertion of bacterial-derived glycolipids within the bilayer.
7. Reduce the particle size of liposome adjuvants through high shear mixing, with the aim of further inclusion of TLR agonists within the system.
8. Conjugate a TLR agonist to lipid and incorporate within the CAF01 liposome system and investigate the subsequent effect on vesicle characteristics, agonist and antigen loading, biodistribution profile and immunogenicity.

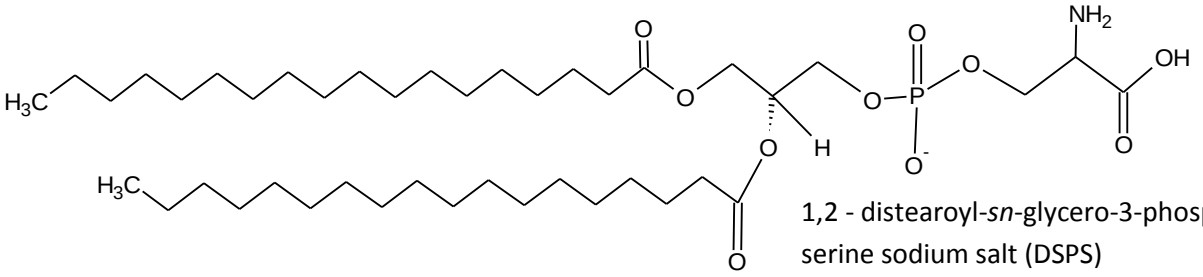
Chapter 2: Materials and Methods

2.1. Materials

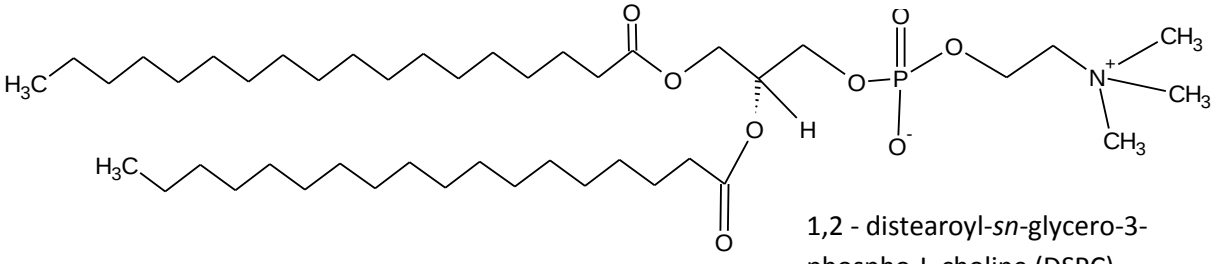
Dimethyldioctadecylammonium bromide (DDA), 1,2 - distearoyl-sn-glycero-3-phospho-L-serine sodium salt (DSPS), 1,2 – disteraoyl-sn-glycero-3-phosphocholine (DSPC), 1,2 - distearoyl-sn-glycero-3-phospho-L-ethanolamine (DSPE) and trehalose 6,6'-dibehenate (TDB) were purchased from Avanti Polar Lipids Inc. (Figure 2.1; Alabaster, USA). Monomycoloyl glycerol (MMG), Ag85B-ESAT6-Rv2660 (H56 TB antigen) and Ag85B-ESAT6 (H1 TB antigen) at concentrations of 1.2 mg/ml and 1.6 mg/mL respectively were obtained from Statens Serum Institute (Copenhagen, Denmark).



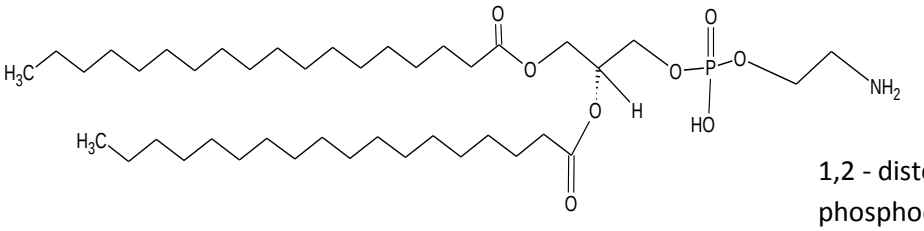
dimethyldioctadecylammonium bromide (DDA)



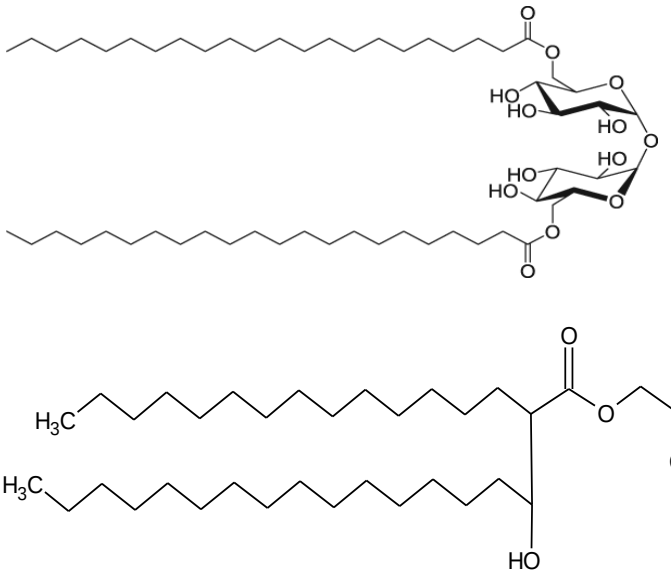
1,2 - distearoyl-*sn*-glycero-3-phospho-L-serine sodium salt (DSPS)



1,2 - distearoyl-*sn*-glycero-3-phospho-L-choline (DSPC)



1,2 - distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)



Trehalose 6,6'-dibehenate (TDB)

Monomycoloyl Glycerol (MMG)

Figure 2-1. Lipids required in studies in order to make liposome formulations.

Lysozyme (from chicken egg white), hydrogen peroxide, resiquimod, Sephadex™ G-75 and G100, bicinchoninic acid protein assay (BCA) components, crystal sky blue (Pontamine blue), Phospho-buffered saline (PBS) tablets, trypsin (from bovine pancreas), succinic anhydride, diisopropyl azodicarboxylate (DIAD), triphenylphosphine (TPP), sodium chloride, protease inhibition mixture, potassium chloride, potassium dihydrogen phosphate, sodium orthophosphate, citric acid, azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [ABTS] tablets, Tween-20, Triton X-100 phosphate-citrate buffer tablets, dimethylsulfoxide (DMSO), sodium azide, bovine serum albumin (BSA), concanavalin A (conA), succinic anhydride, tetramethylbenzidine (TMB) tablets, Brilliant Blue R, glycine, cyclohexane and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich UK.

Glacial acetic acid, diethylether, ethanol, propan-2-ol, methanol and chloroform (both HPLC grade) were purchased from Fisher Scientific UK (Leicestershire, UK). Dialysis tubing (3.5 kDa MWCO, 45 kDa MWCO and 100 kDa MWCO) were obtained from Biodesign and Spectrapor respectively.

The TLR3 agonist poly(I:C) oligodeoxynucleotide (ODN) DNA, agonists was purchased from Source Life Sciences (Nottingham, UK). Ovalbumin (OVA) was bought from Calbiochem (La Jolla, USA). Foetal Calf serum (FCS), and RPMI (without L-glutamine) were obtained from BioSera. Tritium-labelled cholesterol (^{3}H-Cholesterol) was obtained from GE Healthcare (Amersham, UK). IODOGEN® pre-coated iodination tubes were purchased from Pierce Biotechnology (Rockford, IL, USA).

^{3}H-thymidine (tritium-labelled thymidine), ^{125}I (NaI in NaOH solution), ^{32}P- ATP, SOLVABLE™ and UltimaGold™ scintillation fluid were purchased from Perkin Elmer (Waltham, MA, USA).

Tris-base was obtained from IDN Biomedical Inc. (Aurora, OH, USA) and used to make Tris buffer and adjusted to pH 7.4 or pH 4.0 using HCl; unless stated otherwise Tris buffer was used at 10mM, pH 7.4. Pre-cast 12 % and 18 % Tris-glycine gels (12 wells), penicillin streptomycin glutamate (PSG; 100 X liquid] and BenchMark ™ pre-stained protein ladder were bought from Invitrogen Ltd (Paisley, UK). Trehalose [D-Trehalose (99 %) anhydrous] was obtained from Acros Organics. Isotype-specific immunoglobulins (Goat anti-mouse IgG, IgG1, IgG2a and IgG2b) were purchased from AbD Serotec. All plastic wear for use in cell culture experiments and assays was purchased from Greiner BioOne. Mouse duoset capture cytokine analysis kits (IFN-γ, IL17, IL1-β, IL33, IL2, IL5, IL6 and IL10) and ELISA assay components were purchased from RnD (Abingdon, UK). Dried skimmed milk powder was purchased from Marvel. TLC plates and silica were obtained from Merck Chemicals Ltd. Deuteriated chloroform and DMSO were purchased from Cambridge Isotope Laboratories.

2.2. Methods in liposome formulation

2.2.1. Lipid Film Hydration Method for the production of multilamellar vesicles (MLV)

Liposomes were prepared using the previously described lipid film hydration method (Bangham *et al.*, 1965). For individual formulations, weighed amounts of DDA (10 mg/ml), DSPS (10 mg/ml) and TDB (2 mg/ mL) were each dissolved in chloroform/methanol (9:1 by volume). These lipids were added together to a final concentration of either 1.98 mM DDA or DSPS in combination with 0.25 mM TDB (8:1 M/M) added in the case of DDA/TDB and DSPS/TDB liposomes. The organic solvent was extracted using a roto-evaporator followed by flushing with N_2 to form a thin lipid film on the bottom of a round bottom flask (rbf). The lipid film was hydrated using 10 mM Tris buffer at pH 7.4 (unless otherwise stated) and at ~ 20 ˚C above the transition temperature for the main lipid in the formulation; e.g. for DDA and DDA/TDB liposomes the hydration buffer was pre-heated to 60 ºC. For DSPS/TDB liposomes, the hydration buffer was pre-heated to greater than 80 °C in order for these vesicles to assemble. For each of the formulations, the hydrated lipid film was vortexed every 5 minutes (for 20 minutes) in order to form liposomes (Figure 2.2) before being left to cool for 30 minutes. Subsequently, protein antigen, vaccine or TLR agonist could be added in order to investigate vesicle characteristics.

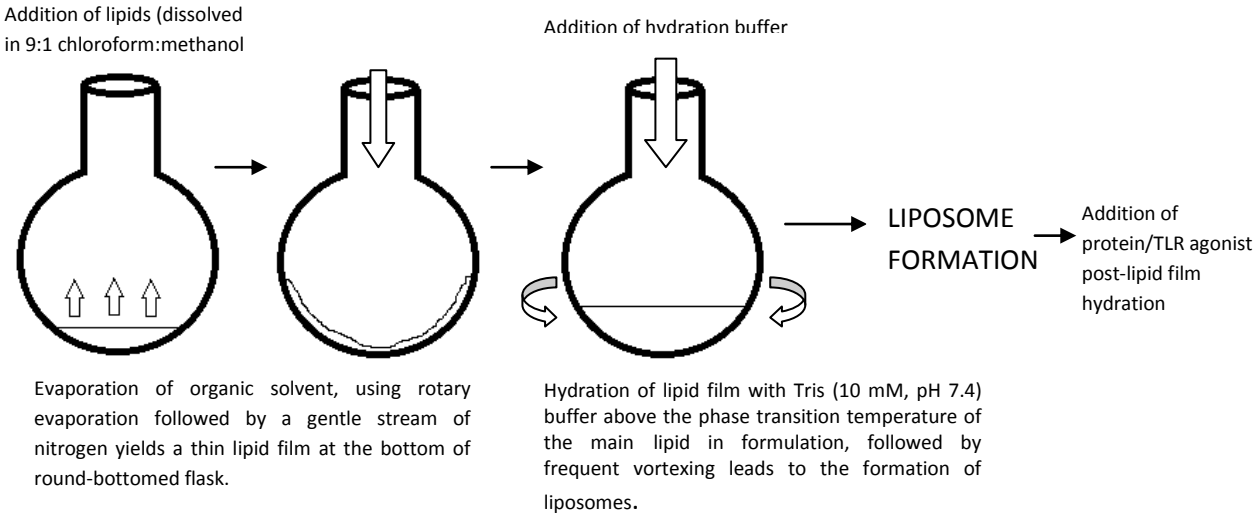


Figure 2-2. Preparation of lipid stocks in organic solvent (9:1 chlorofom/methanol), and preparation of multilamellar vesicles using the lipid film hydration method.

2.2.2. Formulation of small unilamellar vesicles (SUV)

For each liposome formulation, the particle size was reduced using a sonication procedure. The suspension of MLV was sonicated (at 10 psi for 2 minutes) using a titanium probe (MSE PG100 probe) slightly immersed into the solution. This sonication procedure causes the breaking of the lipid bilayer of liposomes, converting a slightly milky suspension of MLV into a clear suspension of SUV (Figure 2.3A). These vesicles were left to stand for 30 minutes before characterisation of the size, polydispersity and zeta potential. These vesicle systems were either characterised empty or with the addition of varying concentrations of protein [either OVA (anionic), lysozyme (cationic) or H56 TB antigens (anionic)] or TLR agonist.

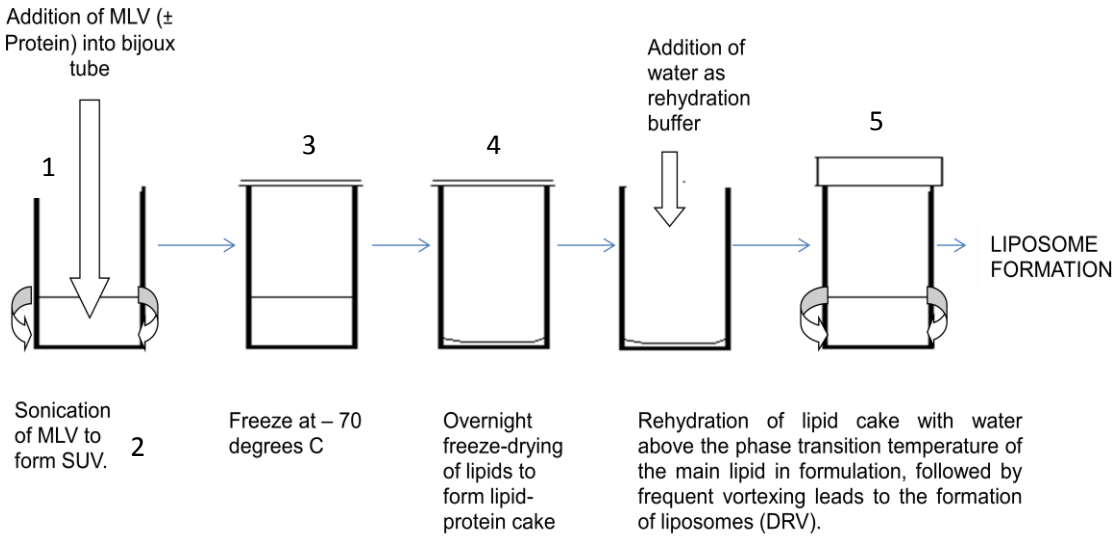
2.2.3. Preparation of dehydration-rehydration vesicles (DRV)

Vesicles were prepared by the dehydration-rehydration method as previously described (Senior and Gregoriadis, 1989, Gregoriadis et al., 1999) at the same composition and molar ratios (8:1 M/M ratio) as for SUV as described in section 2.2.2.

These vesicle suspensions were then placed in glass vials covered with parafilm (with small holes) before being placed in the freezer at - 70 ˚C for 10 minutes. The liposome samples were then placed

in a freeze drier overnight, at - 40 ˚C under vacuum conditions (Figures 2.3A and B) with vacuum to 40 mbars. Controlled rehydration (using deionised water) of the dried powder (lipid cake) led to the formation of antigen containing DRV. Controlled rehydration was achieved by addition of 10% of the final volume which was standardised at 900 µl, twice at 30 min intervals (followed by vortexing), before addition of the remaining volume of deionised water.

A



B

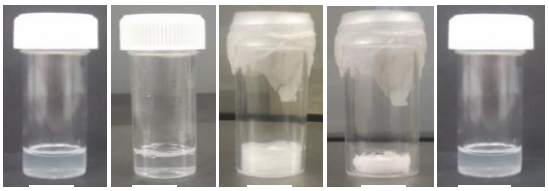


Figure 2-3. (A) Preparation of liposomes by the dehydration-rehydration vesicle (DRV) method [Gregoriadis and Kirby, 1984]; (B) Diagrammatical representation of each stage of the DRV liposome preparation process.

2.2.4. Preparation of double emulsion (DE) vesicles

The same molar ratio was used between formulations, using these various liposome preparation methods, in order to serve as a direct comparison between cationic DDA/TDB and anionic DSPS/TDB liposome formulations. The organic solvent was extracted using a roto-evaporator (at 40 ˚C) followed by flushing with N_2 to yield a thin lipid film on the bottom of a round-bottomed flask or glass vial as described in section 2.2.1. Subsequently, 2.1 ml organic phase (o) consisting of diethylether and cyclohexane (5:1 v/v) was added to this lipid film before being dispersed by tip sonication. To this volume, 350 µl Tris buffer was added (inner water phase, w_1) containing the

desired amount of protein (OVA, lysozyme or H56 antigen) before being sonicated using a titanium probe (MSE PG100 probe; Soniprep 150) slightly immersed into the solution. For *in vivo* formulations, the dose concentration of 5 µg (or 0.1 mg/mL) was added in the w_1 inner water phase.

Under vigorous magnetic stirring, the primary w_1/o emulsion was introduced (using a Hamilton syringe) into a second water phase (w_2) consisting of 10 ml Tris buffer, 10 ml MilliQ water and 8.5 ml of the co-solvent ethanol (30 % v/v) in order to form a double ($w_1/o/w_2$) emulsion (Figure 2.4). Under slow magnetic stirring, this rbf was then placed in a water bath at 35 ˚C in order for solvent evaporation to take place. After a few hours evaporation, a gentle stream of air/N_2 was introduced to facilitate the evaporation process. The organic phase and co-solvent should evaporate after around 5 hours. To further improve the quality of the formulation, the $w_1/o/w_2$ emulsion was sonicated for 2 minutes every 30 minutes during the evaporation process using a bath sonicator (Nordly *et al.*, 2011c).

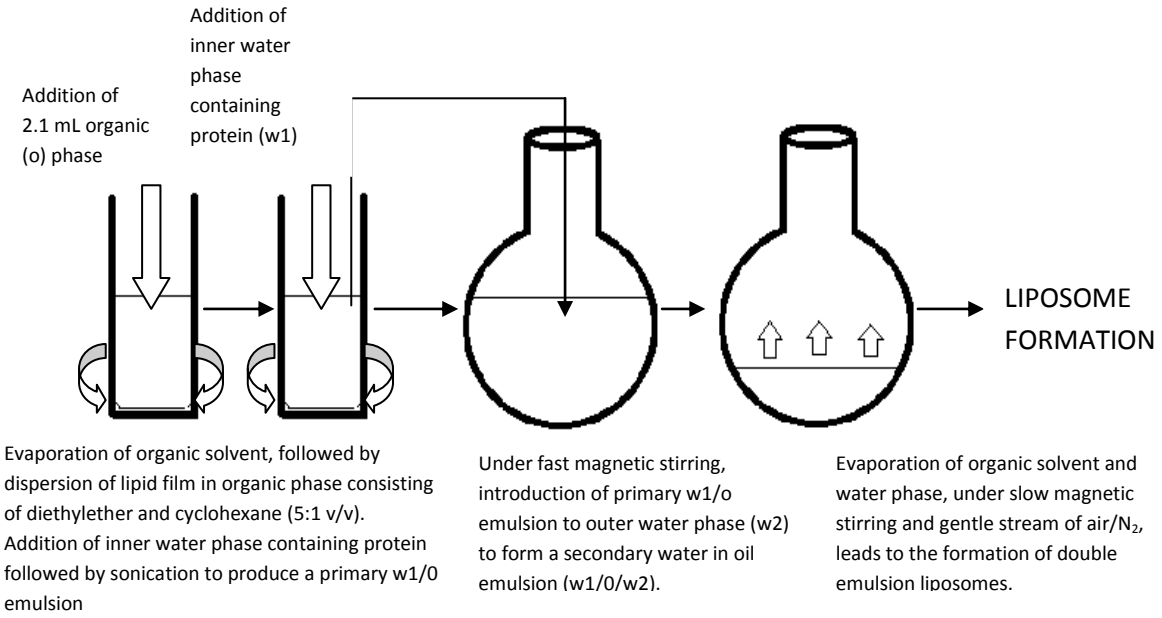


Figure 2-4. Schematic illustration of the double emulsion solvent evaporation method, used in the preparation of double emulsion (DE) liposomes.

2.2.5. Formulation of liposomes by the high shear mixing (HSM) homogenisation method

As with the lipid-film hydration (LH) method, the thin lipid film was formed as described previously in Section 2.2.1. Again Tris buffer (10 mM; pH 7.4) was added to the lipid film, which was then placed and mounted in a 60 °C water bath (for DDA-based formulations). The use of an Ultraturax™ Silent Crusher M at 24,000 rpm (for 15 minutes) was optimal to produce a homogenous population of

vesicles (Figure 2.5). Following formulation of CAF01 (DDA/TDB) or CAF04 (DDA/MMG) by high shear mixing (HSM), subsequently polyI:C was added in steps of 5 µL, in order to form CAF05 or CAF09 respectively. This was followed by high shear mixing at a temperature of 60 °C for 60 seconds. This was continued until the final concentration of poly(I:C) within the formulation was reached.

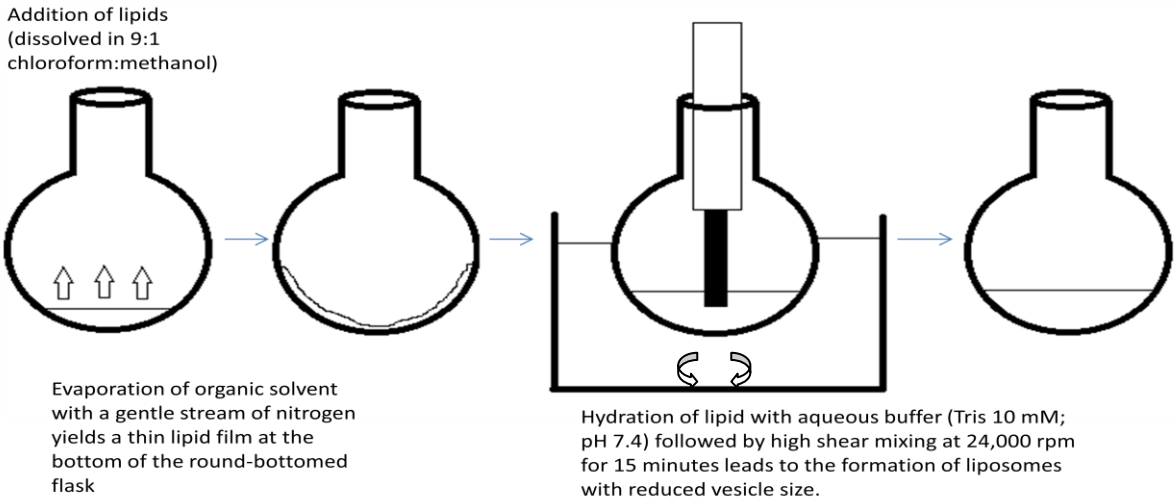


Figure 2-5. Schematic illustration of the high shear mixing homogenisation method used in the formation of liposomes with reduced vesicle size [with or without incorporation of poly(I:C)].

2.2.6. Removal of unincorporated protein from liposome-bound protein

For all liposome formulations (prepared by all of the above methods) with the addition of protein in the preparation process, the liposome-entrapped protein is separated from non-entrapped protein using ultracentrifugation at 125,000 x g (at 4 ˚C for 30 minutes). The supernatant is then decanted, and the liposome pellet is resuspended in Tris buffer (10 mM; pH 7.4).

2.3. Characterisation of liposomal systems

2.3.1. Determination of vesicle size and surface charge, by dynamic light scattering and zeta potential

The particle size distribution and polydispersity (PDI) of the liposome suspensions were determined by dynamic light scattering (DLS). PDI is a measure of the distribution of the particle size. Samples of 100 µL were diluted 10-fold with Tris buffer (1 mM, pH 7.4) before measuring to ensure the concentration of particles was sufficient. Particle size measurement took place using a Malvern

Zetasizer Nano-ZS (Malvern Instruments, Worcs., UK). Triplicate samples were measured three times each at 25 °C. The vesicle surface charge (as indicated by zeta potential) of the formulations were measured using the same instrument at 25 °C in a clear zeta potential cuvette. Each sample was also measured three times (in triplicate).

During a size measurement of the various liposome systems if the particles are illuminated with a laser, the intensity of the scattered light fluctuates at a rate that is dependent upon the size of the particles. Smaller particles are moved further by the solvent molecules and move more rapidly. Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein relationship. These particles in suspension move under 'Brownian motion' which is the motion induced by the bombardment by solvent molecules that themselves are moving due to their thermal energy. During a particle size measurement the laser acts as the light source (at an angle of 90^{o}), therefore light is scattered by the particles at all angles. The scattered light is then able to be converted into electrical pulses by a photon detector which feeds into a digital correlator and gives data analysis of the particle size.

The zeta potential is defined as the electrical potential that exists at the hydrodynamic plane of shear (or slipping plane), which is the area of the diffuse layer where ions can no longer interact. The zeta potential can be used to measure the charge stability of a disperse system and also assist in the formulation of stable products. So, the zeta potential can act as an indirect measurement of the surface charge of a particle (in the case of these studies, liposomes). The zeta potential can be measured by applying an electrical field across the dispersion. During this measurement, particles with a zeta potential will migrate towards the electrode of opposite charge with a velocity proportional to that of the zeta potential.

During this thesis, a number of stability studies were carried out in order to ensure that liposome vesicle size and surface charge characteristics maintained constant over a 28-day period at storage temperatures of 4 ˚C or 25 ˚C. The size, zeta potential and polydispersity of these liposomes (without and with antigen) were measured at a number of different timepoints (Days 0, 3, 7, 14, 21 and 28) in order to show if the liposome formulation remains stable. Also, at each timepoint a visual inspection of the sample was undertaken to check for aggregation, along with a photograph of the specific liposome formulation.

Further stability studies were also undertaken to investigate the long term storage conditions of liposome vaccine formulations over a 6-month study period (Chapter 6). These liposomes were all formulated with the H56 TB vaccine antigen at a concentration of 5 µg (0.1 mg/mL), and also with

and without addition of the TLR3 agonist polyI:C (50 µg). The protein integrity of antigen within these vaccine formulations was also visualised by SDS-PAGE, which will be described in Section 2.3.6.

2.3.2. Cryo-Transmission Electron Microscopy (Cryo-TEM)

Cryo-electron microscopy involves forming a thin aqueous film on a bare specimen grid (3–4 mm thick, with a fine 700 mesh honeycomb pattern of bars) by pipetting 5-10 µl of the liposome suspension onto the grid. After blotting the suspension-coated grid on filter paper, the thin film produced was rapidly (1 s) vitrified by plunging the grid into ethane and cooled to its melting point with liquid nitrogen. Preparation and blotting of thin films was carried out in a controlled environment using a fully automated system (PC-controlled, up to vitrification). The vitrified film was mounted in a cryo-holder (Gatan 626) and observed at 170 °C in a transmission microscope (Philips CM12) operating at 120 kV. Micrographs were taken using low-dose conditions.

2.3.3. Adsorption of protein antigen to liposomal formulations

Once the liposomes (MLV) had been formulated by one of the preparation methods described in Section 2.2, they were mixed with various concentrations of protein/antigen (OVA, lysozyme or H56). Adsorption of protein to these liposomes occurred due to frequent mixing of the eppendorf tube over a time period of 45 minutes. 1 mL of 10 mM Tris buffer (pH 7.4) was added to this mixture before a number of different total protein assays were undertaken. This procedure was also undertaken for the adsorption of radiolabelled protein to liposome formulations, as described in Section 2.4, with the addition of varying concentrations of protein.

2.3.4. Measuring the amount of total protein using a Bicinchonic Acid (BCA) Assay

This assay is based on the colourimetric detection of a purple-coloured BCA-Cu^{+} reaction complex (at 562nm) to determine the total amount of protein in a sample, based on the method carried out by Smith (1985). This protocol combines the reduction of copper ions ($Cu^{2+} \rightarrow Cu^{+}$) by a protein in an alkaline medium (termed the Biuret reaction), with the ability of a single Cu^{+} ion to be able to chelate with two molecules of BCA in order to form the purple-coloured reaction complex.

Protein (present in sample) + Cu^{2+}(green/blue) ⟹ tetradentate-Cu^{+} complex (purple)

This reaction complex has a strong absorbance at 562nm, which increases in a linear fashion over increasing concentrations of protein. During this study, a BCA total protein assay was used to determine the amount of protein which was present in the supernatant fraction from the liposomes (DDA, DDA/TDB, DSPC/TDB and DSPS/TDB) in order to determine the amount of protein which remained unadsorbed to these liposomes. In order to obtain supernatant fractions from the liposomal formulations, these protein-adsorbed liposome samples (as prepared previously in Section 2.2) were centrifuged for 30 minutes at 10,000 x g (using a Sanyo MSE Micocentaur centrifuge). The supernatant fractions for each of the liposomes were then decanted and stored in a new set of eppendorf tubes. The remaining liposome pellet was resuspended in 1 mL Tris 10mM buffer (pH 7.4) and this process was repeated several times for each formulation.

This same protocol was repeated in a subsequent experiment, with the exception that the protein-adsorbed liposome samples were separated from free protein using ultracentrifugation [using a Optima Max-XP Ultracentrifuge (Beckman-Coulter Inc., Fullerton, CA)] for 30 minutes at 125,000 x g at a temperature of 4 °C. Again this step was repeated to gain a series of supernatant fractions. A standard protein curve of 0-1 mg/mL OVA or lysozyme, in triplicate wells, was set up in order to compare to the protein concentration in both these supernatant fractions for various liposome formulations. For these formulations, 25 µL of supernatant, in triplicate wells, were placed in separate wells on a 96-well plate - and then 200 µL of BCA reagent was added to each of these wells. The 96-well plate was then incubated for 30 minutes at 37 °C before the absorbance of this plate was read at an absorbance of 562 nm using a BioRad™ plate reader.

2.3.5. Trypsin Digestion Assay to show the localisation of antigen in regards to the delivery system

Liposomes were prepared as in Sections 2.2, with the addition of protein antigen in the preparation method [lipid-film hydration (LH) method, dehydration-rehydration vesicle (DRV) method or double emulsion (DE) solvent evaporation method]. Post-liposome preparation, these vesicles were subjected to conditions of varying concentrations of trypsin (100, 200 and 400 µg/mL respectively).

Trypsin is a serine protease enzyme located in the digestive system of many vertebrates, where it hydrolyses proteins. This enzyme is able to function by cleaving peptide chains mainly at the

carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. Therefore if the antigen is surface-adsorbed to the liposome delivery system then trypsin will be able to remove this protein. However, if the protein antigen remains entrapped within the delivery system, the trypsin enzyme will be unable to perform its function. This is due to the fact that trypsin is unable to pass through the liposome bilayer.

Post-assay, these preparations will then be separated by ultracentrifuge (125,000 x g; for 30 minutes at 4 ˚C using a Optima Max-XP Ultracentrifuge; Beckman-Coulter Inc., Fullerton, CA) in order to produced a defined liposome pellet. The supernatant (S1) was decanted, and the liposome pellet resuspended in Tris buffer (10 mM; pH 7.4). This process was repeated in order to gain a second supernatant fraction (S2). These fractions were then ran on an SDS-PAGE gel and the protocol was followed as per Section 2.3.6.

In order to quantitatively establish the extent of antigen adsorbed compared with antigen entrapped within the the liposome delivery system, DDA/TDB and DSPS/TDB liposomes (in combination with radiolabelled protein antigen) prepared by either the LH method, DRV method or the double emulsion (DE) solvent evaporation method were subjected to surface digestion of antigen using trypsin and antigen retention measured (Kaur *et al.*, 2011). The % radioactivity within the pellet fraction was quantified as the percentage of adsorbed protein, therefore being a direct measurement of antigen retained with the delivery system, as described in Section 2.4.

2.3.6. SDS-PAGE to determine the adsorption or entrapment of protein antigen to liposomal formulations

Preparation of Tris/ Glycine/ SDS running buffer was achieved by the mixing of Tris (15 g), glycine (72 g) and SDS (5 g) - before being made up to 1 L with deionised H_20. The samples containing the proteins of interest were separated on a 12 % Tris-glycine (Invitrogen) gel, and this was run in Tris/ glycine/ SDS running buffer using Novex Mini Cell gel apparatus (Invitrogen). These samples containing the proteins of interest, from the supernatant and pellet fraction, of various liposomal formulations were compared against 10 µL of a protein marker. Prior to loading, all of these samples were heated at 90 °C for 3 minutes in order to denature the protein present in the sample. A volume of 10 µL of each pellet and supernatant sample were loaded into each specific lane of the gel. The gel was run for about 90 minutes at a constant 30 mA per gel, until the dye had just reached the end of the gel. The gel was subsequently stained using Coomassie Blue overnight, followed by a period of

destaining for several hours. A photograph was then taken of each gel using a GeneFlash gel photoimager (Syngene bioimaging).

2.4. Techniques for Protein, TLR Agonist and Liposome Detection

2.4.1. Preparation of radiolabelled liposomes

To follow the movement of liposomes *in vivo,* a stable radioactive tracer component (^{3}H-Chol) was added to the liposomes which can integrate into the lipid bilayer (Figure 2.9). Each liposome formulation (DDA/TDB or DSPS/TDB) was made in a similar manner (in a 8:1 M/M ratio) as described previously by the lipid film hydration method (Figure 2.2), but tritium-labelled cholesterol (^{3}H-Chol) was added to the rbf at the same time for each separate formulation. ^{3}H-Chol was added at a radioactivity of 2 MBq (100 kBq/ dose) to each liposome formulation (so the equivalent of 20 doses; 1 mL in order to track the *in vivo* fate of liposomes as vaccine components during biodistribution studies. The amount of ^{3}H-Chol added was based on the radioactivity (^{3}H) and the concentration of Cholesterol in the liposomes (0.17 nM) so that the physicochemical liposomal properties were not altered. The high ratio of ^{3}H-Cholesterol was ideal for biodistribution work as both factors - high radioactivity and low levels of cholesterol - were fulfilled. ^{3}H-Cholesterol was incorporated into liposomes at ^{3}H-Cholesterol:lipid weight ratio of 1:10,000. This is equivalent to 25 ng Cholesterol/dose with an approximate radioactivity of between 100-200 kBq.

The membrane stability of liposome delivery systems was determined by means of the incorporation of trace amounts of tritium (^{3}H) radiolabelled cholesterol within the liposome formulation. Therefore, the presence of this radiolabelled lipid either in the liposome or leaked into surrounding media can be measured. With this in mind, dialysis was used to study the retention of ^{3}H-Cholesterol within the liposome membrane. Liposomes were prepared by the lipid-film hydration (LH) method, dehydration-rehydration vesicle (DRV) or double emulsion method as described in Sections 2.2, with the inclusion of trace amounts of ^{3}H-Cholesterol. This liposome preparation was placed in dialysis tubing (MWCO; 3 kDa) and subsequently placed in dialysis media which simulates the in vivo environment (FCS/Tris; 50/50 v/v). Aliquots of dialysis media were removed (1 mL) at various time points (30 minutes, 1 h, 3 h, 5 h, 18 h, 48 h and 96 h). In order to maintain 'sink' conditions, this volume is replaced with new dialysis media. These aliquots were added with 10 mL Ultima Gold scintillation fluid before being measured by standard scintillation counting procedures using a 1600TR Liquid Scintillation Counter.

2.4.2. Radiolabelling of protein antigen

Known volumes of either lysozyme or OVA (both at 10 mg/mL), H56 (at a concentration of 1.2 or 1.6 mg/mL) in combination with ^{125}I were added into an IODO-GEN® tube and left for 1 hour with intermittent swirling to allow the specific protein to be radiolabelled. Typical volumes for the production of 1 mL radiolabelled lysozyme would require the mixing of 100 µl lysozyme (10 mg/mL) with the addition of 9 MBq of ^{125}I. Whereas typical volumes for the production of 1 mL radiolabelled OVA would require the mixing of 100 µL OVA (10 mg/mL) with the addition of 3 MBq of ^{125}I. During subsequent experiments the H56 TB antigen was radiolabelled for used in adsorption and biodistribution studies. This would require the mixing of 0.2 mL H56 antigen (1.6 mg/mL) with 3 MBq ^{125}I. These IODO-GEN® tubes contain an oxidizing reagent, which can convert NaI into a reactive iodine molecule – which can subsequently insert into the tyrosol group of amino acids in all proteins, or the aromatic residues of other molecules (Figure 2.6). Therefore this leads to the protein or antigen being able to be radiolabelled with Iodine-125 (^{125}I). Lysozyme is known to contain 3 tyrosine residues (iodinatable sites), whereas OVA has 9 tyrosine amino acids.

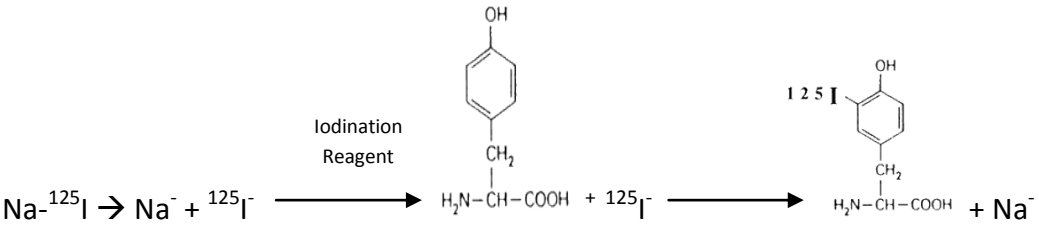


Figure 2-6. Diagram showing iodination of the amino acid tyrosine using Pierce oxidizing reagent present in Pierce iodination tubes. Two possible iodination sites exist on tyrosine at the ortho ring position either side of the hydroxyl group (Adapted from (Salacinski *et al.*, 1981)).

2.4.3. Separation of bound from free I^{125} using a Sephadex gel filtration column

A gel filtration column (with a column volume of 8 cm^3) containing Sephadex G-75 beads was made in order to separate bound from free ^{125}I (Figure 2.7). In order to make the column, 1g of Sephadex G-75 was rehydrated at 90 ˚C for 1-2 hours with 20 mL Tris (10 mM) buffer (at either pH 4.0 for lysozyme or pH 7.4 for OVA and H56 antigen). In order to act as a stopper, a small amount of cotton wool was placed at the bottom of a 5 mL glass pipette – and placed in a clamp and stand. This bead slurry of Sephadex G-75 was then poured into this 5 ml glass pipette, in order to minimise protein binding to the column. The ^{125}I /lysozyme (or OVA) solution (~ 100 µL) or H56 (~ 500 µL) was then added to the column and elucidated using 10 mM Tris buffer (at either pH 4.0 for lysozyme or pH 7.4

for OVA and H56) – this was in order to maintain a steady flow of buffer through the column in order to separate bound from free I^{125}. Aliquots of 0.25 mL were then collected at 1 minute intervals for 40 minutes. These aliquots were collected and analysed for ^{125}I content using a Cobra CPM Auto-Gamma counter. Verification of the presence of protein (lysozyme, OVA or H56) was carried out in separate BCA assays and the absorbance results (at 562 nm) were compared against a standard curve (0-1 mg/mL) for that specific protein which was prepared at the same time (as described previously in Section 2.3.4). The samples with the highest protein content and ^{125}I content (Figure 2.7) were collected, pooled together and diluted for future experimental use as radiolabelled protein in antigen adsorption/entrapment, release and biodistribution studies.

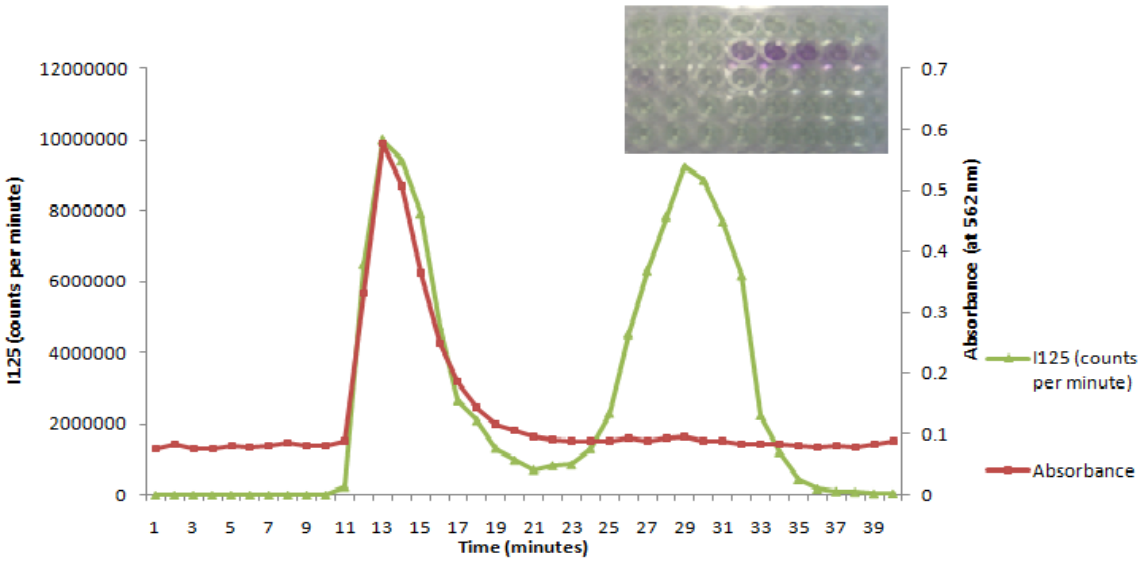


Figure 2-7. Separation of radiolabelled protein (OVA) from free radiolabel using a Sephadex G-75 Column. Aliquots high in protein and gamma counts (radiolabelled protein) are pooled together and diluted for future use.

2.4.4. Adsorption of radiolabelled protein antigen to liposomes

In order to show the degree of protein antigen adsorption to both cationic and anionic liposomes, ^{125}I-labelled lysozyme, OVA or H56 was added to each liposome formulation (DDA/TDB and DSPS/TDB) at an *in vivo* concentration of 5 µg per dose. In order for adsorption to take place this mixture was left for 1 hour with intermittent swirling. This sample was then made up to 1 mL (1:5 dilution) using Tris 10 mM buffer (pH 7.4) and a 100 µL aliquot was used in order to determine the total amount of radioactivity present in the sample. The remaining sample was centrifuged at 45,000 rpm for 45 minutes at 4 °C (using an Optima MAX-XP ultracentrifuge (Beckman Coulter Inc.,

Fullerton, CA)) in order to separate free protein (in supernatant) from bound protein (in liposome pellet). After two washes (with Tris 10 mM pH 7.4), the ^{125}I recovery in the pellet and supernatant was measured using a Cobra CPM Auto-Gamma counter. The percentage of protein adsorbed to liposomal formulations was calculated as a percentage of the radioactivity recovered from both the supernatant (free protein) and pellet (bound protein) as well as the total amount of radioactivity present in the sample.

2.4.5. Protein entrapment by DRV and double emulsion vesicles of cationic DDA/TDB and anionic DSPS/TDB liposomes

In order to measure the proportion of protein, either entrapped within the liposomes (bound protein) or within the supernatant (free protein), radiolabelled (^{125}I) protein was added in the preparation process; either in the inner water phase (for the double emulsion method) or with the sonicated liposomes (for the dehydration-rehydration method). For these liposome preparation methods which involve the entrapment of antigen, an aliquot of the sample was taken (200 µL) and this was made up to 1 mL using Tris buffer (10 mM; pH 7.4) and 100 µL of the sample will be aliquotted to determine the total amount of radioactivity present. The percentage of entrapped (liposome-bound) protein will then be determined as in Section 2.4.4.

The formation of a more pronounced liposome pellet can be achieved using a solution of OVA (1 mg/mL) in Tris buffer (10 mM;pH 7.4) during these studies. This was in order so that supernatant can be efficiently removed without disturbing the liposome pellet, thus enabling efficient measuring of radiolabelled protein associated with each fraction (liposome pellet or supernatant).

2.4.6. Antigen release kinetics in simulated *in vivo* conditions

Antigen release from liposomal formulations (multilamellar vesicles, double emulsion liposomes, small unilamellar vesicles and dehydration-rehydration vesicles) was measured in simulated *in vivo* conditions (FCS/Tris; 50:50 v/v) as well as being measured in control conditions (Tris buffer; 10 mM pH 7.4) over a 96 hour time period. Aliquots of each formulation, consisting of equal volumes of liposome with radiolabelled antigen, were diluted 1:5 in either FCS/Tris (50:50 v/v) or Tris, and incubated in a shaking water bath at 37 °C for 96 hours.

The % antigen loading and release was determined at selected timepoints (0 h, 1 h, 3 h, 5 h, 18 h, 48 h and 96 h) using the centrifuge method as described previously in Section 2.4.4. At these selected time points, the eppendorf tube was removed from the water bath and processed as described previously in Sections 2.4.4 and 2.4.5 in order to determine the % antigen retention with the liposome delivery system.

2.4.7. Radiolabelling of resiquimod and subsequent separation of radiolabelled TLR agonist from free radiolabel

During this investigation, it was required to determine the agonist loading and also to track the biodistribution of TLR agonist *in vivo*. Resiquimod or DSPE conjugated resiquimod is radiolabelled with ^{125}I in an IODO-GEN® tube and left for 1 hour with intermittent swirling (Figure 2.7). Resiquimod can be radiolabelled due to the presence of aromatic residues within this TLR agonist.

Due to the radiolabelling procedure not being 100 % efficient there may still be some free ^{125}I. This was removed by using sodium thiosulphate (Na_2SO_3), which will convert free iodine to iodide ($I_2 \rightarrow 2I^-$). As a control during this study, radiolabelled resiquimod (^{125}I) was added post lipid film-hydration (LH) to the CAF01 formulation (with incorporation of DSPE within the bilayer) in order to determine agonist loading. Also the radiolabelled lipid-TLR conjugate was incorporated within the CAF01 delivery system during the initial process so that TLR agonist can be retained by the delivery system. The percentage loading and release kinetics of resiquimod TLR agonist, when associated within these liposome formulations, can also take place in simulated *in vivo* or control conditions, using the method described in Section 2.4.6. Through radiolabelling of these novel vaccine formulations we were able to track the biodistribution of resiquimod agonist *in vivo* as will be described in Section 2.6.

2.4.8. Radiolabelling of PolyI:C and subsequent separation of radiolabelled TLR agonist from free radiolabel

It was required to radiolabel polyI:C in order to determine whether loading of agonist has a subsequent effect on vesicle characteristics (whole dose or stepwise addition of polyI:C) or whether vesicle size and zeta potential remain constant. In an initial reaction, polyI:C was added with thermosensitive alkaline phosphatise (TSAP), Tris-HCl (70 mM; pH 7.6) and dithreitol (DTT; 5 mM)

before being incubated at 37 °C for 30 minutes. Subsequently polyI:C was radiolabelled through heat inactivation of TSAP in which dephosphorylated polyI:C was reacted with $MgCl_2$ (10 mM), polynucleotide kinase (5 mM) and ^{32}P-ATP (1.35 µM). This reaction mixture was incubated at 74 °C for 15 minutes, before being transferred to a water bath for incubation for 5 minutes (at 37 °C). This was followed by polyI:C purification through repeated centrifugation steps (at 10,000 x g) in order to obtain highly pure radiolabelled polyI:C for use in experiments.

During agonist loading studies, radiolabelled polyI:C can be added either stepwise or at the whole dose to liposomes. Percentage loading can be determined as the quantity of radioactivity retained within the liposome pellet following ultracentrifugation (125,000 x g, 4 °C, 45 minutes). After two washes (with Tris 10 mM pH 7.4), the ^{32}P recovery in the pellet and supernatant was measured by standard scintillation counting procedures using a 1600TR Liquid Scintillation Counter.

2.5. Methods and techniques in chemical synthesis

2.5.1. Design and synthesis of a novel lipid-TLR agonist conjugate

It was proposed to design and synthesise a novel lipid-TLR agonist conjugate consisting of the lipid 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and the TLR7 agonist resiquimod (otherwise known as R848) which possesses anti-viral and anti-tumoral activities. Both lipid and TLR agonist are displayed in Figures 2.8 A and B.

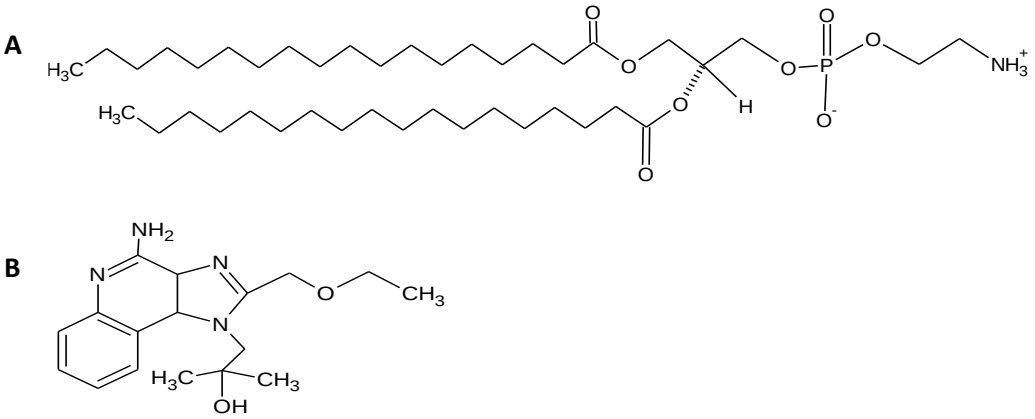


Figure 2-8. Chemical structures of (A) DSPE lipid and (B) Resiquimod.

It was hypothesised these two molecules are able to form a conjugate due to the presence of a hydroxyl (- OH) group on resiquimod which provides an ideal linker molecule in order to bind to the

amine (- NH_3^+) group present on the lipid head group of DSPE. This chemical reaction will require a strong activating agent, termed diisopropyl azodicarboxylate (or DIAD) and succinic anhydride. This study will require two important chemical reactions; firstly DSPE and succinic anhydride will be reacted together (under heat and magnetic stirring) in order to form the DSPE lipid with amide bond and terminal carboxylate. Upon formation of this structure, we will subsequently add the TLR agonist resiquimod [which contains an alcohol group (-OH)] in the presence of the diisopropyl azodicarboxylate (DIAD) activating agent and triphenylphosphine (TPP). This will lead to the formation of an ester (due to the Mitsunobu reaction) made up of DSPE conjugated to the resiquimod TLR7 agonist.

2.5.2. Step 1 Succinylation Reaction

DSPE lipid and succinic anhydride (SA) were dissolved in chloroform/methanol (9:1 v/v) and chloroform respectively (Hermanson, 2008). These starting materials were added together (under magnetic stirring) at a number of different ratios (1:2 M/M, 1:5 M/M and 1:10 M/M) in separate reactions in order to determine optimal reaction conditions to produce the correct reaction product (which was determined to be 1:5 M/M). The temperature was either maintained at room temperature or increased to 40 °C also to determine optimal reaction conditions (which was determined to be at room temperature). This reaction is schematically represented in Figure 2.9. At various time points, aliquots (around 50 µL) of the reaction mixture were kept for future analysis by thin layer chromatography (TLC). Upon completion of the reaction, excess water is 'quenched' by the addition of sodium hydroxide (NaOH) at a 1M concentration before separation of the reaction product using a separation column. Upon separation, dried magnesium sulphate ($MgSO_4$) was added before filtration. The reaction product (in chloroform) was dried using rotary evaporation and placed in the dessicator for future analysis by infra-red (IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy.

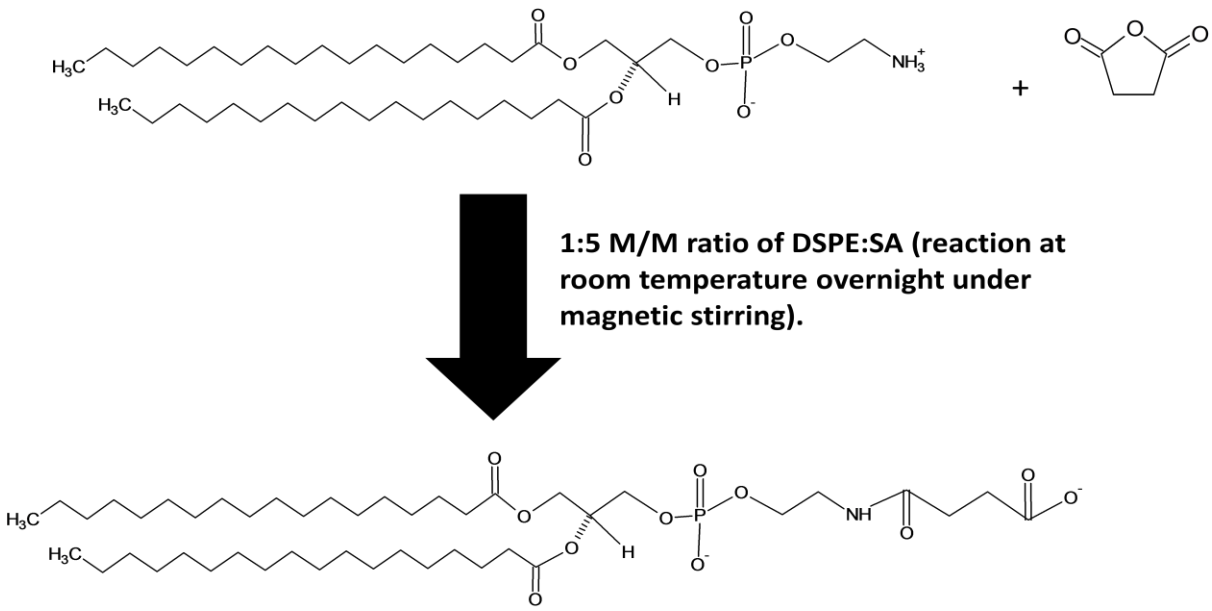


Figure 2-9. Schematic Diagram of the Step 1 Succinylation Reaction.

2.5.3. Step 2 Mitsunobu Esterification Reaction

The product from the Step 1 reaction is added with the TLR agonist resiquimod in the presence of the activation (coupling) agent, diethylazodicarboxylate (DEAD) and triphenylphosphine as part of a Mitsunobu esterification reaction (Mitsunobu and Yamada, 1967, Mitsunobu et al., 1967). As the resiquimod TLR7 agonist contained a tertiary alcohol group, the –OH bond may be sterically hindered therefore a different activation agent may be required, therefore diisopropyl azodicarboxylate (DIAD) was used, in combination with TPP, as the activation agents in this reaction. The starting materials, Step 1 reaction product and TLR agonist resiquimod respectively, were added together during this reaction at a 1:1 M/M ratio as we require 1 molecule of DSPE to be attached or conjugated to 1 molecule of resiquimod. This reaction was taken place at room temperature over several hours, or overnight if required under magnetic stirring. This reaction is schematically represented in Figure 2.10. At various time points (every 1 hour), aliquots of the reaction mixture were kept for future analysis by thin layer chromatography (TLC). Upon completion of the reaction as described previously excess water is 'quenched' by the addition of sodium hydroxide (NaOH) at a 1M concentration before separation of the reaction product using a separation column. Upon separation, dried magnesium sulphate ($MgSO_4$) was added before filtration. The reaction product (in chloroform) was dried using rotary evaporation and placed in the dessicator for future analysis by infra-red (IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy.

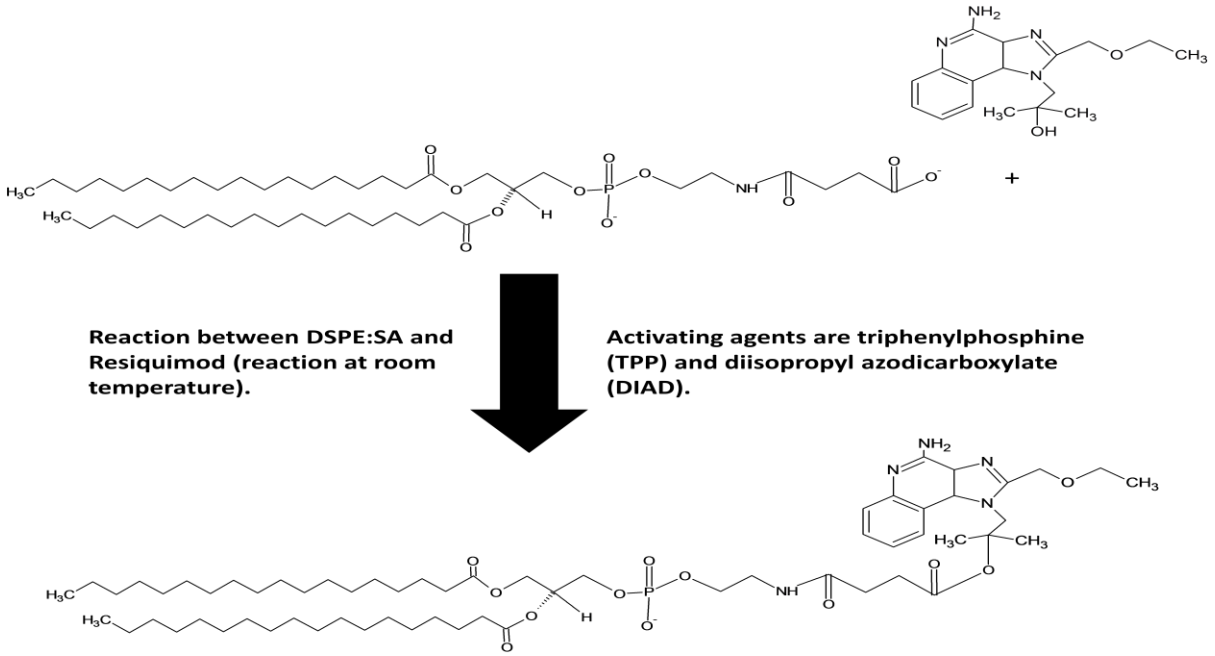


Figure 2-10. Schematic Diagram of the Step 2 Mitsunobu Esterification Reaction.

2.5.4. Thin Layer Chromatography (TLC)

As described previously in Sections 2.5.2 and 2.5.3, aliquots of the reaction mixture were decanted at 1 hour time points ready for analysis by thin layer chromatography (TLC). Samples from these aliquots were taken up by capillary tubes and spotted on to TLC silica-plates (stationary phase). If multiple spotting for each sample was required, then the spot was allowed to dry before the next spot was applied in the same position. Once the plate had been spotted it was allowed to dry before being placed into a sample chamber containing solvent (ethyl acetate) as the mobile phase, which was below the line containing the samples of interest. During experiments, the mobile phase was differed to show if there was any observed effects on the moving of compounds in the reaction mixture (spotted onto the plate).

The TLC plate was allowed to run until the solvent front had been reached. The plate was left to dry before being placed under UV light in order to view the movement of the various molecules (in the reaction mixture) along the plate. These spots were visualised by vanillin staining or taking a camera picture of the plate under UV light. The relative frequency (R_f) of each compound in the various aliquots was determined by the following calculation;

Relative frequency (R_f) = distance travelled by sample (spot) / distance of solvent front.

2.5.5. Column Chromatography

If a number of compounds were present in the same reaction mixture, as determined by TLC, it was required to separate these compounds by column chromatography. This was carried out as described in the methodology by W. Clark Still and colleagues in 1978.

A glass column was set up containing silica and sand. Subsequently the mobile phase was run through the column (same mobile phase as used in TLC analysis). The mobile phase was allowed to run through until reaching just above the silica-sand column. The reaction mixture was then placed on the column and allowed to run through, making sure that the column was never allowed to run dry (constantly supplied with mobile phase). Aliquots (10 mL) were collected in test tubes every 1 minute. These aliquots were subsequently used in TLC analysis in order to identify and isolate the compound of interest from the reaction mixture. Test tubes containing the sample of interest were pooled together and subjected to rotary evaporation and drying procedures. These samples were then analysed by IR and NMR spectroscopy respectively.

2.5.6. Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR)

Upon overnight drying of the sample in the dessicator as described previously in Section 2.5.2, the sample was analysed by ^{1}H-NMR. Each sample (around 5 mg) was dissolved in a deuteriated solvent [either chloroform or dimethylsulfoxide (DMSO)], however this choice of solvent was kept constant between samples (starting materials and reaction product). The sample vial was then placed in a spin magnet and then into an automated sample vial collector. The sample was run for either 16 or 64 scans at room temperature. Upon initiation of the experiment, the sample proceeded to move into the vicinity of the magnet in order to be analysed. Upon completion of the experiment, a specific ^{1}H-NMR spectra was collected and analysed in order to show which functional groups were present in the reaction product or starting materials respectively.

2.5.7. Infra-Red (IR) Spectroscopy

Upon overnight drying of the sample in the dessicator as described previously in Section 2.5.2, the sample was analysed by IR. Initially a background spectrum was run on the spectrometer

(ThermoScientific Nicolet; ID5 ATR). Then a small sample of powder (few milligrams) from either the starting material or reaction product was placed on the ID5 ATR Diamond sample window holder before the pressure screw was placed down upon the sample. The sample was then ran in order to obtain a spectra of the starting material or reaction product in order to show which functional groups were present in the sample mixture.

2.6. *In vivo* studies

2.6.1. Ethics Approvals for *in vivo* studies

BALB/c and C57/BL6 mice were the strain of mice used for research in biodistribution and vaccine studies respectively and were obtained from Charles River Laboratories UK Ltd. (Margate, Kent, UK). Experimentation strictly adhered to the 1986 Scientific Procedures Act (UK) and all protocols were carried out in a designated establishment and are subject to ethical review.

All experimentation undertaken at Statens Serum Institut, Denmark strictly adhered to the regulations of the Danish Ministry of Justice and animal protection committees and was in compliance with European Community Directive 86/609. All protocols were subject to ethical review and were carried out in a designated establishment. CB6/F1 mice were obtained from Harlan Scandinavia, Allerod, Denmark.

During this thesis, a number of biodistribution studies were carried out. The first of which investigated the effect of liposome surface charge on the formation of an antigen depot (either H56 vaccine or the cationic model protein lysozyme) at two sites (SOI and PLN). The second study concentrated on the effect of liposome preparation method and charge on the ensuing liposome and antigen depot (at these same two sites). In further studies, the biodistribution of TLR agonist as well as the delivery system was also measured.

2.6.2. General conditions for mice

BALB/c mice were used for *in vivo* biodistribution studies in order to study the movement of liposomes and antigen from the site of injection (SOI). It was decided that 4 mice/group was sufficient to produce quality and sufficient data. Mice were housed under conventional conditions

(22 °C, 55 % humidity, 12 h day/night cycle) in their experimental groups (4 mice/cage) and were given a standard diet *ad libitum*. All mice were female and purchased at 6-8 weeks of age at the start of the experiment.

2.6.3. Preparation of radiolabelled vaccine formulations for biodistribution studies

Four to seven days prior to injection with the radiolabeled vaccine (liposome) formulation, mice were injected subcutaneously (s.c) into the scruff of the neck with 200 µL (Figure 2.11) pontamine blue [Sigma Aldrich; 0.5% w/v in PBS buffer (10 mM; pH 7.4)]. Pontamine blue stain is phagocytosed by monocytes and therefore a suitable marker for aiding location of lymph nodes during dissection (Tilney, 1971).

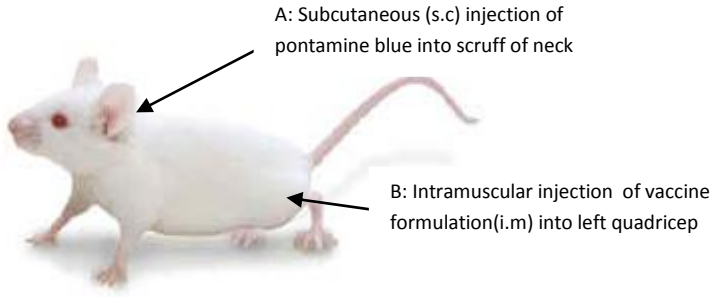


Figure 2-11. Routes of administration for injection during biodistribution studies [A: Subcutaneous injection (s.c) into scruff of neck; B: intramuscular (i.m) injection into left quadricep].

Biodistribution studies were conducted with ^{3}H-labelled liposomes (radiolabelled with ^{3}H-Cholesterol) and ^{125}I -labelled protein antigen. Due to the dilution of antigen during the gel filtration step after iodination, the concentration of the liposomes had to be increased 2-fold so that upon mixing of equal volumes of protein and liposome the final concentration remained 1.25 mg lipid/mL equivalent to 250 µg lipid:dose. Furthermore, the final concentration of the liposomes and antigen had to be further concentrated as whilst this dosage volume (200 µL) was suitable for subcutaneous (s.c.) injection, the recommended volume for intramuscular (i.m) injection was 50 µL (Figure 2.11). Liposomes were actually rehydrated in a volume of buffer 8-fold less than usual, followed by the addition of an equal volume of antigen at a concentration 2-fold higher than required so that upon dilution the correct in vivo concentration is achieved (0.1 mg/mL). Approximately 1 hr prior to injection, radiolabelled (^{3}H-Chol) liposome and protein antigen (I^{125}) were mixed together and allowed to adsorb to each other (Figure 2.12). Each dose contained 250 µg lipid, 50 µg TDB and 5 µg

protein antigen (lysozyme or H56). Vaccines were given by the *i.m* route into the left quadricep with a dose volume of 50 µL (Figure 2.12). In order to provide isotonicity within the vaccine formulation, trehalose was added at a final concentration of 10 % w/v. Upon rehydration of the lipid film, trehalose was added at a concentration of 20 % w/v so that upon dilution of liposomes the final concentration was 10 % w/v.

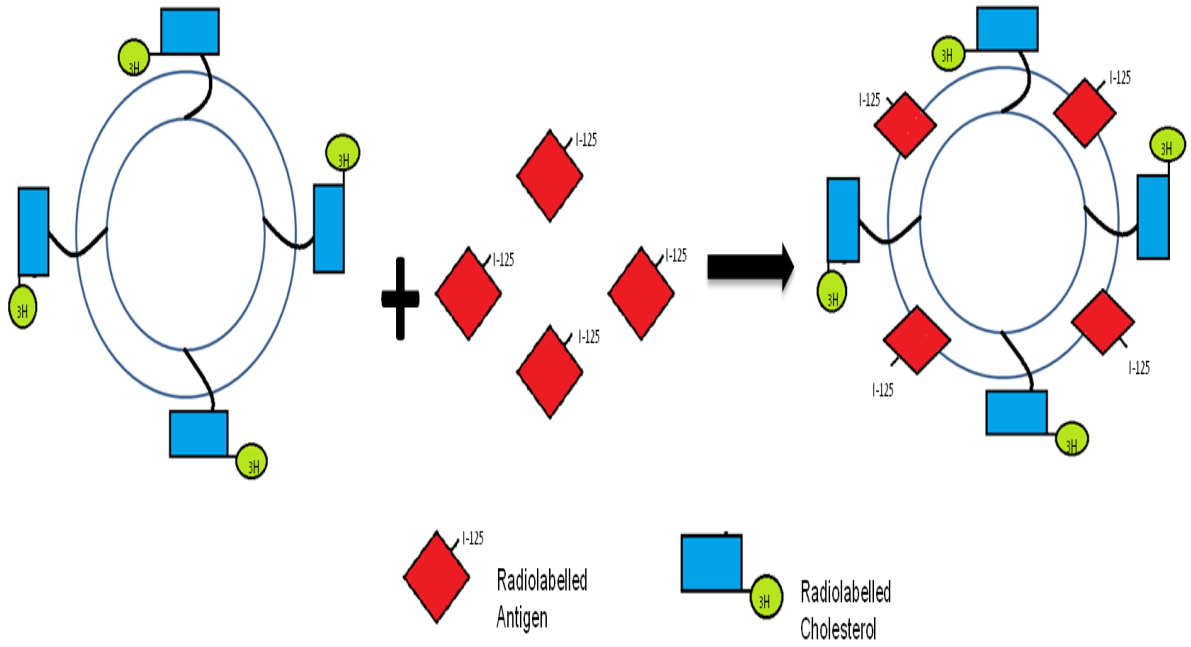


Figure 2-12. Schematic Diagram for the preparation of radiolabelled liposome vaccine formulations

In order to prepare radiolabelled dehydration-rehydration vesicle (DRV) vaccine formulations, trehalose was added (at a concentration of 10 % w/v) in the final controlled rehydration step in order to maintain isotonicity within the radiolabelled liposome vaccine formulations. Also, in order to prepare radiolabelled double emulsion (DE) vaccine formulations, trehalose was added at a concentration of 10 % (w/v) at the end of the preparation process in order to maintain isotonicity within these radiolabelled liposome vaccine formulations.

2.6.3.1. Processing of tissues

Mice were terminated at the relevant time points during this study (24 hours, 4 days and 8 or 14 days) by cervical dislocation and a number of various tissues were collected [muscle from the site of

injection (left quadricep) and popliteal lymph nodes (PLN)]. All tissues were processed in the same manner as described in a previous study (Henriksen-Lacey *et al.*, 2010a).

The major steps of this tissue processing protocol were solubilisation of tissues (muscle from vaccination site, spleen and draining lymph nodes), quantification of ^{125}I, bleaching with hydrogen peroxide (H_20_2) followed by quantification of H^3 (Figure 2.13). Each tissue sample was weighed into a γ-vial and Solvable™ (1.5 mL) added to each sample before quantifying ^{125}I presence using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA). During data analysis the percentage dose per mg of tissue from the draining lymph nodes was quantified.

Figure 2-13. Schematic diagram showing the processes involved in tissue processing to detect ^{125}I and ^{3}H presence following injection of dual-radiolabelled components (Henriksen-Lacey et al, 2010).

As described by Henriksen-Lacey et al (2010); to verify that the presence of undigested tissue and whole organs did not affect the count rate, all samples from original experiments were counted for ^{125}I prior and post-tissue digestion with no difference in the results noted (results not shown).

All tissue samples were solubilised using Solvable™, and heated at 50 °C until tissues were fully solubilised. These samples were then quantified for the percentage of ^{125}I-antigen retention. Following which these samples were allowed to cool and then transferred into 20 mL plastic scintillation vials. Hydrogen peroxide (200 µL) was added to each sample and once fully bleached (sometimes additional heating of samples was required to ensure complete digestion), Ultima Gold™ scintillation fluid (10 mL) was added to each sample and ^{3}H presence quantified using a standard ^{3}H detection protocol using a 1600TR Liquid Scintillation Counter (Packard).

2.6.3.2. Quantification of the proportion of vaccine components in various tissues

For the determination of liposome (^{3}H) and antigen (^{125}I) in the different tissues, the data was presented as a proportion of the dose (% dose) at a number of sites (vaccination site, spleen and draining lymph nodes). The results were presented as percentage dose per mg tissue at the draining lymph nodes. To calculate the % dose, triplicate samples of the original dose were processed simultaneously to the tissue processing. In some cases, these doses represented a fraction of the whole dose and relevant dilution factors were considered after obtaining the cpm values. Therefore the specific % dose was calculated by dividing the radioactivity counts (for antigen or liposome in a specific tissue) divided by the total amount of radioactivity (per dose) multiplied by 100.

For all samples, the cpm values derived from the Cobra™ CPM Auto-Gamma® counter (relating to ^{125}I presence) did not need additional processing as ^{3}H scintillation counts cannot be detected by the gamma counter (Figure 2.13) without the presence of a scintillant; solely the removal of background values (average of three Solvable™ samples) was undertaken.

2.6.3.3. Preparation of ^{125}I standard curve in order to factor out ^{125}I counts measured on the scintillation counter

For the determination of actual ^{3}H (tritium) counts, the amount of ^{125}I (from radiolabelled antigen) had to be considered as ^{125}I counts detectable on a scintillation counter. There is no such problem measuring gamma (γ) counts, as this is the only radiation detected on a gamma counter. A method was devised whereby the ^{125}I could be factored out by the use of a standard curve (communication with Malou Henriksen-Lacey). Triplicate samples of ^{125}I starting at an activity equivalent to the dose administered *in vivo* (~ 100 kBq) were diluted 2-fold until background levels (~ 20 cpms) were reached (therefore about 18 dilutions).

These samples were counted using a γ-counter and then transferred and processed as the tissue samples would be. The samples were then counted on a scintillation counter using the same detection protocol as used for the tissue samples (as described previously in Section 2.6.3.1 and Figure 2.13). For each sample, a plot of the cpm (counts per minute) values derived from the gamma counter (x-axis) was plotted against the cpm values from the scintillation counter (y-axis) was made. The line of best fit and equation for samples below 50,000 cpm (~ 2 % of the dose or less) and those above 50,000 cpm (~ 2 % of the dose or more) was carried out. The two equations (derived from these standard curves) were used to calculate the effective interference that the ^{125}I would have on

the ^{3}H values determined by scintillation counting. So therefore, uncorrected scintillation counts were put into the equation of choice (depending on the number of gamma counts) in order to determine the actual number of scintillation counts.

2.6.4. Immunisation Studies in Mice

The first and final immunisation studies were conducted at Aston University. Whereas, the second *in vivo* study was carried out in collaboration with Statens Serum Institute (Copenhagen, Denmark). These immunisation studies have been subject to ethical review and carried out in a designated establishment. The number of mice per experimental group and allocated per researcher was not constant between experiments – Table 2.1 outlines the experimental design for each of these in vivo studies.

The aim of Study 1 was to investigate the effect of liposome surface charge on the ensuing immunogenicity of vaccine formulations. The H56 TB subunit vaccine was adsorbed to cationic DDA/TDB and anionic DSPS/TDB (at an *in vivo* concentration of 5 µg or 0.1 mg/mL). During Study 2, liposomal vaccine formulations were prepared at Statens Serum Institute by either the lipid-film hydration (LH) method, double emulsion (DE) solvent evaporation method or the dehydration-rehydration vesicle (DRV) method with the liposomes adsorbing Ag85B-ESAT-6-Rv2660c (H56) antigen to a final concentration of 0.1 mg/mL (5 µg/vaccine dose). These formulations were prepared with the addition of trehalose (10 % w/v) to the hydration buffer in order to maintain isotonicity. During the DE preparation process the H56 antigen was added in the primary water phase whereas during the DRV preparation process the antigen was added to small unilamellar vesicles (SUVs) prior to freezing (- 70 °C), overnight freeze drying (at - 40 °C) and rehydration the following day. All mice were immunised intramuscularly (i.m.) into the left quadriceps with the proposed vaccine (50 µL/dose) three times (at days 0, 14 and 28), with two week intervals between each immunisation.

The aim of the final study 3 was to show the effect of inclusion of TLR agonists within the delivery system on the resulting immune response. The TLR 7 agonist, resiquimod, was included within these formulations.

Table 2-1. Summary of vaccine immunisation studies carried out during this thesis

FORMULATION	STUDY 1 (ASTON)	STUDY 2 (SSI)	STUDY 3 (ASTON)
1	MLV DDA/TDB:H56 (5)	MLV DDA/TDB:H56 (6)	H56 (5)
2	MLV DSPS/TDB:H56 (5)	MLV DSPS/TDB:H56 (6)	MLV DDA/TDB/DSPE: Resiquimod: H56 (5)
3	H56 (5)	DRV DDA/TDB:H56 (6)	MLV DDA/TDB/DSPE= Resiquimod:H56 (5)
4	PBS (5)	DRV DSPS/TDB:H56 (6)	Resiquimod alone (5)
5		DE DDA/TDB:H56 (6)	MLV DDA/TDB: H56 (5)
6		DE DSPS/TDB:H56 (6)	
7		H56 (6)	
8		Naive (6)	

2.6.4.1. Preparation of vaccine formulations for studies

The liposome-based vaccine formulations were prepared as in Sections 2.2 for multilamellar vesicles (MLV), dehydration-rehydration vesicles (DRV) and double emulsion (DE) vesicles. The only difference in the liposome preparation stage was with the use of trehalose (at a final concentration of 10 % w/v) in the rehydration buffer in order to maintain isotonicity. When PBS buffer alone was used as a control in experiments there was no need to add trehalose as this buffer is already isotonic in nature. Mice were immunised with a dose volume of 50 µL into the left quadriceps (Figure 2.14).

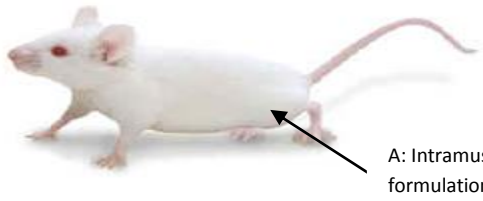


Figure 2-14. Routes of administration for injection during vaccine immunisation studies [A: intramuscular (i.m) injection into left quadricep.

2.6.4.2. Antibody analysis

Serum samples were collected for the detection of IgG, IgG1 and IgG2b antibodies (AbD Serotec, Oxford, UK). Blood (50 µL) was collected using capillary tubes dipped in 1 % heparin (Sigma Aldrich, UK). The blood was diluted 10-fold in PBS; 10 mM, pH 7.4 (50 µl blood + 450 µl PBS) and centrifuged for 5 minutes (at 10,000 x g) to obtain serum. The serum was then decanted in order to be analysed

for the presence of antibodies and cytokines. In order to achieve an initial 1/400 dilution, the blood sera was subsequently diluted 1/20 using PBS if the volume ratio of sera:red blood cells is 1:1. Blood sera was subsequently diluted down the ELISA plate in order to determine the log10 reciprocal endpoint dilution where the absorbance was greater than 0.2.

Standard ELISA protocol was used to detect antibodies. Briefly, plates were coated overnight with H56 antigen (5 µg/mL), blocked for 1 hour (with Marvel milk; 4 % w/v in PBS) and 100 µL serially diluted serum added. Serum (10 µL) was added to the appropriate wells of row A and mixed well – a 50 µl sample was removed and added to the wells in row B. Again, this was thoroughly mixed and the serial dilutions continued until lastly 50 µL of diluted serum was removed from the wells of row A and H so that the total volume of serially diluted serum in all wells was 100 µL. Each sample was investigated in duplicate, and were allowed to incubate for 2 hours. Goat anti-mouse IgG (1:500), IgG1(1:4000) and IgG2b (1:4000) were added to wells for 1 hour followed by addition of ABTS substrate (in citrate buffer); samples were detected by reading the absorbance at 405 nm and the results expressed as the $\log_{10}$ of the reciprocal of the end-point dilution giving an optical density (O.D) of ≥ 0.2. Between each of these stages, the contents of each of the wells are aspirated before the plate is washed three times using PBST wash buffer (40 g NaCl, 1 g KCl, 1 g KH_2PO_4, 7.2 g $Na_2HPO_4(2H_2O)$, 0.4 mL Tween 20, 5 L ddH2O).

2.6.4.3. Proliferation of restimulated splenocytes and lymph nodes *ex vivo*

Spleens and popliteal lymph nodes (PLN) from individual mice were removed on day 49 of the vaccination study and cell suspensions obtained. Each spleen was placed in a 7 mL bijoux tube containing 5 mL PBS, making sure that this solution is kept ice-cold. Whereas for the PLN, these are pooled together for each immunisation group (also making sure that this solution is kept ice-cold).

Spleens and PLN were mashed and strained using a cell strainer into a 50 ml centrifuge tube before the addition of 10 mL cold RPMI 1640 solution without L-glutamine (pH 6.9-7.2), containing 10 % FBS and 1 % PSG. The cell debris was allowed to settle in the 50 mL centrifuge tube for 5 minutes before the supernatant (13 mL) was transferred into a 15 mL centrifuge tube. This tube was further centrifuged at 1200 rpm for 10 minutes (15 °C) and the supernatant discarded before a further wash and centrifugation step using 10 mL cold RPMI solution. The final pellet was resuspended in 5 mL or 400 µL cold RPMI (for splenocyte cells and lymph node cells respectively) before performing a cell

count, and the cell number was subsequently adjusted to 0.2-1 x 10^7 cells/ mL for splenocytes and LN (making sure that the same cell number was used for each mouse/organ).

Determination of cell number for experiments was achieved via tryphan blue exclusion whereby 20 µL of resuspended cells were removed and mixed with 80 µL of tryphan blue. Using a hemocytometer, cells excluding tryphan blue were quantified visually by microscopy (Figure 2.15) and the number of cells/ml was calculated using the following equation:

No of cells/ml = no. cells/sq x dilution factor x 10^4

Where: no. cells/sq is the average of 5 squares in the hemocytometer .
Dilution factor is 5 if resuspended cells and tryphan blue are used in 1:5 v/v ratio.
10^4 is the multiplication factor related to the volume of the hemocytometer grid.

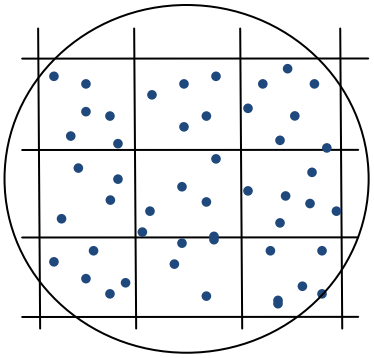


Figure 2-15. Visualisation of cells via microscopy. Viable cells are stained using tryphan blue exclusion.

Splenocytes and PLN cells were plated in 96-well plates (0.2-1 x 10^7 cells/mL) and stimulated with either ConA (2 µg/mL) as a positive control, medium only (as a negative control) or H56 antigen (Ag85B-ESAT-6-Rv2660c) at a concentration of 0.05, 0.5 and 5 µg/mL in cold RPMI in order to make a total volume of 200 µL in each well of the plate. These splenocyte and lymph node cells were incubated (37 °C, 5 % CO_2, 95 % humidity) for 48 hours (with 6 wells per mouse, formulation and restimulation condition), following which the supernatants were decanted for future analysis. If not being used immediately, these supernatants were frozen at – 20 °C.

In a separate plate, splenocytes were incubated (37 °C, 5 % CO_2, 95 % humidity) for 72 hrs after which 18.5 kBq ^{3}H-thymidine (GE Healthcare) was added per well. Incubation occurred for a further 24 hrs before the cells were harvested using a cell harvester (Titertek) with quartz filter mats and ^{3}H detected using standard scintillation counting procedures (as described previously) with each mat

being placed into a plastic scintillation vial with the addition of 5 mL Ultima Gold™ scintillation fluid per sample. Each spleen was assayed in triplicate under each condition.

2.6.4.4. Cytokine analysis from ex vivo restimulated splenocytes

Splenocyte suspensions restimulated with ConA or Ag85B-ESAT-6 were prepared as outlined in Section 2.6.4.3. After incubation, supernatants were removed and pooled according to spleen and restimulation condition. Duoset® sandwich ELISAs were used to detect IL-2, IL-5, IL-6, IL-10 and IFN-γ (RnD, Abingdon, UK) in the supernatants.

In brief, ELISA plates were coated with cytokine-specific capture antibody (IFNγ – 4 μg/mL; IL17 – 2 μg/mL; IL2 - μg/mL; IL5 – 1 μg/mL; IL10 – 4 μg/mL; IL6 – 2 μg/mL; IL1β – 4 μg/mL; IL33 – 0.8 μg/mL; IL18 – 4 μg/mL), blocked with 1 % bovine serum albumin (BSA) and then samples and serially diluted standards added. After 2 hrs incubation, plates were washed followed by addition of cytokine-specific detection antibody (IFNγ – 100 ng/mL; IL17 – 400 ng/mL; IL2 – 400 ng/mL; IL5 – 100 ng/mL; IL10 – 300 ng/mL; IL1β – 2.5 μg/mL; IL18 – 2.5 μg/mL; IL33 – 200 ng/mL) , streptavidin-HRP conjugate (in dark), TMB substrate solution (in dark) and stop solution. The OD at 450 nm was measured and a 4-parameter sigmoidal standard curve for each cytokine standard created using GraphPad Prism to determine cytokine concentrations in unknown samples accordingly. Between each of these stages, the contents of each of the wells are aspirated before the plate is washed three times using PBST wash buffer (40 g NaCl, 1 g KCl, 1 g KH_2PO4, 7.2 g $Na_2HPO_4(2H2O)$, 0.4 mL Tween 20, 5 L ddH2O).

2.6.4.5. Cytokine analysis at the site of injection

To determine the effect of vesicle charge and preparation method on the production of IL-1β, IL-18 and IL-33 at the site of injection (SOI), the method described by Sharp et al was used (Sharp *et al.*, 2009). Muscle from the SOI was excised 3 weeks after the final injection. The quadriceps muscle was removed and 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, 1 % v/v Sigma protease inhibition mixture and 0.02 % NaN_3). If not processing the tissue straight away, the legs could be flash frozen using liquid N_2 and transferred into a labelled bijoux tube and placed in the freezer at – 70 °C.

Samples were sonicated (2 x 15 s) and centrifuged (3600 rpm, 20 min, 4 °C) before removal of 1.4 mL of the supernatant layer into an eppendorf tube. These samples were then used for the detection of IL-1β, IL-18 and IL-33 cytokines using a standard ELISA protocol (as described previously in Section

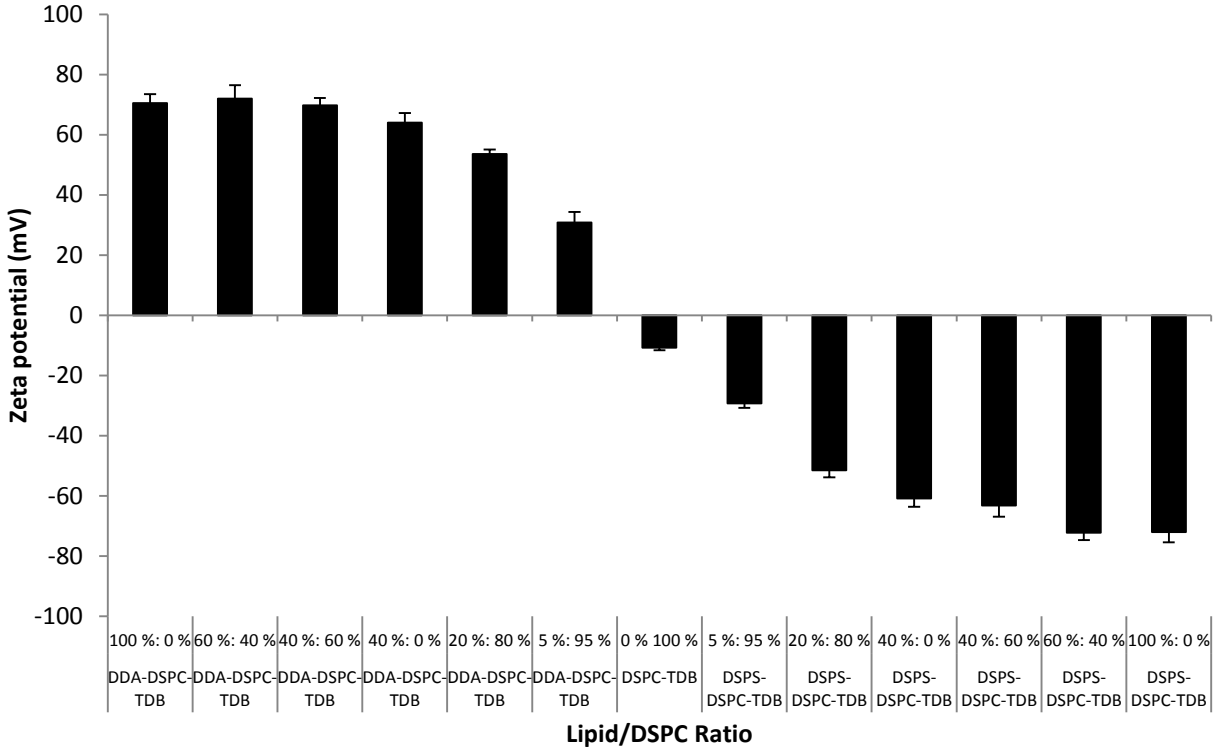
2.6.4.4) and all of these experiments were carried out in duplicate. The OD at 450 nm was measured and a 4-parameter sigmoidal standard curve for each cytokine standard created using GraphPad Prism to determine cytokine concentrations in unknown samples accordingly.

2.6.5. SSI Studies: The effect of liposome preparation method on vaccine immune response

This study was carried out as described previously in Section 2.6.5. Mice immunised with liposome vaccine formulations were measured for their cytokine response (from *ex vivo* restimulated splenocytes and lymph nodes) and antibody response (in the blood sera). Blood samples were drawn from the cheek one day before the second immunisation in order to measure antibodies (in the blood sera) by ELISA assay. Also 3 weeks after the last immunisation, blood samples were drawn from the cheek (50 µL) in order to measure antibodies in the blood sera. Also spleens and popliteal lymph nodes (PLN) were collected (3 weeks after the last immunisation) for experimental use in order to measure T-cell responses (by ELISA assay) as described previously (Section 2.6.3). Cells from these organs, following restimulation with antigen, were restimulated for 72 hours (rather than 48 hours) in accordance to the same protocol; which also took place for vaccine studies in Chapter 7 in order to maintain similar protocol.

2.7. Statistical analysis

During analysis of data and results, statistical analysis was carried out by one-way ANOVA (analysis of variance) at a significance level of 0.05. This was followed by statistical analysis by the post-hoc Tukey's test, in order to compare the mean differences of results between formulations. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) software (Version 15.0 for Windows). Statistically significant differences are noted in Microsoft Excel Graphs (***, $P < 0.001$; **, $P < 0.005$; *, $P < 0.05$). Each experiment was carried out in triplicate with the average mean result recorded ± the standard deviation of this set of results (represented as error bars).



Some of the results presented in this chapter have been published in the paper:

Hussain, M.J., Wilkinson, A., Bramwell, V., Christensen, D., Perrie, Y. (2013) "Th1 immune responses can be modulated by varying dimethyldioctadecylammonium and distearoyl-sn-glycero-3-phosphocholine content in liposomal adjuvants (manuscript accepted; Journal of Pharmacy and Pharmacology, November 2013).

3.1. Aims and Objectives

The aim of the work reported in this chapter was in the initial characterisation and product specification of liposomes based on dimethyldioctadecylammonium bromide (DDA), with and without the addition of the immunostimulatory glycolipid trehalose 6,6' – dibehenate (TDB). The physicochemical effects of the choice of hydration buffer and dilution buffer were also presented. A novel liposome system consisting of the anionic lipid 1,2 - distearoyl-*sn*-glycero-3-phospho-L-serine (DSPS) in combination with TDB was also formulated and observed for its measured physicochemical characteristics. The stability of both of these formulations was addressed following storage at two different temperature conditions (4 °C and 25 °C respectively). Further investigations were carried out with the aim of modulating various parameters of the delivery system, including vesicle size and surface charge, through increasing the concentration of the neutral lipid 1,2 - distearoyl-*sn*-glycero-3-phosphocholine (DSPC) within the formulation. Therefore through these initial studies the aim was to produce a product specification for various liposome adjuvants, including cationic DDA/TDB and anionic DSPS/TDB liposomes. These liposomes were to be used as platforms or vehicles for the delivery of vaccine antigen.

3.2. Introduction

A key advantage of liposome-based vaccine delivery systems is their flexible and versatile nature. Lipid constituents can be altered or tailored in order to formulate liposomes with desired physicochemical parameters. Liposomes can be formulated due to the amphiphilic nature of phospholipids, with lipids being made up of a non-polar hydrophobic tail group and a charged, polar lipid head group. Therefore due to the charged nature of the lipid head groups, this will confer the surface charge of the delivery system itself therefore formulations can be cationic (positive), neutral and anionic (negative) respectively. Within our laboratory, we have used the cationic liposome system DDA/TDB which has been shown to have proven efficacy as a vaccine adjuvant.

In terms of liposome formulation, various other factors are important in the formation of these vesicles, including the lipid transition temperature. The transition temperature can be defined as the temperature above which the lipid physical state is modified from the ordered gel phase to the disordered liquid crystalline phase. The lipid transition temperature depends on a number of criteria,

in which the carbon chain length and its degree of saturation can have a subsequent effect on liposome properties such as membrane bilayer fluidity (Perrie and Rades, 2010).

3.3. Results and Discussion

3.3.1. Investigating the effect of hydration buffer on the characteristics of cationic DDA/TDB and anionic DSPS/TDB liposomes

During this initial investigation, DDA and DSPS were investigated due to their opposing charge, being cationic and anionic respectively, as well as the fact that they have tail groups which are both double-chained and are similar in length. Also the immunostimulatory glycolipid, TDB, was incorporated within these respective delivery systems at the same ratio. Therefore these initial experiments will serve as direct comparison to show the effect of lipid charge on the subsequent formulation of liposome delivery systems.

Preliminary studies were undertaken in order to show the effect of hydration buffer on the subsequent characteristics and assembly of DDA/TDB and DSPS/TDB liposomes. Both distilled water and PBS (10 mM PBS; pH 7.4) were used as the aqueous hydration buffer in the formation of liposomes, as well as Tris buffer (10 mM; pH 7.4). This was carried out in order to help to gain initial liposome characterisation data, before model protein antigens were combined with the liposome delivery system (Chapter 4). The liposome characteristics (size, polydispersity and zeta potential) of DDA/TDB and DSPS/TDB liposomes were measured. The results are displayed in Table 3.1.

Table 3-1. Liposome characteristics of DDA/TDB and DSPS/TDB liposomes prepared by the LH-method; either hydrated in Tris buffer (10 mM; pH 7.4), distilled water or PBS buffer (10 mM; pH 7.4). Results are the mean of triplicate experiments ± SD. Significance, compared to respective Tris-buffer hydrated counterparts, was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

Formulation	Hydration buffer	Size (nm)	PDI	Zeta potential (mV)
DDA/TDB	Tris (10 mM; pH7.4)	533.8 ± 39.6	0.435 ± 0.201	80.4 ± 18.7
DDA/TDB	Distilled water	1246 ± 244.2 (***)	0.341 ± 0.043	74.1 ± 11.9
DDA/TDB	PBS (10 mM; pH 7.4)	2988 ± 337.7 (***)	0.682 ± 0.044	71.4 ± 12.1
DSPS/TDB	Tris (10 mM; pH7.4)	468.8 ± 42.2	0.730 ± 0.012	-59.7 ± 24.4
DSPS/TDB	Distilled water	369.9 ± 23.2	0.486 ± 0.060	-52.1 ± 14.5
DSPS/TDB	PBS (10 mM; pH 7.4)	659.2 ± 214.0 (**)	0.464 ± 0.063	-77.4 ± 20.0 (*)

These studies demonstrate that the choice of hydration buffer used in formulation of liposomes had a significant effect on the liposome characteristics for DDA/TDB, when compared to anionic DSPS/TDB liposomes. This was due to the varying interaction between the polar (charged) lipid head group (DDA or DSPS) with the hydration buffer in the formation and assembly of these liposomes. The use of either distilled water or PBS buffer in the hydration of DDA/TDB liposomes (Table 3.1) resulted in a significant ($P < 0.01$ and $P < 0.001$ for distilled water and PBS respectively) increase in the vesicle size (1246 ± 244.2 nm and 2988 ± 337.7 nm respectively) compared to hydration using Tris buffer (533.8 ± 39.6 nm; Table 3.1). In terms of polydispersity of cationic DDA/TDB formulations, PBS-buffer hydrated vesicles showed a significant increase compared to water and Tris, which may show that the vesicle population is more heterogenous upon using PBS as the hydration buffer (Table 3.1). This increase in vesicle size of the cationic DDA/TDB liposomes (in PBS buffer) could be caused by salt-induced aggregation of these vesicles; the presence of neutralising counterions in saline buffers has been primarily suggested to be the major cause of vesicle aggregation, due to the reduced repulsion between adjacent vesicles (Yan and Huang, 2009).

In contrast there were no such pronounced changes in the vesicle characteristics of DSPS/TDB liposomes when hydrated using distilled water (369.9 ± 23.2 nm) or PBS buffer (659.2 ± 214.0 nm) compared to Tris buffer-hydrated liposomes (468.8 ± 42.2 nm) as displayed in Table 3.1. However of these formulations, PBS-hydrated liposomes showed significant increase in vesicle size ($P < 0.05$) compared to their distilled water and Tris buffer-hydrated counterparts. In contrast in terms of the polydispersity of DSPS/TDB liposomes, Tris-hydrated vesicles showed more heterogeneity than their distilled water and Tris buffer hydrated counterparts (Table 3.1).

Overall, these results suggested that the use of hydration buffer caused an important effect in the assembly of MLV liposome delivery systems. The role of liposome surface charge (in relation to aqueous hydration buffer) has also been demonstrated in the fact that positively-charged (cationic) liposomes showed an increased size when hydrated using a salt-containing buffer such as PBS and to a lesser extent with water and Tris buffer respectively (Yan and Huang, 2009). The formation of liposomes was shown to be dependent on the temperature, lipid concentration, critical packing parameter (cpp) as well as the electrostatic interactions of the polar lipid head groups with the solvent and solute molecules in the hydration buffer (Perrie and Rades, 2010). During liposome formation, the shape of the vesicle produced during formulation can be defined by its cpp. Therefore, these initial studies suggested that buffer choice had a significant impact upon the measured characteristics of liposome delivery systems, which was due to the presence of the charged lipid head group and hydrophobic tail region. The effect of buffer on these vesicles was not

due to their charge, but was rather due to the interaction of the charged lipids with the electrolytes present probably causing the formation of bridges with the charged lipid head groups. This has been shown in the previous literature, which has shown that cationic liposomes can double in size in the presence of PBS; this was due to the phosphate head groups of PBS acting as 'binders' and effectively 'bridging' the cationic polar lipid head groups (Ciani *et al.*, 2007). Further studies have also shown that cationic liposome systems can aggregate when there is a high presence of salt within the formulation which was due to reduced electrostatic interactions between liposome systems hence leading to aggregation and the formation of larger liposomes (Wasan *et al.*, 1999). Flexible bilayer vesicles were increasingly likely to be formed as the hydrophobic tail region of the lipid (present in the formulation) increased, which occurred with double-chained lipids such as DDA, DSPS and DSPC which had a critical packing parameter between ½ and 1 (Perrie and Rades, 2010), whereas trehalose 6,6'- dibehenate (TDB) can be inserted into the bilayer in order to stabilise the liposome delivery system (Davidsen *et al.*, 2005).

3.3.2. Investigating the effect of buffer concentration on the characteristics of cationic and anionic liposomes

Given that the choice of buffer used in the formulation of liposomes was shown to impact on the vesicle characteristics, further investigations were conducted to consider the role of buffer concentration. Therefore, liposomes were diluted in a set of serially diluted buffer solutions (concentrations of 10 mM, 1 mM, 0.1 mM and 0.01 mM, as well as distilled water) before the various liposome characteristics (size, polydispersity and zeta potential) were measured (Figure 3.1).

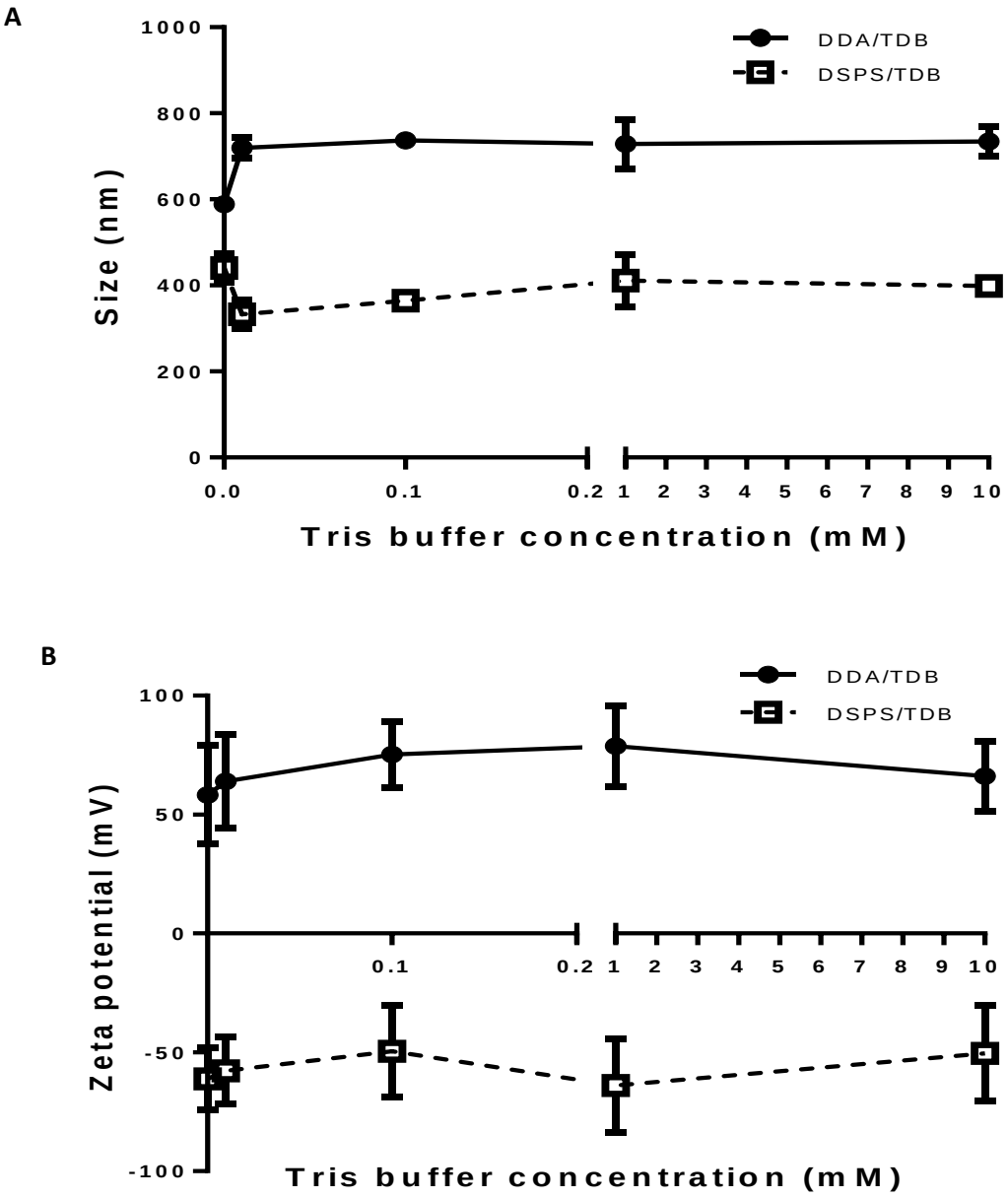


Figure 3-1. Variation in liposome characteristics (A – size; B – zeta potential) for multilamellar vesicles (MLV) of cationic DDA/TDB and anionic DSPS/TDB liposomes (hydrated in Tris buffer; 10 mM, pH 7.4) with dilution in various buffer concentrations. Results are the mean of triplicate experiments ± SD.

The results in figure 3.1 show that the impact of electrolytes present in the buffer occurs at low concentrations, with an increase in vesicle size for DDA liposomes occurring at 0.01 mM (with liposomes increasing from ~600 to over 700 nm in size; Figure 3.1A) but further increases in concentration made no impact. In contrast, the DSPS vesicles showed a drop in measured vesicle size when prepared in Tris buffer at 0.01mM concentration (from around 400 nm to 320 nm; Figure 3.1A) and again, further changes in buffer concentration had no impact on size. In both

formulations, there was no obvious trend in polydispersity with buffer concentration, which may be due to the more heterogeneous nature of the vesicles at all concentrations making differences difficult to detect (results not shown).

The cationic nature of DDA confers the positive surface charge of these liposomes, with this charge being most prominent in the case of DDA/TDB liposomes diluted in Tris buffer (1 mM; pH 7.4) in which a zeta potential of 78.8 ± 14.8 mV (Figure 3.1B) was measured. The anionic nature of DSPS confers the negative charge of these liposomes, with this charge being most prominent in the case of DSPS/TDB liposomes diluted in 1 mM Tris buffer (pH 7.4), in which a zeta potential of – 63.9 ± 19.8 mV was displayed (Figure 3.1B).

These results showed that variation in Tris buffer concentration (in the dilution of liposomes at a range of concentrations) had no major effect on the zeta potential or vesicle size of the liposome, therefore this buffer did not affect the electrical double layer or the assembly of this delivery system. Therefore we used Tris (10 mM; pH 7.4) as the hydration buffer for further formulations, whilst measuring liposomes in a weak dilution (1 mM) of the same buffer.

The vesicle size remains unaffected by the range of concentrations of dilution buffer tested throughout this study during measurement of liposome characteristics (Figure 3.1A); therefore this suggested that the hydration buffer alone determined the molecular shape and structure of the liposome delivery system. During liposome formation the hydrophobic tail regions were located on the inner surface of the membrane bilayer, whereas around this on the outer surface of the liposome contained a ‘concentric shell of hydrophilic head groups’ (Florence and Attwood, 1998) which was termed the ‘Stern Layer’. Surrounding the charged ‘Stern Layer’ was located the diffuse, Gouy-Chapman electrical double layer which neutralises the charge of the ’stern layer’ (in this case, the liposome delivery system). Therefore during these studies, positively-charged (cationic) DDA/TDB liposome delivery systems were the main focus of study and these liposomes will be surrounded by a negatively-charged Gouy-Chapman electric double layer. The thickness of this double layer was known to differ depending on the ionic strength of the solution, with increased concentrations of electrolyte leading to the compression of this diffuse layer (Florence and Attwood, 1998). However, during these studies, the low ionic strength buffer, Tris, was used in which even at high concentrations (up to 10 mM) there were no subsequent effects on vesicle characteristics (Figure 3.1).

Zeta potential analysis enabled the indirect measurement of the liposome surface charge and was measured at the plane of closest approach of ions to the liposome surface, termed the ‘shear plane’

(Delgado et al., 2005, Kaszuba et al., 2010). The zeta potential is also a key parameter that controls electrostatics in particle dispersions so was therefore important in understanding the stability of colloidal dispersions (Kaszuba *et al.*, 2010) and also in predicting long-term stability (Shaw, 1992).

3.3.3. Investigating the stability of DDA/TDB and DSPS/TDB liposomes

Liposomes composed of solely dimethyldioctadecylammonium bromide (DDA) have been demonstrated to be physically unstable and aggregate during prolonged storage (Hilgers and Snippe, 1992, Davidsen et al., 2005). During previous studies, the incorporation of immunostimulatory molecules such as trehalose 6,6'-dibehenate (TDB) and monomycolyl glycerol (MMG) were demonstrated to effectively stabilise these DDA-based liposomes with a consistent vesicle size being displayed, at temperatures of 4 ˚C and 25 ˚C (Davidsen et al., 2005, Nordly et al., 2011b). During this investigation, both cationic DDA/TDB and anionic DSPS/TDB liposomes (either hydrated in Tris buffer (10 mM; pH 7.4), distilled water or phosphate-buffered saline (PBS: 10 mM; pH 7.4) were assessed by their stability and liposome characteristics (size, polydispersity and zeta potential) over a 28-day time period.

3.3.3.1. Vesicle size

The effect of hydration buffer on the vesicle size of DDA/TDB and DSPS/TDB formulations was assessed during this investigation (Figure 3.2). Over the 28 day time period, at temperatures of 4 ˚C and 25 ˚C, cationic DDA/TDB liposomes (hydrated in Tris buffer) displayed a vesicle size in the region of 550 nm at the day 0 time point (Figure 3.2A), with these liposomes showing an increase in size over this 28-day study (~ 750 nm). This change in vesicle size was significant ($p < 0.01$). The anionic liposome formulation DSPS/TDB (hydrated in Tris buffer) displayed no significant increase in particle size, over this 28-day study, with size measurements of between 400-500 nm being displayed at both storage temperatures (Figure 3.2B) at all these various time points. By the day 28 time point (at a temperature of 25 ˚C), DSPS/TDB liposomes were measured to be in the region of 330 nm in size, suggesting a slight reduction in vesicle size however this was not significant.

When cationic DDA/TDB liposomes were hydrated using distilled water and the size characteristics were measured, these delivery systems were in the region of 1-1.2 µm over the course of this 28-day

study (Figure 3.2C) irrespective of the storage temperature (4 °C or 25 °C). Anionic DSPS/TDB liposomes (hydrated in distilled water) displayed size characteristics of between 400-500 nm over the time course of this study, at both storage temperatures of 4 ˚C and 25 ˚C respectively (Figure 3.2D).

Upon hydration of cationic DDA/TDB liposomes in PBS buffer, this gave rise to a significant increase in vesicle size of these formulations ($P < 0.001$) over their Tris- and distilled water counterparts and also over the time course of this study. The increased particle size displayed by these liposomes was indicative of increased instability of PBS-hydrated liposomes over this time period (Figure 3.2E). Also, for anionic DSPS/TDB liposomes, hydrated in PBS buffer, these vesicles showed a slight increase in vesicle size, at 4˚C, with this effect being increased at 25 ˚C over the time-course of this study ($P < 0.001$), from 659 nm (at day 0) to ~800 nm (at 4 ˚C at day 28) or ~ 1.3 µm (at 25 ˚C at day 28; Figure 3.2F).

Therefore these studies have shown that variation in aqueous buffer for the hydration of liposomes had a significant effect on the short-term stability of liposome formulations. Hydration of liposomes with salt-containing buffers, such as PBS, had a greater effect on the cationic DDA/TDB liposomes in comparison to the anionic DSPS/TDB formulation in terms of vesicle size. This has been discussed in the previous literature in which liposomes with higher transition temperatures appear to be more stable in PBS both at temperatures of 4 °C or 37 °C, which therefore suggested that the increased acyl chain length (and therefore transition temperature) was directly proportional to the stability of the liposome formulation (Andersen and Omri, 2004). During these studies the use of various other buffers in the hydration of liposomes, such as low-salt containing buffer (Tris 10 mM; pH 7.4) and distilled water, had a lesser effect on initial vesicle characteristics and the subsequent stability of these formulations when compared to PBS-hydrated liposomes. During these short term stability trials, the stability of these vesicles was due to their small size and highly charged nature (either cationic or anionic) upon hydration with Tris buffer (10 mM; pH 7.4). The high stability of these vesicles was due to the insertion of the double-chained glycolipid trehalose – 6,6’ dibehenate (TDB) within the lipid bilayer (Christensen *et al.*, 2008). It was shown that the insertion of trehalose dimycolate (TDM) and TDB within the bilayer inhibits the fusion between phospholipid vesicles (Spargo et al., 1991, Crowe et al., 1994) thus causing a reduction in vesicle aggregation. Therefore during these studies the incorporation of TDB within the liposomal system will increase the hydration of the membrane, thereby preventing the dehydration of the quaternary ammonium ion head groups of the lipids present within the formulation, which would otherwise lead to reduction in charge repulsion and aggregation of vesicles (Christensen *et al.*, 2008) thus leading to enhanced

vesicle stability (Davidsen *et al.*, 2005). However even the insertion of the stabilising TDB is not enough to counteract the effect of PBS buffer on the stability of these formulations. Therefore this hydration buffer will not be taken forward into further studies.

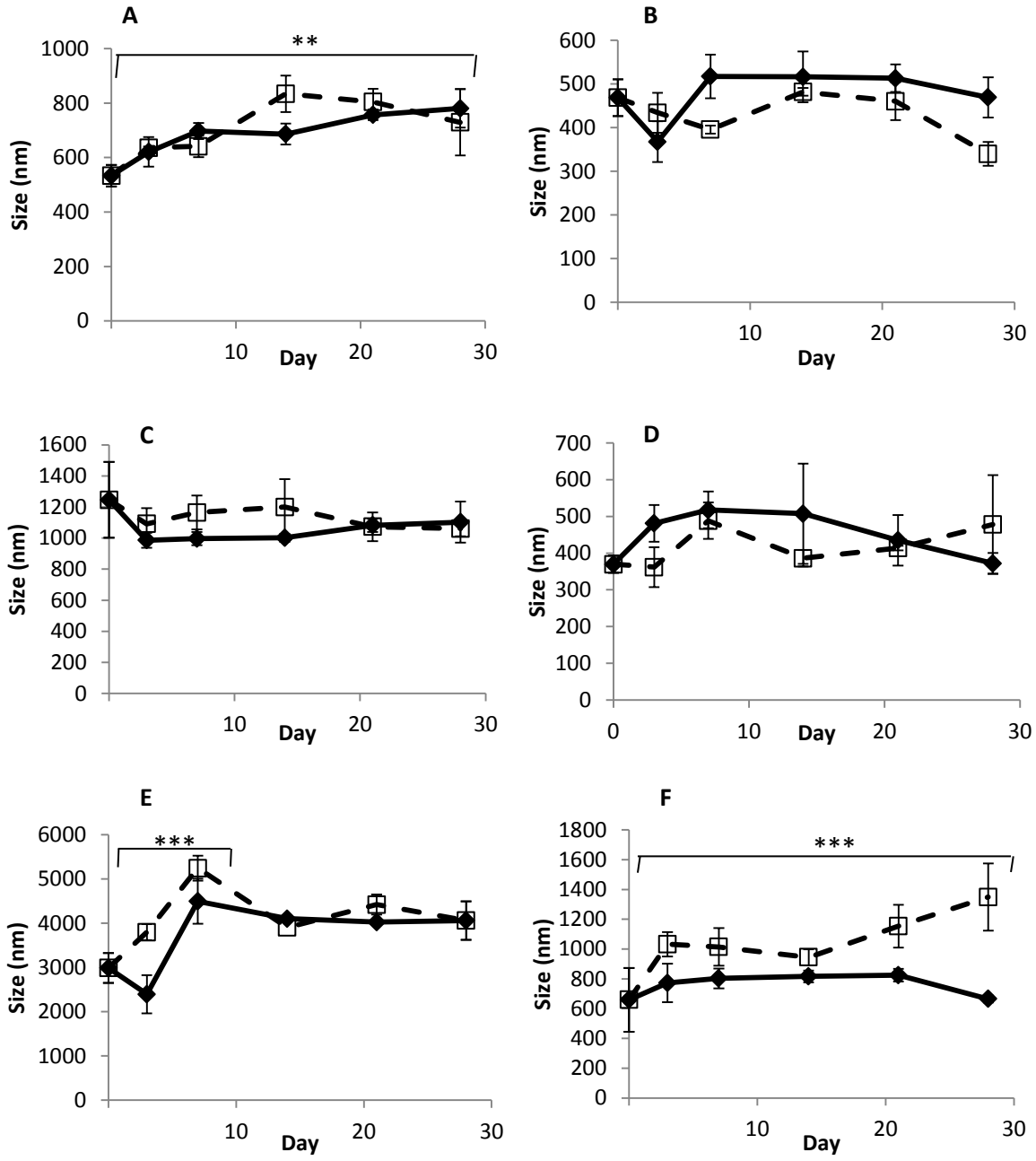


Figure 3-2. Time development of the average mean particle size of DDA/TDB (A, C and E) and DSPS/TDB (B, D and F) liposomes hydrated in Tris buffer (10 mM; pH 7.4 – A and B), distilled water (C and D) and PBS buffer (10 mM; pH 7.4 – E and F) at storage temperatures of 4 ˚C and 25 ˚C. Results are the mean of triplicate experiments ± SD. Significance between these characteristics between Day 0 and Day 28 was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$). Solid and dotted lines represent storage at 4 ˚C and 25 ˚C respectively.

3.3.3.2. Zeta potential

During these studies it was also required to show the effect of hydration buffer on the zeta potential of DDA/TDB and DSPS/TDB formulations. Over the 28 day time point, at temperatures of 4 ˚C and 25 ˚C, cationic DDA/TDB liposomes (hydrated in Tris buffer) exhibited a zeta potential in the range between 60-80 mV, with no dramatic change being observed over this study (Figure 3.3A). The maintenance of a highly positive zeta potential over this study suggested that these liposomes remained relatively stable. Also, the anionic liposome formulation DSPS/TDB (hydrated in Tris buffer) revealed no major changes in the surface characteristics of liposomes stored at both 4 ˚C and 25 ˚C (Figure 3.3B) with measurements of between - 60 to - 80 mV being displayed throughout this study.

The zeta potential analysis of the cationic liposome formulation DDA/TDB (hydrated in distilled water) showed that no significant changes in the zeta potential were displayed at both storage temperatures (Figures 3.3C) with measurements of between 55-70 mV being displayed throughout this study at all shown time points. Likewise, for the anionic DSPS/TDB liposome formulation (when hydrated in distilled water) this showed that no significant changes in the liposome surface charge are displayed over this 28-day study, at storage temperatures of 4 ˚C and 25 ˚C respectively (Figures 3.3D). The initial zeta potential of these anionic liposomes was shown to be in the region of - 50 mV. These liposomes were less stable than their Tris-hydrated counterparts (at 25 ˚C), with a reduction in zeta potential being measured at the day 28 time point. Whereas when these liposomes were stored at 4 °C, they displayed a zeta potential of ~ -65 mV (Figure 3.3D).

When studying the zeta potential of cationic DDA/TDB liposomes (when hydrated in PBS buffer) this showed that over the 28-day time period of this study there was a marked reduction ($P < 0.01$) in the liposome surface charge from ~ 70 mV to ~ 40 mV by day 7 (Figure 3.3E) following storage at 25 ˚C. Whereas following storage at 4 ˚C, there were no significant changes over the initial 7 days, however there was a further reduction to around 40 mV by day 28 of the stability study. This was indicative of the salt-induced aggregation of these liposomes as was also indicated by the increased particle size at each of these time points at both storage temperatures (Figure 3.2E). However for anionic DSPS/TDB liposomes (hydrated in PBS buffer), these vesicles also showed a reduction in zeta potential of DSPS/TDB over the time period of this 28-day study, with a reduction from -77.4 mV to ~ -60mV ($P < 0.05$) being displayed (Figure 3.3F). This reduction in zeta potential was not as pronounced as that for DDA/TDB liposomes, therefore indicating that hydration using PBS buffer did not affect the stability of these negatively-charged liposomes as much as their positively-charged counterparts.

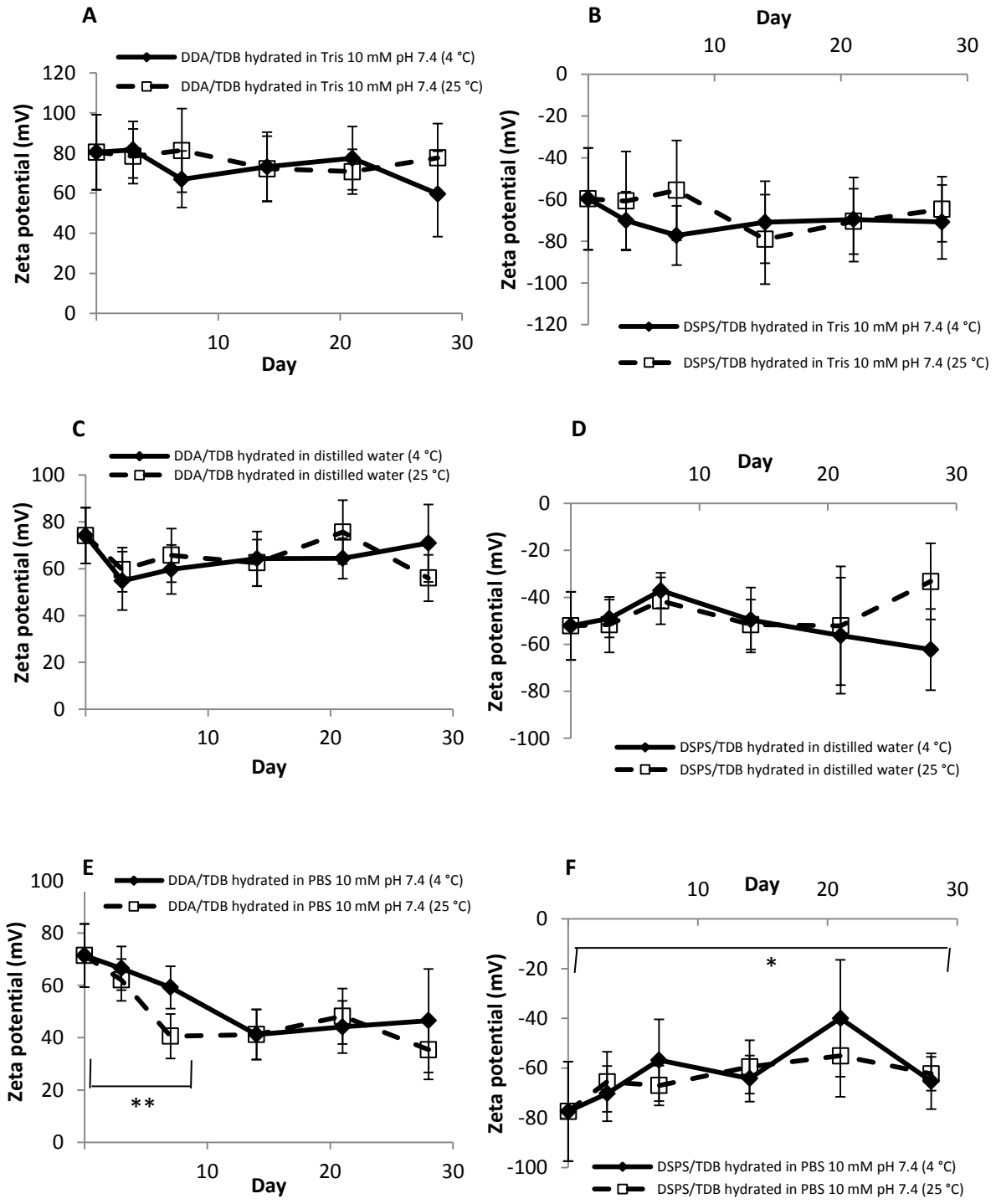


Figure 3-3. Time development of the average zeta potential of DDA/TDB (A, C and E) and DSPS/TDB (B, D and F) liposomes hydrated in Tris buffer (10 mM; pH 7.4 – A and B), distilled water (C and D) and PBS buffer (10 mM; pH 7.4 – E and F) at storage temperatures of 4 ˚C and 25 ˚C. Results are the mean of triplicate experiments ± SD. Significance between these characteristics between Day 0 and Day 28 was measured by one-way ANOVA (** $p<0.01$; * $p<0.05$).

As described previously for vesicle size, over this short-term stability study, PBS-hydrated liposomes showed relative instability in comparison to their distilled water and Tris-hydrated counterparts (Figure 3.2). As described previously the high presence of salt within the formulation could lead to vesicle aggregation due to reduced electrostatic interactions between the systems (Wasan *et al.*, 1999). The impact of vesicle aggregation over time, for PBS-buffer hydrated liposomes, also appeared to have an effect on the zeta potential of the formulation, with a noticeable reduction in zeta potential being shown for both DDA/TDB and DSPS/TDB liposome formulations. It has previously been discussed that increasing electrolyte concentration led to the condensing of the 'electrical double layer' and hence resulted in reduction in the zeta potential on a charged surface (Florence and Attwood, 1998), which may explain the reduction in zeta potential of both cationic DDA/TDB and anionic DSPS/TDB liposome systems over this short term stability study.

3.3.3.3. Visual stability

During these studies, the visual stability of these liposome systems was observed. The physical instability of DDA/TDB liposomes prepared using PBS buffer was seen in Figure 3.4. These liposomes were stored at 4 ˚C and 25 ˚C and results (Figure 3.4) showed clear phase separation even after 3 days, with this effect becoming more pronounced over the 28-day time period. This was in contrast to DDA/TDB liposomes, prepared using Tris buffer or distilled water, which remained notably more homogenous even after a 28-day storage period (at both storage temperatures; Figure 3.4) which was evident by no significant changes in both vesicle size and zeta potential (Figures 3.2 and 3.3 A and C).

In contrast DSPS/TDB liposomes prepared using PBS buffer remained more stable than their cationic DDA/TDB counterparts hydrated in PBS buffer, with sedimentation of liposomes appearing on the bottom of the vial appearing after just 3 days post liposome formation (Figure 3.5). This explained the slight increase in vesicle size and reduction in zeta potential measured over the 28-day storage period of this study (Figures 3.2F and 3.3F) Therefore, the difference in charge of the lipid head groups of both cationic DDA/TDB and anionic DSPS/TDB liposome delivery systems and the hydration buffer used in the study had a direct effect on the assembly and characteristics of these vesicles (including the size and zeta potential). This was in contrast to vesicle characteristics of DSPS/TDB liposomes prepared using either distilled water or Tris buffer, which remained fairly homogenous even after 28 days (at 4 ˚C or 25 ˚C) as shown in Figure 3.5.

The difference in visual stability of the liposomes, between either phase separation or sedimentation for cationic DDA/TDB and anionic DSPS/TDB therefore showed that charged lipids interact differently with aqueous salt-containing buffers in the assembly of liposomes. Vesicle aggregation, in presence of PBS buffer, was therefore hypothesised to be dependent on the charge of the liposome formulation. As described previously, Ciani and colleagues showed that cationic liposomes can at least double in size in the presence of PBS, with this buffer causing a bridging effect between cationic lipid head groups thus causing vesicle aggregation (Ciani *et al.*, 2007) and hence phase separation (Figure 3.4). However as described in these studies, anionic DSPS/TDB liposomes showed slightly less vesicle aggregation, especially in 4 °C temperature conditions, which suggested that the anionic lipid head groups interact to a lesser extent with the electrolytes present in PBS buffer.

The stability of liposomes was a crucial issue for the use of these vesicles as vaccine delivery systems. The storage and handling of these liposomes is extremely important in order to maintain the ability to act as vaccine adjuvants, and also in being able to transport them globally for use in vaccination. Since most vaccines are thermally unstable, there is an important need to create stable vaccine formulations which can be transported to third world countries. The distribution of vaccines requires “cold-chains” with functional freezers, as well as reliable transport links (Christensen *et al.*, 2007a) therefore making vaccine stability a key criteria in the development of these delivery systems.

These results have suggested that both DDA/TDB and DSPS/TDB liposomes hydrated in Tris buffer (10 mM; pH 7.4) displayed sub-micron vesicle size over the time-course of these studies, irrespective of the storage temperature. Therefore hydration of liposomes, using this aqueous buffer, was carried forward into further studies throughout this thesis.

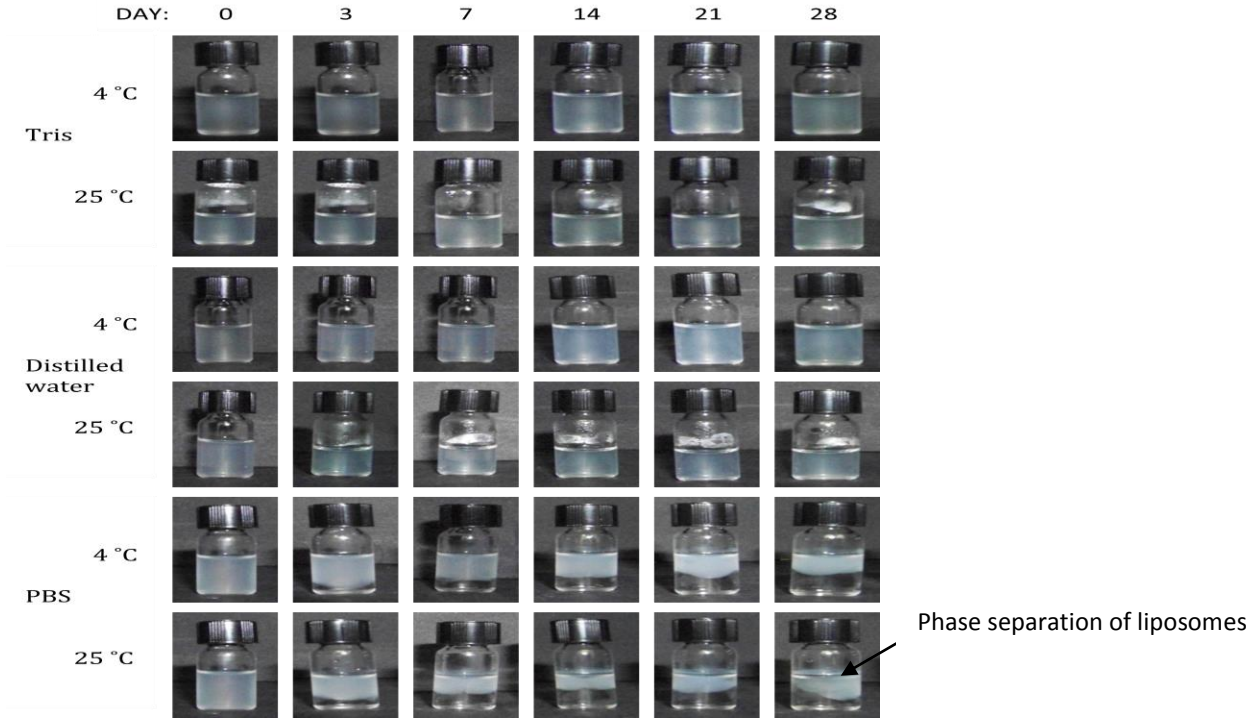


Figure 3-4. DDA/TDB liposome samples made in either Tris buffer (10 mM; pH 7.4), distilled water or PBS buffer (10 mM; pH 7.4) were stored for 28 days at 4 ˚C and 25 ˚C.

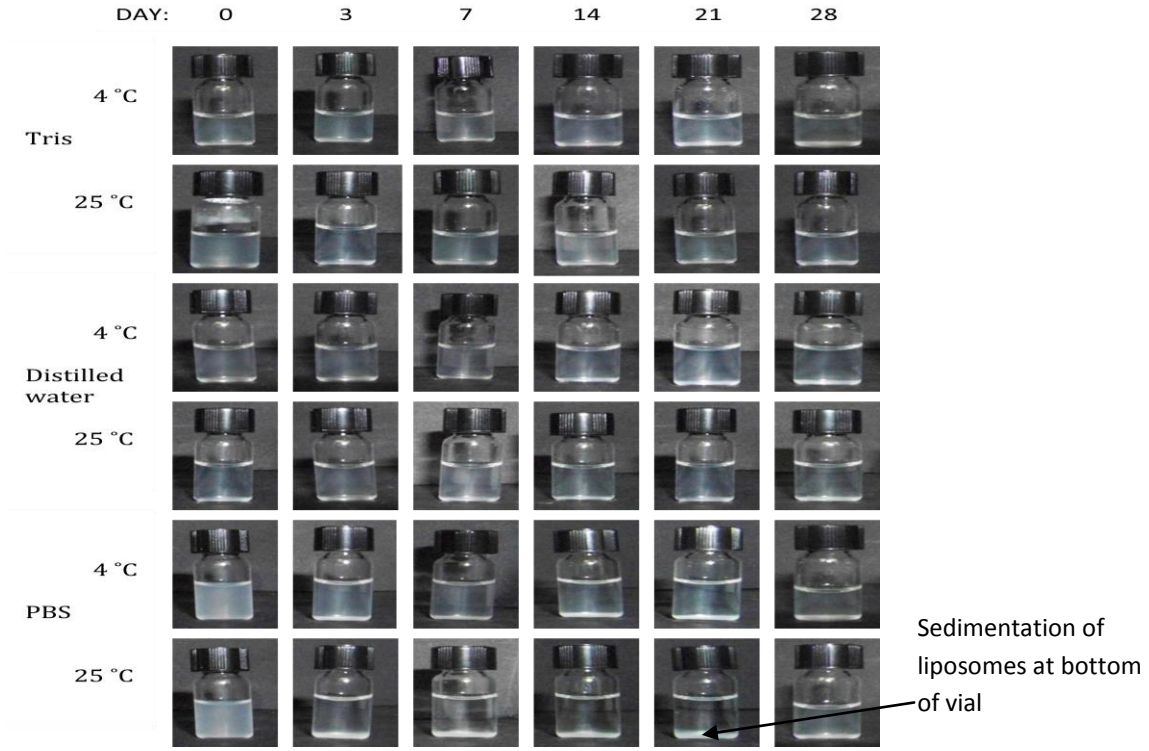


Figure 3-5. DSPS/TDB liposome samples made in either Tris buffer (10 mM; pH 7.4), distilled water or PBS buffer (10 mM; pH 7.4) were stored for 28 days at 4 ˚C and 25 ˚C.

However further results have suggested that the storage of formulations in an aqueous format has the possibility to lead to longer-term storage issues, especially using salt-containing buffers such as PBS. However, the stability of these liposomal-based vaccines can be improved due to the formation of a stable, sterile freeze-dried formulation This process of freeze-drying can be further enhanced by the addition of sugars, such as trehalose and sucrose, which act as cryoprotectants for biopharmaceuticals (Christensen *et al.*, 2008). The addition of a cryoprotectant during the freeze-drying of liposomes leads to the prevention of fusion of the lipid membranes from occurring. Due to the freezing and rehydration of these liposomes it was then possible for these liposomes to maintain their adjuvant effect and ability to maintain an immune response. The inclusion of TDB within liposomal-based vaccine delivery systems leads to an increased vesicle stability (Davidsen *et al.*, 2005) of DDA-based liposomes. The inclusion of TDB within liposomes leads to an enhanced stabilisation of the lipid membrane due to an increased hydration of the membrane surface (Christensen *et al.*, 2008).

During liposome formulation, the optimisation of the preparation process including the temperature, ionic strength of the buffer as well as the addition of stabilising components within the bilayer (including cholesterol and immunostimulatory lipids) can lead to increased stability of the delivery system also (Grit and Crommelin, 1993, Zuidam et al., 1995, Davidsen et al., 2005).

3.3.4. Investigating the effect of additional phospholipid content in the DDA and DSPS liposomal systems.

In a further investigation, the well characterised cationic adjuvant formulation DDA/TDB (or CAF01) was formulated at the optimal ratio between DDA and TDB (5:1 w/w or 8:1 M/M) which has been discovered to provide the optimal immune response in characterisation and vaccination studies (Davidsen *et al.*, 2005). Further liposome formulations were prepared (Table 3.2) in order to investigate the effect of increasing DSPC lipid content on the liposome characteristics (size, zeta potential and polydispersity) of this liposome system, as well as the anionic liposome formulation DSPS/TDB. These liposome systems were formulated with and without the *in vivo* dose of the latent H56 TB antigen (0.1 mg/mL).

Table 3-2 - Liposome formulations including cationic or anionic lipids, with increased replacement with the neutral lipid DSPC. For all formulations, the molar concentration of TDB is 11 mol%, apart from where noted by # where the molar concentration of TDB is increased to 25 mol %.

FORMULATION	[Lipid (DDA or DSPS)] (mg/mL)	[DSPC] (mg/mL)	Lipid:DSPC ratio	[TDB] (mg/mL)
Lipid/DSPC/TDB	1.25	0	100%:0%	0.25
Lipid/DSPC/TDB	0.75	0.5	60%:40%	0.25
Lipid/DSPC/TDB	0.5	0.75	40%:60%	0.25
Lipid/DSPC/TDB	0.5	0	40%:0%	0.25 (#)
Lipid/DSPC/TDB	0.25	1	20%:80%	0.25
Lipid/DSPC/TDB	0.05	1.20	5%:95%	0.25
Lipid/DSPC/TDB	0	1.25	0%:100%	0.25

The data collected during this study was summarised in Figures 3.6 to 3.8. These results showed that modification of the lipid molar ratio in the formulation of liposome delivery systems had a significant effect upon vesicle characteristics as well as their subsequent ability to load the latent TB subunit vaccine, H56. During these studies, liposomes were diluted with Tris buffer (1 mM; pH 7.4) as previous investigations have revealed that this choice of dilution buffer had no significant effect on vesicle characteristics (Figure 3.1).

Zeta potential analysis of these liposome formulations showed a trend in which increasing DSPC content within the formulation led to a reduction in zeta potential, hence neutralisation of the surface charge. This difference becomes significant ($P < 0.01$) for DDA/DSPC/TDB (0.05 mg/ml: 1.2 mg/mL : 0.25 mg/mL; Figure 3.6). Therefore the surface charge can be modulated by increased replacement of DDA with DSPC within the formulation. Therefore upon increasing the content of the DSPC lipid, as well as decreasing the DDA content within the formulation led to modulation of the liposome surface charge due to neutralisation of the surface charge. The main lipid constituent present within the formulation was therefore shown in the resultant liposome surface charge, due to the charged nature of polar lipid head groups present within the bilayer.

DSPS/TDB liposomes displayed a zeta potential in the region of – 60 to -70 mV (as has been displayed previously in this Chapter). However, upon replacement of the anionic DSPS lipid with neutral DSPC within the liposome formulation this led to the neutralisation of the surface charge as shown by a significant reduction ($P < 0.001$) in the zeta potential (Figure 3.6). This again showed that liposome surface charge could be modulated upon anionic lipid (DSPS) replacement with DSPC.

Vesicle size analysis of these liposome formulations (Figure 3.7), in combination with the anionic H56 TB antigen (at the proposed *in vivo* dose of 5 µg or 0.1 mg/mL) was carried out. Upon surface adsorption of the anionic H56 antigen, the particle size of all the cationic liposome formulations was measured to be in the region of 700-800 nm. The sole exception was with complete cationic replacement with the neutral DSPC lipid in which the vesicle size was measured to be close to 2 µm both with and without antigen (Figure 3.7). So upon complete DDA replacement with the neutral lipid DSPC within the formulation (without antigen) this resulted in a significant increase in initial vesicle size ($P < 0.001$).

Anionic DSPS/TDB liposomes displayed a vesicle size of around 450 nm (which was similar to previously measured values; Table 3.1). Upon incorporation and replacement of DSPS, with neutral DSPC, this resulted in vesicles ranging in size from 300-400 nm. Whilst complete lipid replacement to form neutral DSPC-TDB liposomes, led to vesicles in the region of ~ 2 µm (Figure 3.7) which were significantly higher in size ($P < 0.001$).

Antigen loading results showed that liposome formulations which were highly cationic (positive) in charge have the ability to electrostatically bind with anionic (negatively charged) antigen at the liposome surface, with antigen loading values in the region of 80 % being measured (Figure 3.8). The increasing replacement of cationic DDA with DSPC, or full replacement with DSPC or DSPS, within the formulation resulted in significant reduction ($P < 0.001$) in loading of the H56 TB antigen (Figure 3.8). This is due to lesser interactions between antigen and liposome components, which led to lower antigen loading and hence no significant change in the vesicle size and zeta potential for DSPC/TDB and DSPS/TDB (upon addition of H56 TB antigen). Due to the similarity in antigen loading values between DSPS/TDB and DSPC/TDB the loading values between these two formulations (increased anionic lipid replacement) were not measured. The effect of liposome charge in the loading and biodistribution of various antigens has been studied in the previous literature (Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2010c), with electrostatic interaction between oppositely-charged antigen and delivery system being important with regards to high antigen loading.

Therefore as described previously cationic liposomes are able to highly load anionic vaccine antigens, such as the TB vaccine candidate H1 (Henriksen-Lacey et al., 2011a, Kaur et al., 2011, Perrie et al., 2013) with these studies showing that the formation of a strong 'antigen depot' was important in the potentiation of a strong Th1 immune response (Perrie *et al.*, 2013).

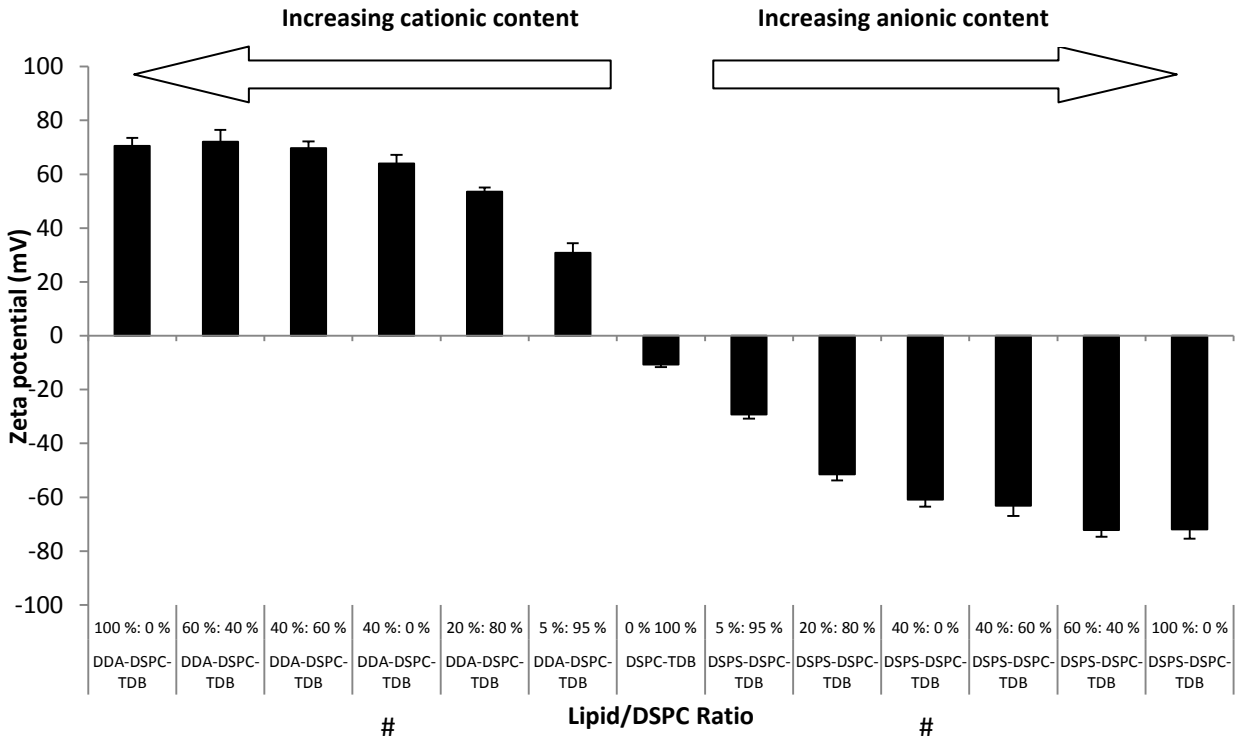


Figure 3-6. The effect of lipid molar ratio on the zeta potential of liposome formulations. All formulations are prepared with the addition of the *in vivo* dose of H56 antigen (5 µg per dose; 0.1 mg/mL). Results are the mean of triplicate experiments ± SD. For all formulations, the molar concentration of TDB is 11 mol%, apart from where noted by # where the molar concentration of TDB is increased to 25 mol %.

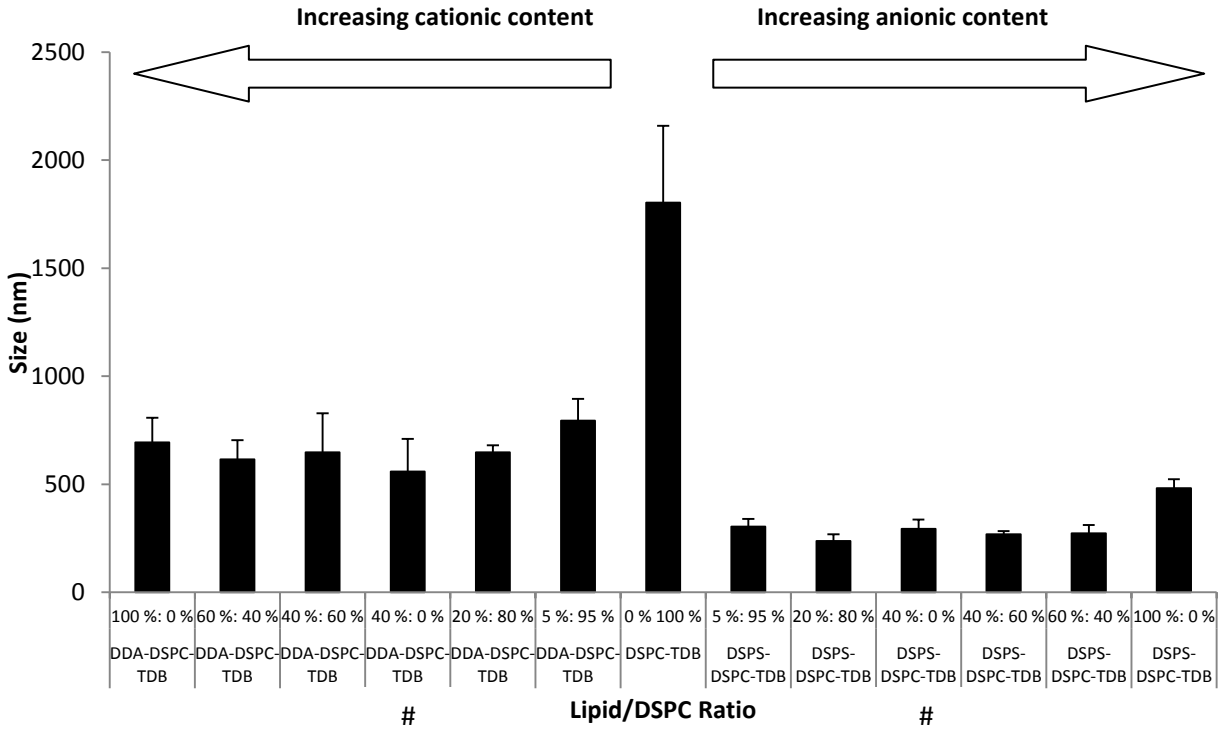


Figure 3-7. The effect of lipid molar ratio on the vesicle size of liposome formulations. All formulations are prepared with the addition of the *in vivo* dose of H56 antigen (5 µg per dose; 0.1 mg/mL). Results are the mean of triplicate experiments ± SD. For all formulations, the molar concentration of TDB is 11 mol%, apart from where noted by # where the molar concentration of TDB is increased to 25 mol %.

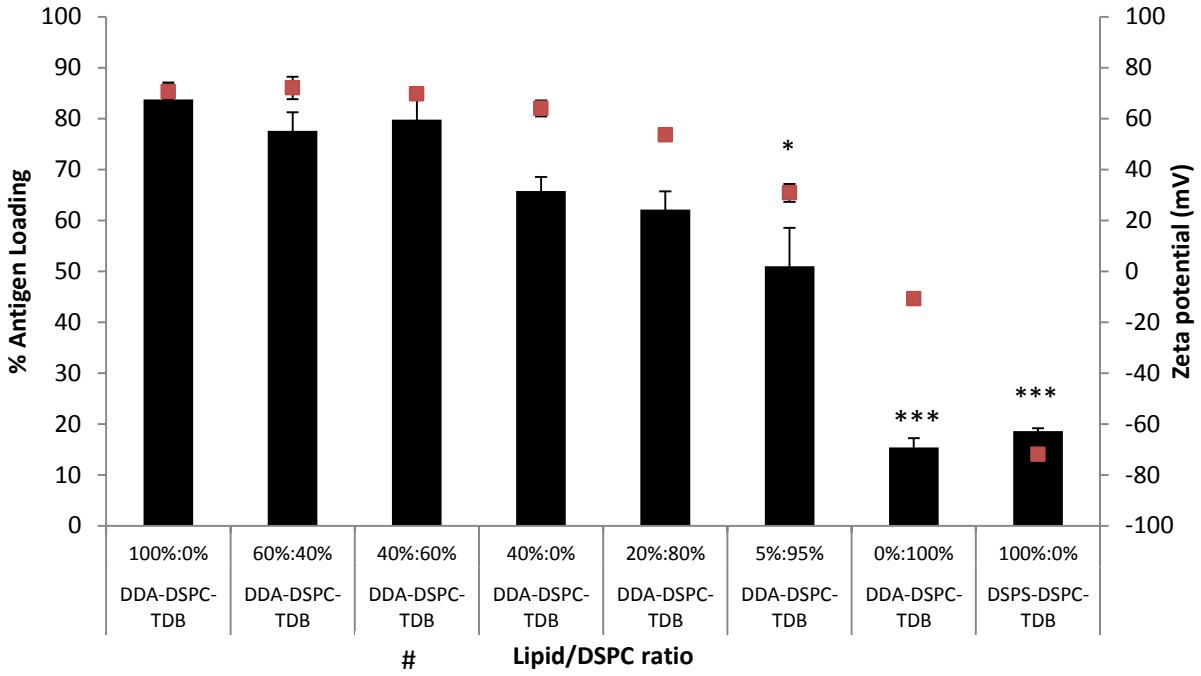


Figure 3-8. Percentage (%) antigen loading (represented by bar columns) and zeta potential (represented by squares) of a series of liposome formulations with increasing cationic lipid replacement with neutral DSPC and anionic DSPS. Results are the mean of triplicate experiments ± SD. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$; * $p<0.05$) as compared to DDA-DSPC-TDB (100%:0%). For all formulations, the molar concentration of TDB is 11 mol%, apart from where noted by # where the molar concentration of TDB is increased to 25 mol %.

3.3.4.1. Immunogenicity of liposome vaccine formulations upon cationic lipid replacement with DSPC

The immunological capability of these liposomal adjuvants (in combination with H56 vaccine antigen), with increasing DDA replacement with DSPC, has been assessed during *in vivo* vaccine studies (Hussain et al., 2013). Upon immunisation, partial and complete DDA replacement with DSPC reduced the levels of antigen-specific IgG2b antibody production, as well as a significant reduction in the levels of splenocyte proliferation. This reduction in splenocyte proliferation correlated with lower levels of Th1 cytokine production (IFN-γ and IL-2). However DSPC-enriched liposomal adjuvants induced significantly higher levels of Th2 cytokines (IL-5 and IL-10), therefore this suggested that the Th2 immune responses obtained were independent of the surface charge of the adjuvant which correlated with the previous literature (Henriksen-Lacey *et al.*, 2010c).

3.3.5. Product specification for liposome vaccine adjuvants

As a result of these studies a product specification has been set up for both cationic DDA/TDB anionic DSPS/TDB liposome formulations. These are displayed in Table 3.3. These studies were therefore used to validate formulations prepared in Chapter 4 of this thesis, for use in further in vivo studies.

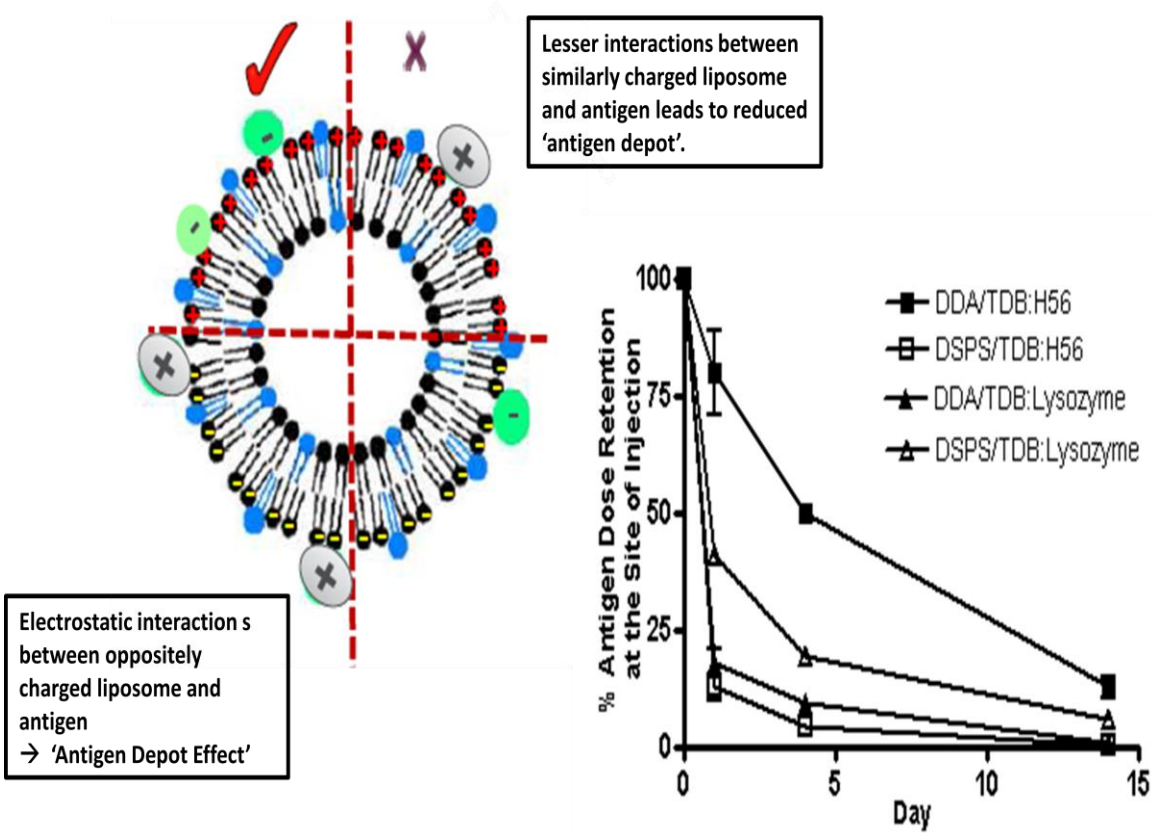
Table 3-3. Production specification of cationic DDA/TDB and anionic DSPS/TDB liposome adjuvants

Formulation	DDA/TDB	DSPS/TDB
Hydration buffer	Tris (10 mM; pH 7.4)	Tris (10 mM; pH 7.4)
Dilution buffer	Tris (1 mM; pH 7.4)	Tris (1 mM; pH 7.4)
Expected vesicle size	500-700 nm	350-500 nm
Expected zeta potential	65 to 80 mV	-60 to -80 mV
Expected PDI	0.3 to 0.5	0.4 to 0.7
Expected H56 antigen loading (antigen added at 0.1 mg/mL)	80-90 %	15-25 %

3.4. Conclusions

The work in this chapter showed that a range of liposomal systems can be prepared over the full range of charge using DDA and DSPS. The charged lipid content was shown to impact on the measured zeta potential and in some cases the vesicle size. The choice of buffer used in these liposome systems was also shown to be important, with high electrolyte buffers such as PBS shown to cause flocculation and aggregation of the DDA-based liposomes which resulted in phase separation. Similarly the DSPS-based liposome systems were shown to sediment over time, suggesting that for longer term storage of these systems, the process of freeze-drying may be required. Furthermore from these initial experiments, product specifications have been developed for both the DDA- and DSPS-based formulations. These will be used to validate any further formulations used prior to *in vivo* studies.

Chapter 4: The role of liposome surface charge in vaccine delivery and formulation



Some of the results presented in this chapter have been published in the paper:

Wilkinson A., Kaur R., Henriksen-Lacey M., Christensen D., Mohammed AR, Perrie Y. "Investigating the role of surface charge on the ability of liposome vaccine formulations to form an antigen depot and subsequently induce an immune response" (manuscript in preparation).

4.1. Aims and Objectives

The aim of the work reported in this chapter was to further investigate the effect of liposome surface charge and liposome-antigen interactions, defined by electrostatic interaction, on the production of a liposome and vaccine antigen depot at the injection site. Cationic DDA/TDB and anionic DSPS/TDB liposomes with various antigens were investigated (formulated by the lipid film-hydration method) and physicochemical characteristics measured, as well their ability to load and retain antigen. The issue of stability of these systems was considered. In vivo biodistribution and immunisation studies in mice were undertaken in order to investigate the effect of liposome surface charge on the subsequent immunogenicity of the formulation, in terms of immune responses such as cytokine analysis at the spleen, leg (site of injection) as well as antibody production in the blood sera.

4.2. Introduction

Liposomes are an ideal vaccine delivery system due to their particulate nature, versatility and flexibility in formulation and it is these parameters that can be used to promote a range of immune responses (Foged *et al.*, 2004). This is due to the ability of liposomes as delivery systems to render soluble antigen into a particulate form, thereby leading to the lengthening of their in vivo half life (Henriksen-Lacey *et al.*, 2011c). The importance of charge, antigen isoelectric point (pI) and buffer pH for liposomes offers an important platform for antigen adsorption due to electrostatic interaction between oppositely charged liposome and antigen components.

This 'depot effect' has been shown to be the mechanism of action for cationic liposomes (Vangasseri et al., 2006, Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2010c, 2011a). The liposome composition, such as the lipid head-group structure (conferring the surface charge) and membrane fluidity as well as the cationic surface charge have been shown to play an important role in the retention of vaccine at the site of injection (SOI) in order to initiate a vaccine-specific immune response (Henriksen-Lacey *et al.*, 2011a). The surface charge of liposomes has also been shown to have a positive effect on the immune response with cationic DDA-based liposomes having an advantage over their neutral and anionic counterparts (Henriksen-Lacey et al., 2010c, 2011a) due to their ability to electrostatically bind anionic antigen. This has been attributed to their ability to form a 'depot' at the SOI, due to their interaction with negatively-charged serum proteins following s.c

and i.m injection, hence leading to monocyte influx and local activation of dendritic cells (Korsholm *et al.*, 2007). Through association with DCs, such as antigen presenting cells (APCs) this release of antigen can then drain to the lymph nodes in order to and initiate T-cells to activate a Th1 cell-mediated immune response.

A number of other research studies have investigated the variation in surface charge of liposomes and the subsequent effect on the generated immune response in mice. For example, Nakanishi et al showed that encapsulation of ovalbumin (OVA) or diphtheria toxin in cationic liposomes (PC:Cholesterol:SA) elicited the highest anti-OVA serum IgG1 responses (Nakanishi *et al.*, 1999) when compared to anionic and neutral liposomes (PC:Cholesterol:PA and PC:Cholesterol respectively). Therefore these studies support the conclusion that cationic liposomes promote stronger Th1 immunity, as well as antigen-specific serum antibody responses when compared to otherwise similar anionic and neutral formulations, although these responses are not always specifically correlated.

However, in contrast, original studies by Allison and Gregoriadis in 1974 demonstrated the use of diphtheria toxoid in combination with negatively-charged liposomes which caused a significant elevation in the levels of antibody in comparison to positively-charged liposomes (Allison and Gregoriadis, 1974). The use of liposomes of varying charge has also been investigated by Yanasarn et al, in which some negatively-charged liposomes (including the anionic lipid DOPA) are able to generate strong and functional antibody responses, as well as upregulating gene expression related to the activation and maturation of dendritic cells (Yanasarn *et al.*, 2011). This occurred due to the simple mixing of pre-formed negatively charged liposomes with antigens. However, these studies did not compare between cationic and neutral liposomes. The general trend during the studied literature is for cationic liposomes having greater potential and efficacy as vaccine adjuvants, in comparison to their anionic and neutral counterparts.

Therefore, to consider these factors, it was required to directly compare between cationic and anionic liposomes in terms of their characterisation, antigen loading ability, biodistribution and their ability to act as a vaccine adjuvant. Of interest during this study is the immunostimulatory capability of DSPS/TDB liposomes, in comparison to the well characterised CAF01 vaccine adjuvant (DDA/TDB), which is currently in clinical trials for the TB subunit vaccine, H1 (NCT 00922363).

4.3. Results and Discussion

4.3.1. Physicochemical characteristics including quantification of antigen loading

Liposomes composed of DDA and TDB at a 5:1 weight ratio expressed physicochemical characteristics as described in Chapter 3; displaying a vesicle size of ~500 nm and a zeta potential of ~63 mV (summarised in Table 4.1). DSPS/TDB liposomes were made at an 8:1 molar ratio (therefore mimicking the DDA/TDB formulation to serve as a direct comparison): DSPS/TDB liposomes had an average vesicle size of around 400 nm and polydispersity of 0.5 (Table 4.1). DSPS/TDB liposomes were slightly reduced in size ($P < 0.05$) in comparison to their counterpart DDA/TDB liposomes (Table 4.1). This size similarity was confirmed by cryo-TEM imaging (Figure 4.1). DSPS lipid has an anionic (negatively charged) head group and therefore liposomes produced of DSPS with TDB express a strongly anionic zeta potential. Therefore, substitution of DDA with DSPS results in a decrease in zeta potential from approximately +63 mV to – 65 mV (Table 4.1).

Table 4-1. Physical characterisation of cationic DDA/TDB liposomes and anionic DSPS/TDB liposomes formulated by the lipid-film hydration (LH) method. Results are the mean ± SD of triplicate experiments.

	DDA/TDB liposomes	DSPS/TDB liposomes
Size (nm)	508 ± 100	419 ± 80
Polydispersity (PDI)	0.516 ± 0.040	0.464 ± 0.150
Zeta potential (mV)	63 ± 10	-65 ± 6

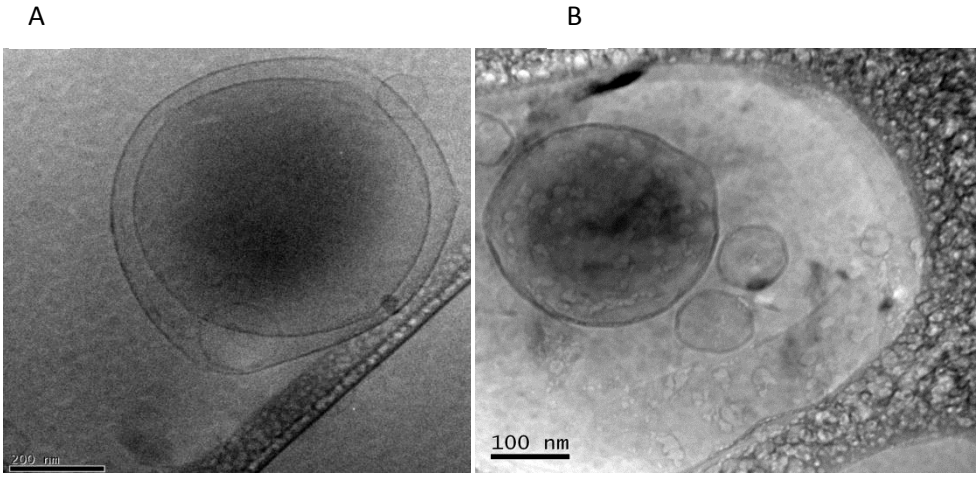


Figure 4-1. Cryo-TEM micrograph of (A) DDA/TDB and (B) DSPS/TDB liposomes prepared by the lipid-film hydration method. The long dark structures are the carbon grid the sample was deposited on before analysis.

4.3.2. Effect of antigen charge on the physicochemical characteristics of liposomal adjuvants

To consider the ability of these systems to deliver antigens, multilamellar vesicles (MLV) of cationic DDA/TDB and anionic DSPS/TDB liposomes were prepared and their characteristics considered, both with and without antigen (cationic lysozyme or anionic OVA). DSPS/TDB liposomes were formulated at the 8:1 molar ratio, thereby mimicking the DDA/TDB formulation (as described previously in Chapter 3).

Prior to addition of protein, the cationic DDA/TDB and anionic DSPS/TDB liposomes had zeta potential values of 67.3 ± 5.8 mV and – 58.3 ± 8.9 mV, respectively (Figure 4.2A). The addition of lysozyme to DDA/TDB resulted in minimal to no adsorption (1.78 ± 0.5 %), which correlates with no significant change in the zeta potential (59.2 ± 3.3 mV). The addition of OVA (1 mg/mL) to DDA/TDB liposomes (post-lipid film hydration) resulted in a significant ($P < 0.001$) reduction in zeta potential, suggesting high adsorption of anionic OVA to cationic DDA/TDB by electrostatic interactions at the liposome surface, which thus reduced the cationic charge. In contrast, the outcome with the anionic DSPS/TDB formulation (Figure 4.2A) is reversed with electrostatic binding and the subsequent significant changes ($P < 0.001$) occurring only with lysozyme (-21.2 ± 3.1 mV and 79.1 ± 10.3 % adsorption) and not with OVA (-59.3 ± 10.3 mV and 0.35 % adsorption).

The results in Figure 4.2B showed that DDA/TDB liposomes displayed a vesicle size in the region of 700 nm and a polydispersity of 0.53 ± 0.05. Whereas DSPS/TDB liposomes (prepared at the same molar ratio) had similar size characteristics; being around 400 nm in size (Figure 4.2B), but slightly less polydisperse (0.39 ± 0.17). The addition of ovalbumin (OVA) to DDA/TDB (at a final concentration of 1 mg/mL) resulted in a significant ($P < 0.001$) increase in vesicle size and polydispersity (Figure 4.2B). In contrast, the addition of OVA had no significant effect on the liposome characteristics of DSPS/TDB. The addition of cationic lysozyme to DSPS/TDB liposomes resulted in a significant ($P < 0.001$) increase in vesicle size and polydispersity, with the addition of lysozyme to DDA/TDB having no significant effect on liposome characteristics. These results indicated that the addition of oppositely charged liposome and antigen components leads to vesicle aggregation (as shown by an increased vesicle size and polydispersity; Figure 4.2B) due to high antigen adsorption to the liposome surface by electrostatic interactions (Figure 4.2A) which caused subsequent neutralisation of the liposome surface charge (Figure 4.2A).

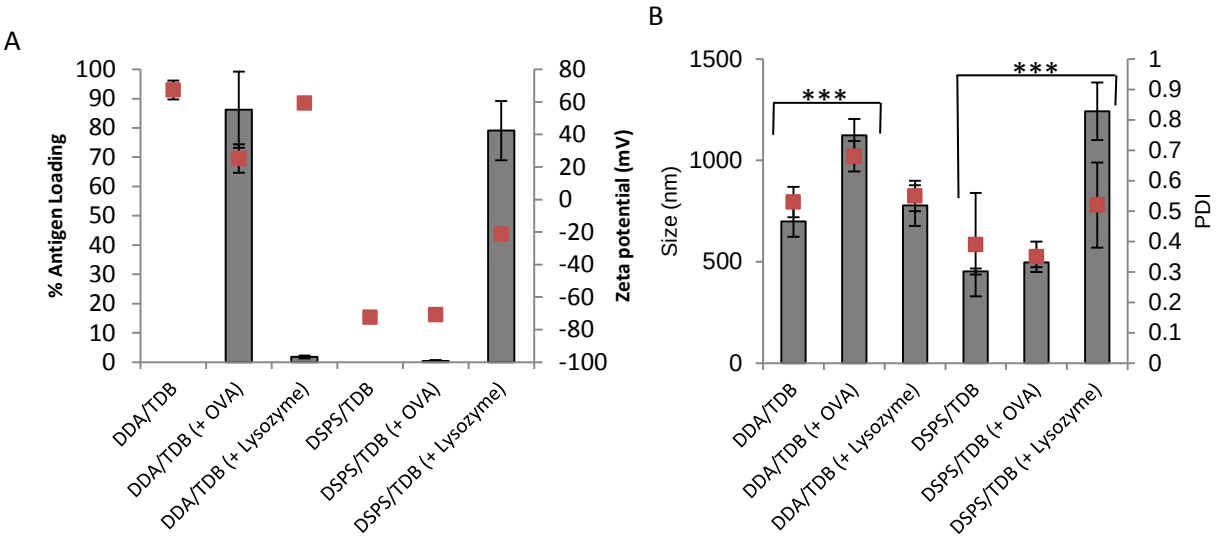


Figure 4-2. Liposome characteristics [A: antigen adsorption (columns) and zeta potential (squares); B: size (columns) and polydispersity (squares)] of cationic DDA/TDB and anionic DSPS/TDB liposomes with or without the addition of 1 mg/mL protein (OVA or lysozyme). Antigen adsorption is measured as total protein recovered in the supernatant fraction (following analysis by BCA assay). Results are the mean of 3 experiments ± SD. Significance between liposome characteristics was measured by one-way ANOVA (*** $p<0.001$).

Table 4-2. Summary table of proteins used during this investigation

PROTEIN	BUFFER	MOLECULAR WEIGHT (kDa)	ISOELECTRIC POINT (pI)
Ovalbumin (OVA)	10 mM Tris (pH 7.4)	45.0	4.5
Lysozyme	10 mM Tris (pH 4.0)	14.7	11.2
H56 (Ag85B-ESAT6-Rv2660c)	10 mM Glycine (pH 9.2)	46.0	4

DDA-based liposomes were shown to display a cationic (positive) surface charge, whereas DSPS/TDB liposomes displayed an anionic (negative) charge. OVA and lysozyme have a pI (isoelectric point) value of 4.5 and 11.2 units, making them either positively or negatively-charged at pH 7.4 (Table 4.2) respectively. Therefore, upon addition of oppositely-charged liposome and antigen components, electrostatic-mediated adsorption of protein antigen to the liposome surface can occur.

The addition of protein antigen (at a concentration of 1 mg/mL) was undertaken in order to investigate the subsequent effects on liposome characteristics including vesicle size, zeta potential and antigen adsorption. Electrostatic interactions have been shown to play a role in the interaction between liposome and antigen, so when these components are opposing in charge this results in charge-related interactions at the liposome surface as described in previous studies (Shek and Heath, 1983, Tsuruta et al., 1997, Korsholm et al., 2007, Henriksen-Lacey et al., 2010c). Antigen adsorption to the liposome results in an increased shielding of the liposome surface, hence the observed increase in vesicle size and reduction in zeta potential. The electrostatic interactions between the cationic surface of the liposomes with the anionic protein structures (and vice versa,

with anionic liposomes and cationic protein) results in charge neutralisation and hence a reduction in the measured zeta potential of the vesicles

Electrostatic interactions between liposome and antigen have been shown to occur during these studies, in which this interaction leads to vesicle aggregation due to the adsorption of protein at the liposome surface. This causes the increase in vesicle size and reduction in zeta potential, which increases at higher concentrations of oppositely-charged (to the liposome) protein antigen (see Sections 4.3.2.1 and 4.3.2.2). However, when liposome and antigen are similar in charge (cationic DDA/TDB and cationic protein lysozyme) no electrostatic binding will occur, as shown by the low levels of antigen adsorption and no notable changes in vesicles characteristics measured. Such interactions have previously been shown with other systems, most commonly in gene therapy systems where cationic liposomes are used to deliver DNA. For example, the addition of increasing concentration of DNA to cationic liposomes leads to vesicle aggregation with increases in vesicle size and reduction in zeta potential (Moghaddam *et al.*, 2011). However addition of DNA to pre-formed anionic liposomes has been previously shown to lead to minimal loading and hence no measured changes in vesicle characteristics (Perrie and Gregoriadis, 2000).

4.3.2.1. The effect of the addition of anionic protein antigen on vesicle characteristics

Given the electrostatic interactions of antigen with the above liposome formulations, the effect of +/- and charge ratios were considered and the characteristics of both cationic and anionic liposomes were investigated after the addition of varying concentrations of these model antigens (Table 4.2).The physicochemical characteristics of both cationic DDA/TDB and anionic DSPS/TDB liposomes were investigated after the addition of varying concentrations of OVA. The addition of increasing concentrations of OVA (between 0 and 10 mg/mL) resulted in no significant difference in the liposome characteristics of anionic DSPS/TDB liposomes, with the vesicle size and polydispersity remaining around 400 nm and between 0.5-0.6 respectively (Figure 4.3A).

Zeta potential analysis also showed no significant effect on anionic DSPS/TDB upon addition of increasing concentrations of OVA, with measurements of between -70 to -80 mV being recorded (Figure 4.3B). Both antigen and delivery systems are net anionic in charge which suggests that even at high OVA concentrations, there is no electrostatic binding between these similarly charged liposome and antigen components (both anionic in charge) over a wide concentration range.

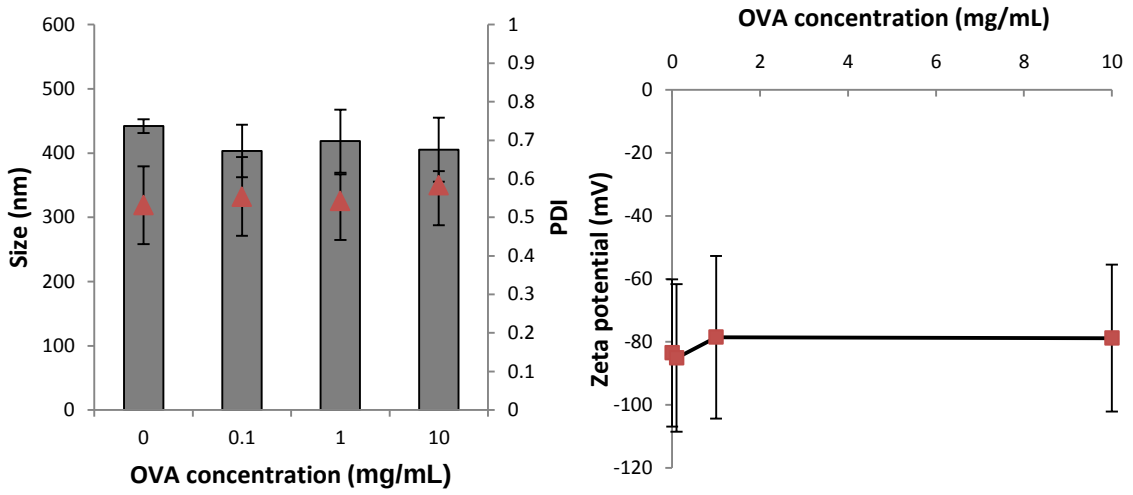


Figure 4-3. Variation in liposome characteristics of anionic DSPS/TDB liposomes, A: size (columns) and polydispersity (points), B: zeta potential; with the addition of increasing concentrations of OVA. Results are the mean of triplicate experiments ± SD.

Upon addition of increasing concentrations of OVA to cationic DDA/TDB liposomes, there was a significant ($P < 0.001$) increase in vesicle size and neutralisation of the surface charge when compared to empty DDA/TDB liposomes. The binding of anionic OVA to these cationic liposomes occurred via electrostatic interactions (indicated by high OVA adsorption in Figure 4.2A); at an in vivo dose concentration of 5 µg (0.1 mg/mL), there are no significant changes in liposome characteristics for DDA/TDB. Therefore, this indicates that at low protein concentrations, liposome characteristics can be maintained (Figure 4.4). The vesicle size increased from 595.8 ± 41.1 nm (empty vesicle) to ~ 2 µm (at concentrations of 1 and 10 mg/mL OVA – Figure 4.4A). Zeta potential analysis has showed that there was a highly significant reduction in surface charge ($P < 0.001$) from 76.5 ± 20.9 mV to –23.9 ± 4.3 mV (after addition of 10 mg/ml OVA) as displayed in Figure 4.4B. At these lower protein concentrations (0.1 mg/mL), the levels of antigen loading were assessed by the radioactivity method (Table 4.3). These studies further concluded the importance of electrostatic interactions between cationic DDA/TDB and negatively charged antigen (OVA), therefore resulting in high levels of antigen loading/adsorption occurring at the liposome surface (Table 4.3). At the *in vivo* antigen concentration (0.1 mg/mL), this level of antigen loading had no effect on the resulting liposome characteristics whereas at a higher antigen dose (1 and 10 mg/ml), the addition of protein resulted in increased binding and lowering of the cationic charge combined with an increased vesicle size as vesicle aggregation occurs (Figures 4.4 A and B). This correlated with previous work in the literature with the addition of DNA to liposomes which has shown that as the ratio between lipid and DNA within the formulation is modified this can lead to a reversal in zeta potential from positive to

negative, with subsequent increase in the vesicle size (Perrie and Gregoriadis, 2000, Perrie et al., 2001, Moghaddam et al., 2011).

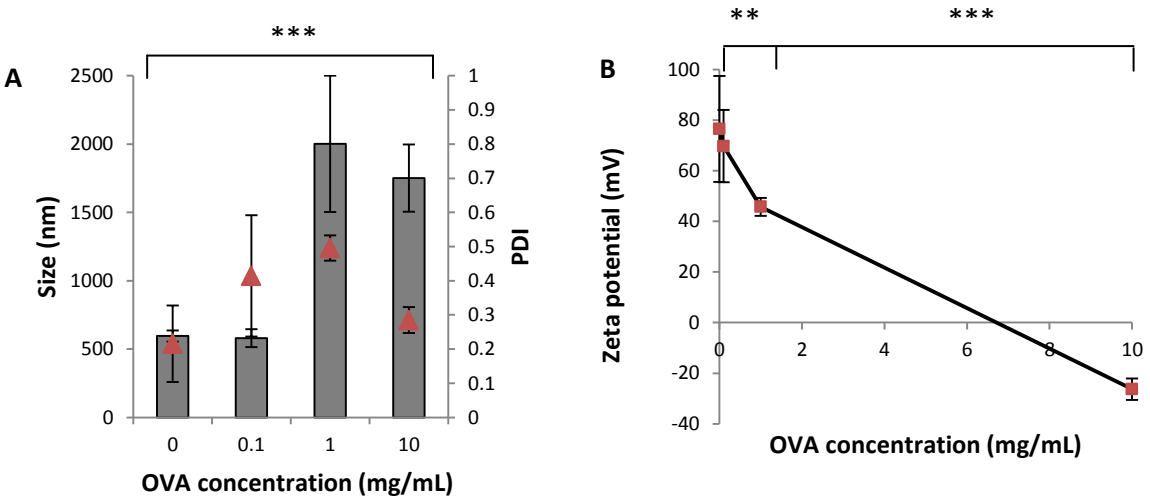


Figure 4-4. Variation in liposome characteristics of cationic DDA/TDB liposomes, A; size (columns) and polydispersity (points), B; zeta potential; with the addition of increasing concentrations of OVA. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$).

Table 4-3. Antigen Loading Characteristics for multilamellar vesicles (MLV) of DDA/TDB and DSPS/TDB liposomes [either empty or combined with OVA. OVA is added at an in vivo dose concentration of 5 µg (0.1 mg/mL).

Formulation	% Antigen Loading	Antigen Loading (µg)
DDA/TDB:OVA	93.3 ± 1.9	4.67 ± 0.1
DSPS/TDB:OVA	23.1 ± 7.4	1.16 ± 0.37

% Antigen Loading is proportional to the total recovery of radiolabelled antigen within the liposome pellet. Results are the mean of 3 experiments ± SD.

In subsequent studies, the tuberculosis subunit vaccine antigen H56 (Ag85B-ESAT6-Rv2660c) was included in combination with both cationic DDA/TDB and anionic DSPS/TDB liposomes in order to show the effect of this antigen addition on the liposome characteristics of MLV. Due to the OVA concentration range that can be produced, this protein is an ideal protein to study protein-induced changes on liposomal systems due to its low cost and availability. However, OVA is not a disease model antigen therefore we moved towards the use of the above subunit protein antigens which have been developed for immunisation against active and latent tuberculosis respectively (Statens Serum Institut, Copenhagen, Denmark).

H56 has a molecular weight of ~ 45-50 kDa and has a pI of around 4.0 units, making it similar in size and pI to OVA. The pI of H56 favours its association via electrostatic interactions with cationic DDA/TDB liposomes (Table 4.4). For in vivo studies, each liposome dose (250 μg lipid, 50 μg TDB) contained 5 μg Ag85B-ESAT6-Rv2660 (H56) antigen. The association of the H56 antigen with both DDA/TDB and DSPS/TDB liposome delivery systems was investigated and the vesicle characteristics for MLVs (size, zeta potential and polydispersity and antigen loading) are displayed in Figure 4.5 and Table 4.4, respectively.

At the in vivo experimental dose of 5 μg H56/dose (0.1 mg/mL), the addition of antigen made no significant difference to the physico-chemical characteristics of these liposomes compared to their antigen-free counterparts (Figure 4.5). The adsorption of the H56 antigen (at an in vivo dose of 5 μg) to both DDA/TDB and DSPS/TDB has been assessed by the radioactivity method (as described previously) with levels of ~ 80 % and ~ 20 % being measured respectively (Table 4.4). These studies have indicated a high level of adsorption of this anionic (negatively charged) antigen with cationic DDA/TDB in comparison with low level antigen adsorption to anionic DSPS/TDB (Table 4.4). This again demonstrated the importance of electrostatic interactions between oppositely charged liposome and antigen components.

Table 4-4. Antigen loading for multilamellar vesicles (MLV) of DDA/TDB and DSPS/TDB liposomes [either empty or combined with an *in vivo* dose of H56 antigen (5 μg)].

Formulation	Antigen Loading (%)
DDA/TDB (+ H56)	77.3 ± 4.6
DSPS/TDB (+ H56)	20.3 ± 5.2

Results are the mean of triplicate experiments ± SD.

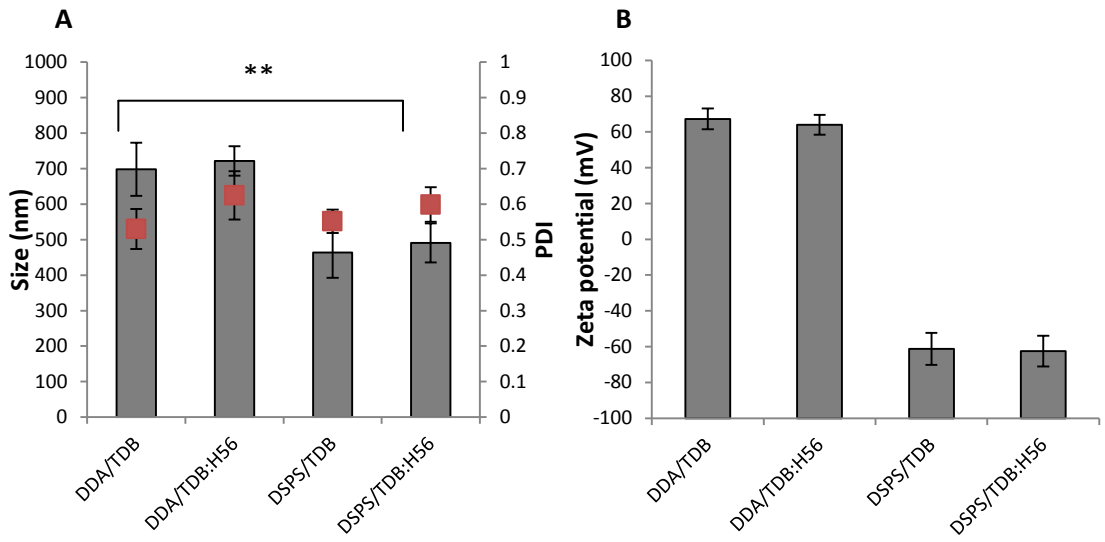


Figure 4-5. Variation in liposome characteristics [size, polydispersity (A; columns and points respectively) and zeta potential (B)] for DDA/TDB and DSPS/TDB with and without an in vivo experimental dose of H56 antigen (5 µg)]. Results are the mean of 3 experiments ± SD. Significance was measured by one-way ANOVA (** $p<0.01$).

These studies have demonstrated that interaction will occur by charge-related electrostatics of H56 antigen binding to the liposome surface as is demonstrated by the high loading to the liposome surface of cationic DDA/TDB (Table 4.4). This will be confirmed by means of a trypsin digestion assay in Section 4.3.4.

4.3.2.2. The effect of the addition of cationic protein on vesicle characteristics

Given the electrostatic interactions of antigen with the above liposome formulations, the effect of -/+ and +/+ charge ratios were considered and the characteristics of both cationic and anionic liposomes were investigated after the addition of varying concentrations of the cationic protein lysozyme.

The addition of increasing concentrations of lysozyme resulted in no significant changes in the characteristics of DDA/TDB, with liposomes displaying a vesicle size of ~500-600 nm in this study (Figure 4.6A). Zeta potential analysis (Figure 4.6B) of these cationic delivery systems showed a highly positive surface charge between 70-80 mV which remained constant (depending on lysozyme concentration) for all formulations. Both delivery system and protein are cationic in charge,

therefore upon post-LH addition of lysozyme to the formulation this results in no marked changes in vesicle characteristics.

As shown previously (Figure 4.2A and B), the addition of lysozyme (at a concentration of 1 mg/mL) had a significant effect on the liposome characteristics of DSPS/TDB. As the concentration dose of protein was increased (between 0 and 10 mg/mL) there was a significant ($P < 0.001$) increase in vesicle size (from 445.7 nm to 932.7 nm) and neutralisation of the surface charge due to interaction between these oppositely charged liposome and antigen components (Figure 4.7). At the in vivo experimental dose of 5 µg (0.1 mg/mL), there were no significant differences in the size or zeta potential of anionic DSPS/TDB liposomes, however significant lysozyme loading occurs at this dose (Table 4.5).

Zeta potential analysis shows that at lysozyme concentrations reaching up to 10 mg/mL, that a slightly positive surface charge is displayed (+ 16 mV) for DSPS/TDB. The addition of the cationic protein lysozyme will hence lead to neutralisation of the surface charge, which led to a significant ($P < 0.001$) reduction in the zeta potential of these anionic delivery systems (Figure 4.7B). These changes are therefore caused by high adsorption of cationic lysozyme at the negatively-charged surface of DSPS/TDB liposomes, causing the anionic surface charge to be masked and becoming weakly cationic due to neutralisation of the surface charge.

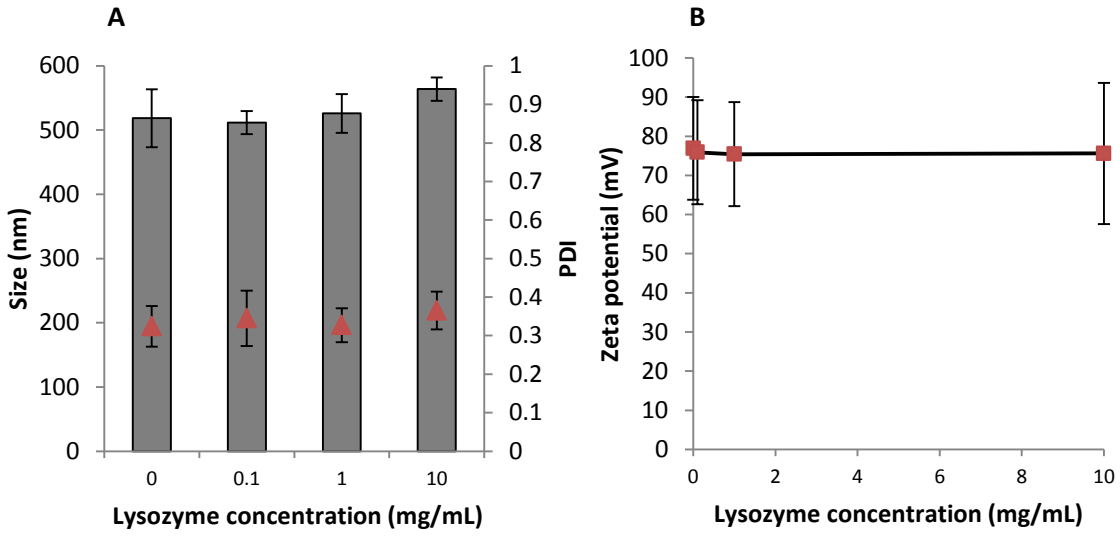


Figure 4-6. Variation in liposome characteristics of cationic DDA/TDB liposomes, A; size (columns) and polydispersity (points), B; zeta potential; with the addition of increasing concentrations of lysozyme. Results are the mean of triplicate experiments ± SD.

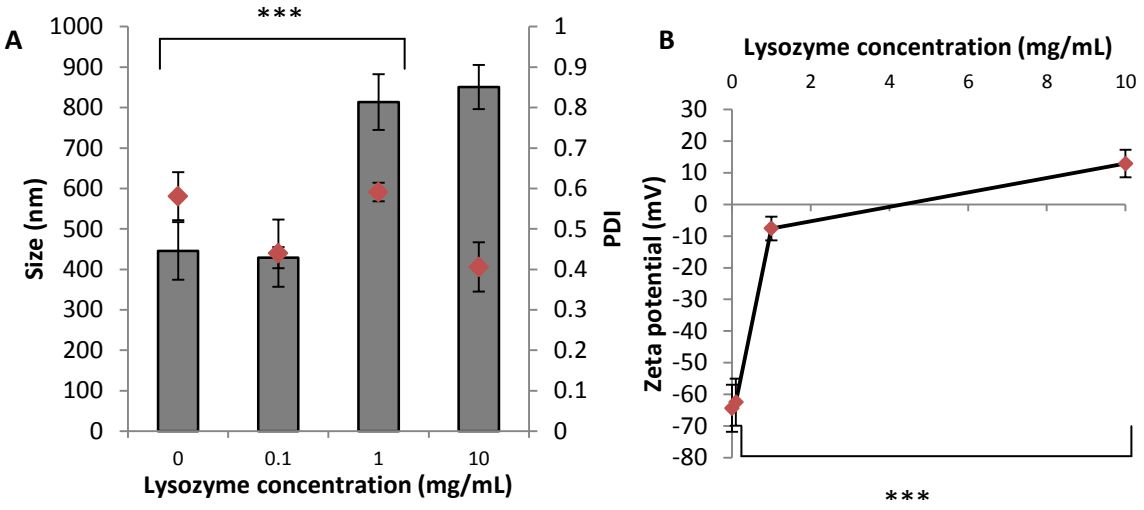


Figure 4-7. Variation in liposome characteristics of anionic DSPS/TDB liposomes, A; size (columns) and polydispersity (points), B; zeta potential; with the addition of increasing concentrations of lysozyme. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$).

Table 4-5. Antigen Loading Characteristics of DDA/TDB and DSPS/TDB liposomes, either empty or combined with lysozyme.

Formulation	% Antigen Loading	Antigen Loading (µg)
DDA/TDB: Lysozyme	27.9 ± 7.1	1.4 ± 0.36
DSPS/TDB: Lysozyme	82.6 ± 3.5	4.1 ± 0.2

Lysozyme is added at an in vivo dose concentration of 5 µg (0.1 mg/mL). % Antigen Loading is proportional to the total recovery of radiolabelled antigen within the liposome pellet. Results are the mean of 3 experiments ± SD.

At these lower lysozyme concentrations (0.1 mg/mL), the levels of antigen loading were assessed by the radioactivity method. These studies further concluded the importance of electrostatic interactions between anionic DSPS/TDB and cationic protein (lysozyme), therefore resulting in high levels of antigen loading or adsorption (82.6 %) occurring at the liposome surface (Table 4.5). This was in comparison with DDA/TDB, which displayed low to minimal levels of lysozyme loading (27.9 %) which is due to these components being similarly charged and therefore both liposome and antigen components are unable to electrostatically interact at the liposome surface (Figure 4.6). At this antigen dose this adsorption of antigen has no subsequent effect on liposome characteristics (such as size and zeta potential), whereas at higher doses of antigen this results in enhanced shielding of the liposome surface charge. This resulted in a significant effect on liposome characteristics, such as an increased vesicle size (Figure 4.7A) and reduction or neutralisation of the surface charge (as reflected by a reduction in zeta potential: Figure 4.7B).

These studies have concluded that using MLV (prepared by the LH method) for cationic DDA/TDB and anionic DSPS/TDB liposome delivery systems with increasing concentrations of protein (anionic OVA and cationic lysozyme) leads to a marked change in liposome characteristics when these components are opposite in charge (high antigen adsorption). Therefore, this demonstrates the mode of association between liposome and antigen to occur at the liposome surface and the mechanism of action between these oppositely charged components is by electrostatic (charge-related) interactions (Henriksen-Lacey *et al.*, 2010c). The high adsorption of protein (lysozyme by anionic DSPS/TDB, or OVA by DDA/TDB) at the liposome surface results in vesicle aggregation as indicated by an increased vesicle size, as well as subsequent neutralisation of the surface charge of the liposome delivery system. The presence of some antigen loading between similarly charged antigen and liposome components during this study demonstrates that electrostatic interactions may not be the only crucial factor in determining antigen-liposome interaction. The presence of hydrophobic and hydrophilic regions within the protein may demonstrate interactions between liposome and antigen. Also, the protein may have a net cationic charge (for example, lysozyme) but contain several smaller regions which are anionic in charge which therefore may favour the interaction with a positively charged (cationic) liposome delivery system at the surface of the vesicle. Also from the chemical structures of the two main lipids present in liposome formulations (DDA and DSPS) even though containing an overall net cationic and anionic charge respectively, these lipids contain areas of the opposing charge. This can be seen within the head group of the DSPS lipid which is overall anionic in charge, but contains an ammonium ion ($-NH_3$) head group therefore this may favour the binding of a small amount of anionic protein antigen to the anionic delivery system. This phenomenon may be reversed with the minimal binding of lysozyme to cationic DDA/TDB liposomes.

These findings in respect of protein antigen have been discussed previously with the addition of DNA to both cationic and anionic liposomes, in which increasing the concentration of DNA with pre-formed cationic liposomes led to increase in vesicle size and the reduction in the zeta potential of vesicles (Perrie and Gregoriadis, 2000).

4.3.2.3. Stability of Antigen-Loaded Multilamellar Vesicles

Cationic DDA/TDB and anionic DSPS/TDB (with the addition of the in vivo dose of lysozyme or H56 antigen at a concentration of 0.1 mg/mL) liposome vaccine delivery systems were placed into stability trials in order to assess stability (in terms of size, zeta potential and polydispersity) over a 28-day period. These stability studies have shown that both cationic DDA/TDB and anionic DSPS/TDB liposomes (in combination with an in vivo dose of H56 antigen) remain homogenous and exhibit no phase separation even after a 28-day storage period (at both temperatures of 4 ˚C and 25 ˚C; Figure 4.8). Over the storage period DDA/TDB liposomes exhibited a vesicle size of ~ 550-700 nm whereas the zeta potential remained highly cationic throughout this study, remaining in the region of + 60-70 mV (Figures 4.9 and 4.10). This is in correlation with previous data (Chapter 3) of DDA/TDB and DSPS/TDB liposomes, after hydration using Tris buffer (10 mM; pH 7.4). Similarly DSPS/TDB liposomes retained their zeta potential throughout the time period of this study (after the addition of an in vivo dose of H56 antigen), in the region of -70 to -80 mV, whilst displaying a vesicle size which remained constant at about 400-500 nm throughout this storage period (Figures 4.11 and 4.12).

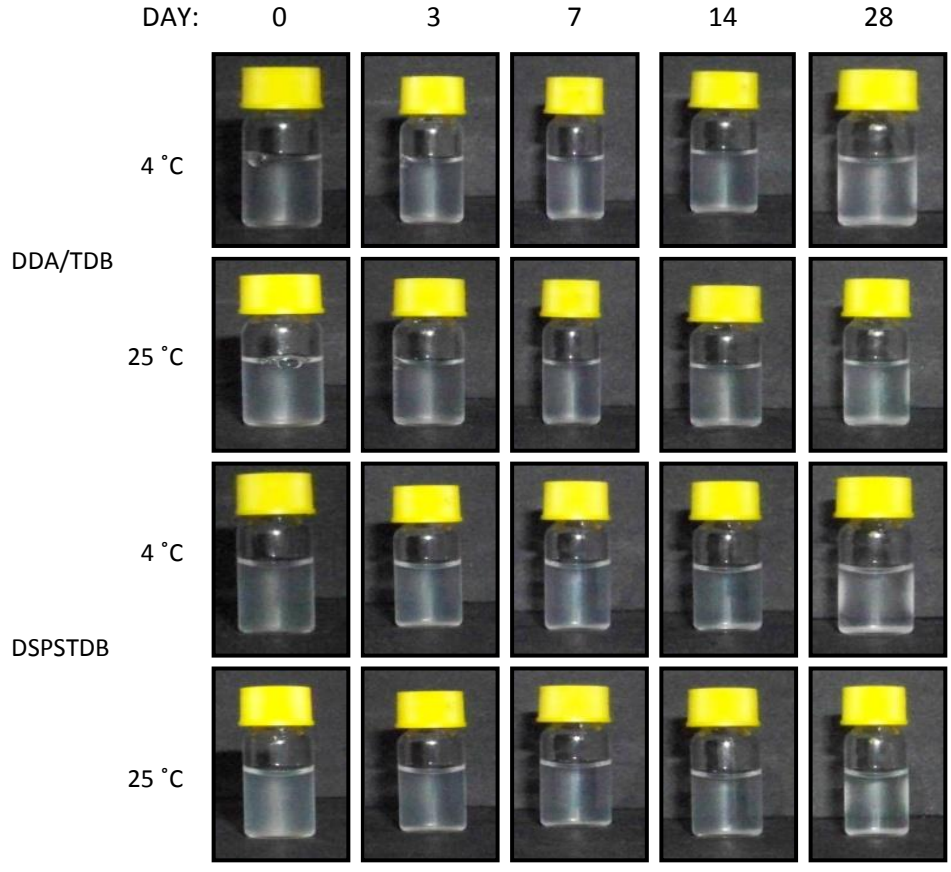


Figure 4-8. Time development of the stability of DDA/TDB and DSPS/TDB liposome formulations (in combination with the *in vivo* dose of H56) following storage for 28 days at 4 ˚C or 25 ˚C.

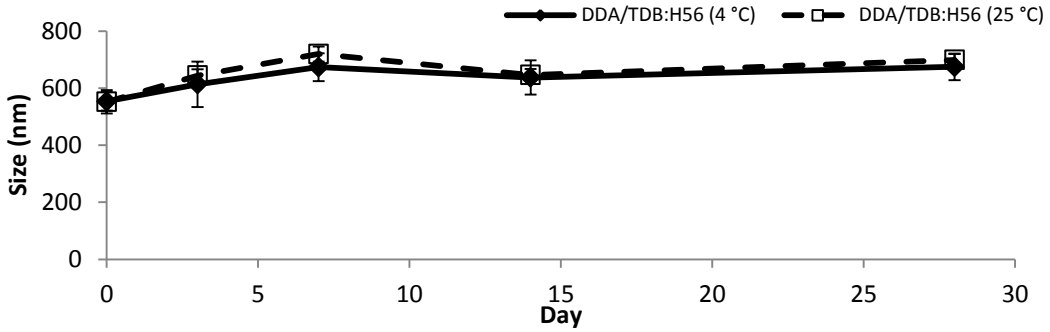


Figure 4-9. Time development of liposome size characteristics for MLV DDA/TDB (with the addition of H56 at an in vivo dose) at a storage temperature of 4 ˚C or 25 ˚C. Results are the mean of triplicate experiments ± SD.

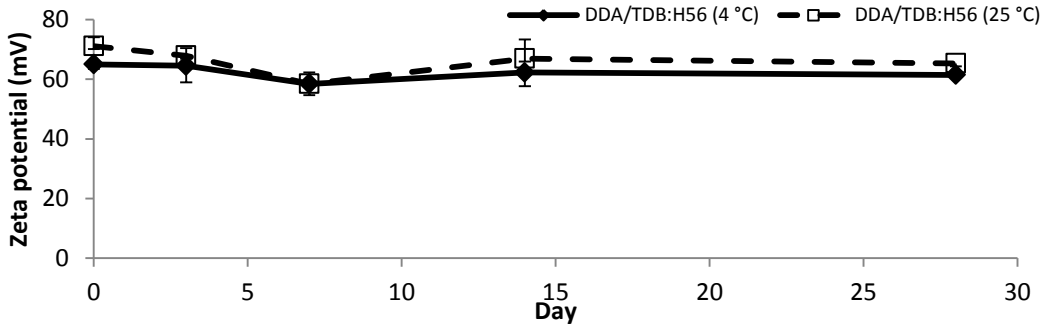


Figure 4-10. Time development of liposome characteristics (zeta potential) for MLV DDA/TDB (with the addition of H56 at an in vivo dose) following storage at 4 and 25 ˚C. Results are the mean of triplicate experiments ± SD.

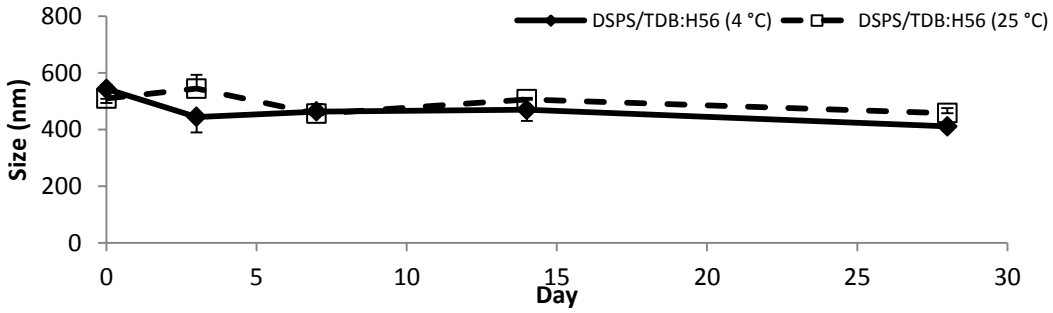


Figure 4-11. Time development of liposome size characteristics for MLV DSPS/TDB (with the addition of H56 at an in vivo dose) following storage at 4 and 25 ˚C. Results are the mean of triplicate experiments ± SD.

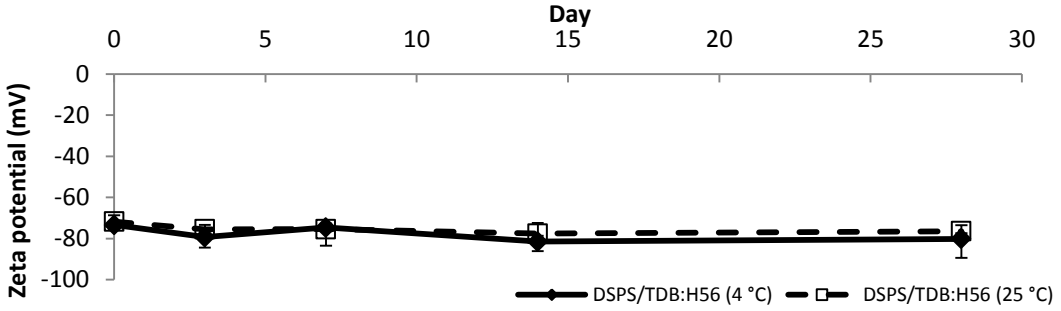


Figure 4-12. Time development of liposome characteristics (zeta potential) for MLV DSPS/TDB (with the addition of H56 at an in vivo dose) following storage at 4 and 25 ˚C. Results are the mean of triplicate experiments ± SD.

These liposome systems (cationic DDA/TDB and anionic DSPS/TDB respectively), in combination with antigen (lysozyme), were also measured for various characteristics including size, polydispersity and zeta potential at a number of time points throughout this study (Figures 4.13 to 4.16). Throughout these studies, DDA/TDB liposomes (in combination with an *in vivo* dose of lysozyme; 0.1 mg/mL) displayed vesicles with sizes in the region of 600-650 nm (Figure 4.13). Zeta potential analysis of these systems showed that these systems displayed a cationic charge in the region of 70 mV (Figure 4.14) irrespective of the storage temperature. As shown by no significant changes in characteristics over the 28-day period, these vesicles remained relatively stable which is a prerequisite for the use and development of liposomal-based vaccine formulations.

Whereas, anionic DSPS/TDB liposomes showed an average vesicle size of around 500 nm and a zeta potential between -70 to – 80 mV over the 28-day study period (Figures 4.15 and 4.16), therefore demonstrating these systems remained stable during this study period. These results showed that both of these liposome delivery systems, at both storage temperatures, remained stable in terms of their liposome characteristics as was confirmed by visual stability (Figure 4.17).

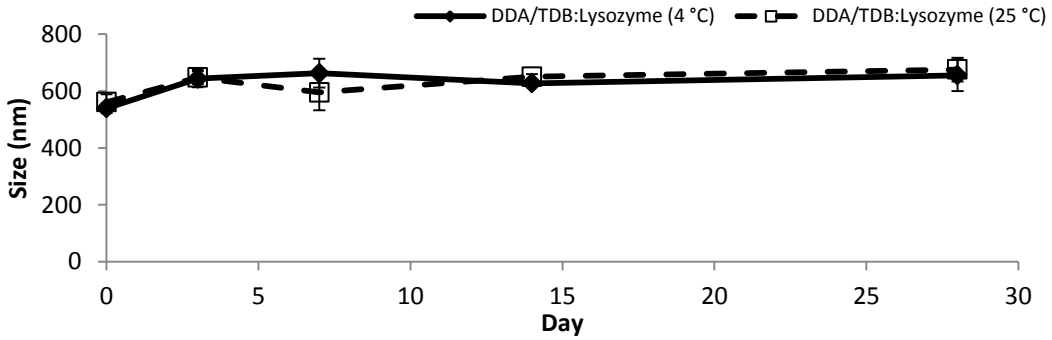


Figure 4-13. Time development of liposome size characteristics for MLV DDA/TDB (with the addition of lysozyme at an *in vivo* dose) following storage at 4 and 25 ˚C. Results are the mean of triplicate experiments ± SD.

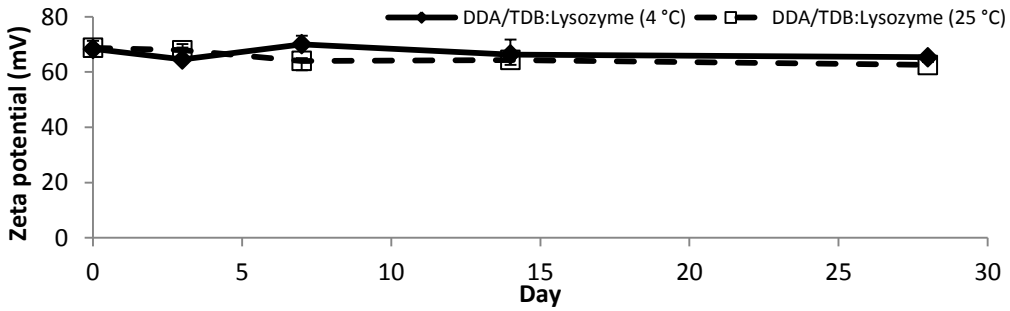


Figure 4-14. Time development of liposome characteristics (zeta potential) for MLV DDA/TDB (with the addition of lysozyme at an *in vivo* dose) following storage at 4 and 25 ˚C. Results are the mean of triplicate experiments ± SD.

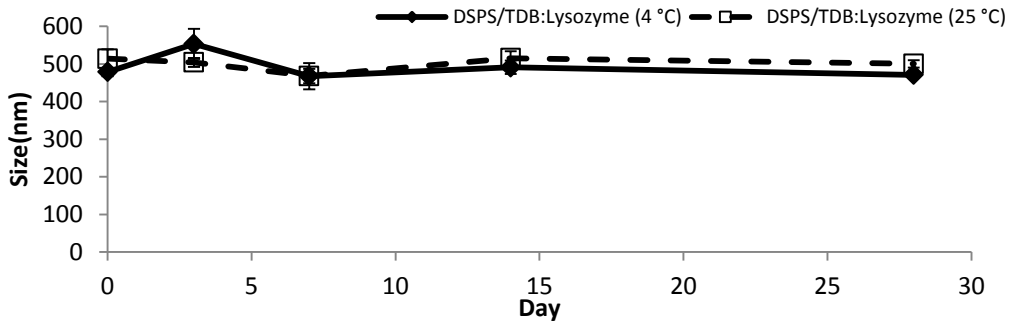


Figure 4-15. Time development of liposome size characteristics for MLV DSPS/TDB (with the addition of lysozyme at an *in vivo* dose) following storage at 4 and 25 ˚C. Results are the mean of triplicate experiments ± SD.

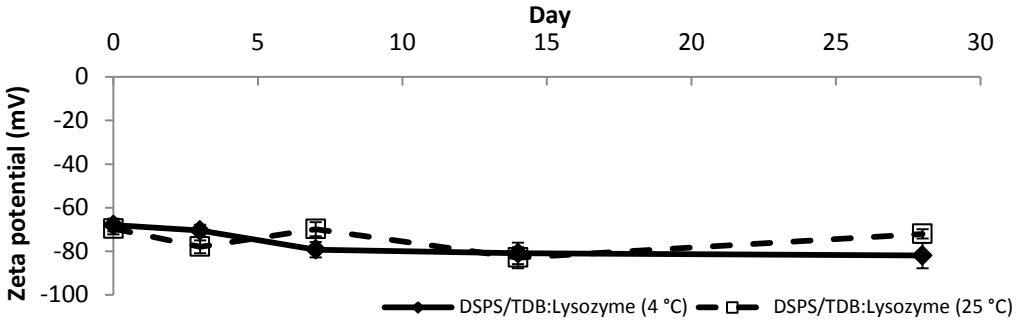


Figure 4-16. Time development of liposome characteristics for MLV DSPS/TDB (with the addition of lysozyme at an *in vivo* dose) following storage at 4 and 25 ˚C. Results are the mean of triplicate experiments ± SD.

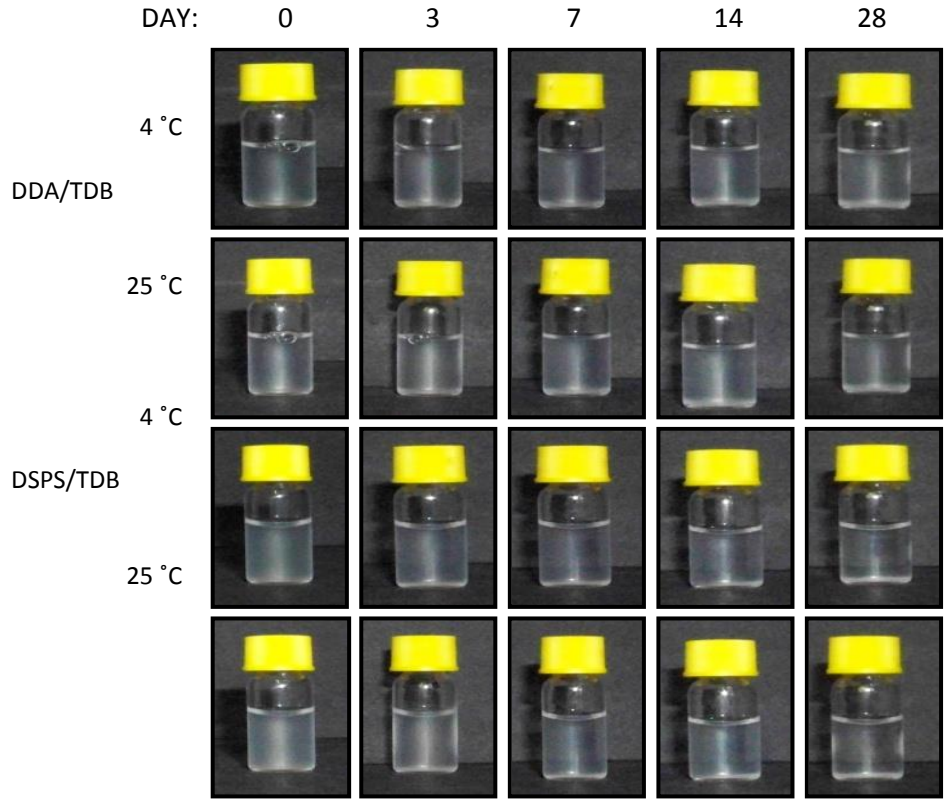


Figure 4-17. Time development on the stability of DDA/TDB and DSPS/TDB liposome formulations (in combination with the *in vivo* dose of lysozyme) following storage for 28 days at 4 ˚C or 25 ˚C.

The relative stability of these vesicles over this time period is hypothesised to be due to their small size and highly charged nature (either cationic or anionic) upon rehydration with Tris buffer (10 mM; pH 7.4). The high stability of these vesicles is due to the insertion of trehalose – 6,6' dibehenate within the lipid bilayer (Christensen *et al.*, 2008). Therefore incorporation of TDB within the liposomal system will increase the hydration of the membrane, thereby preventing dehydration of the quaternary ammonium ion head groups which would otherwise lead to reduction in charge repulsion and aggregation of vesicles (Christensen *et al.*, 2008) thus leading to vesicle stability (Davidsen *et al.*, 2005). These vesicles, in combination with both antigens, are stable in both the 'cold chain' conditions (4 °C) and at 25 °C in terms of their vesicle size and surface charge (as reflected by the zeta potential). This is important for the transportation and delivery of vaccines to third-world countries, in which the vaccines may be subjected to different temperatures upon storage.

4.3.3. Optimisation and quantification of antigen adsorption

4.3.3.1. Indirect quantification of antigen adsorption through use of total proteins assays

During these investigations, it was possible for antigen adsorption to be quantified indirectly by calculating the percentage of free (un-adsorbed) protein present in the supernatant (by the BCA total protein assay; Figure 4.18). This was then confirmed by showing the presence of protein (OVA or lysozyme) in the liposome pellet or supernatant fraction, via SDS-PAGE, therefore demonstrating whether the protein antigen associates with the antigen delivery system.

These results from the BCA assay showed that using multilamellar vesicles (MLV) for cationic DDA/TDB and anionic DSPS/TDB liposomes enabled interaction between oppositely charged liposome and antigen components (due to surface adsorption) resulting in high antigen adsorption, as measured indirectly by the presence of a reduced amount of protein antigen present in the supernatant (as shown by the BCA assay; Figure 4.18). These results therefore indicate high adsorption of anionic OVA to DDA/TDB liposomes (Figure 4.18A), with the reversed effect occurring with cationic lysozyme which is able to highly adsorb to anionic DSPS/TDB liposome delivery systems (Figure 4.18B). These results also suggested that only two wash steps [with Tris buffer (10 mM; pH 7.4)] are required to remove all the protein ($P < 0.001$) from the supernatant fraction of the liposome formulation (Figures 4.18 A and B), so during future experiments the liposome pellet was only be resuspended two times in order to minimise time during the preparation process.

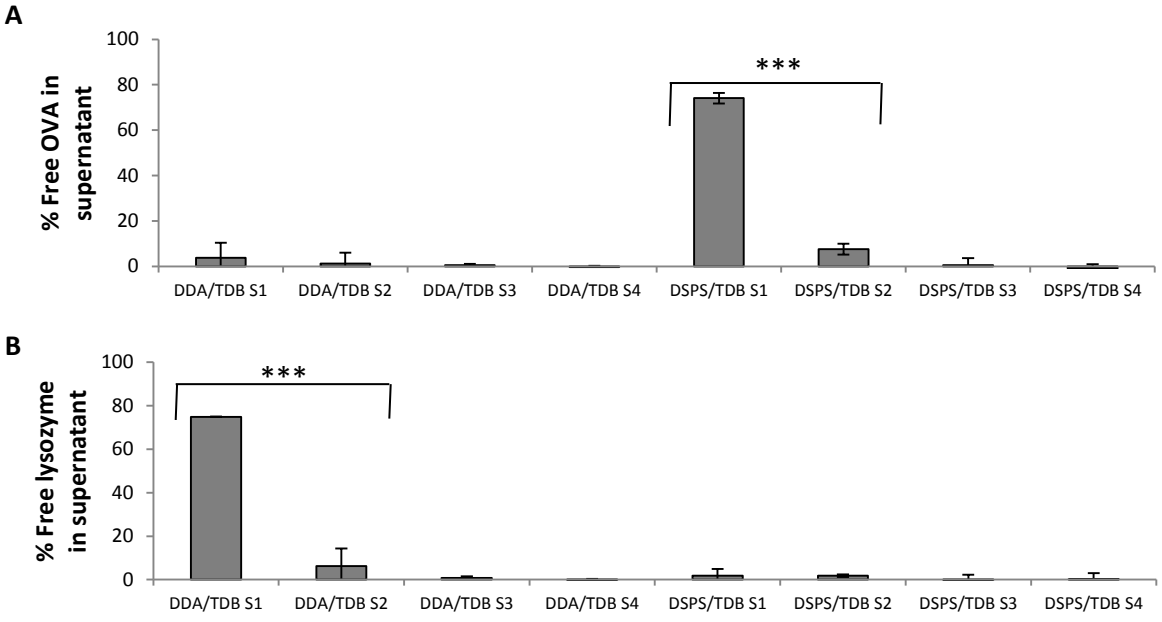


Figure 4-18. BCA assay to show the amount (%) of protein (A; OVA, B; lysozyme) in a series of supernatant fractions for DDA/TDB and DSPS/TDB liposomes. Results are the mean of triplicate experiments ± SD. Significance between the % of protein found in a series of supernatant fractions was measured by one-way ANOVA (*** $p<0.001$).

A SDS-PAGE gel was carried out to show the presence of lysozyme either in the pellet (liposome) or supernatant fractions, for both cationic DDA/TDB and anionic DSPS/TDB liposomes (Figure 4.19A). The aim was to confirm whether the cationic lysozyme was being bound by DDA/TDB or DSPS/TDB liposomes.

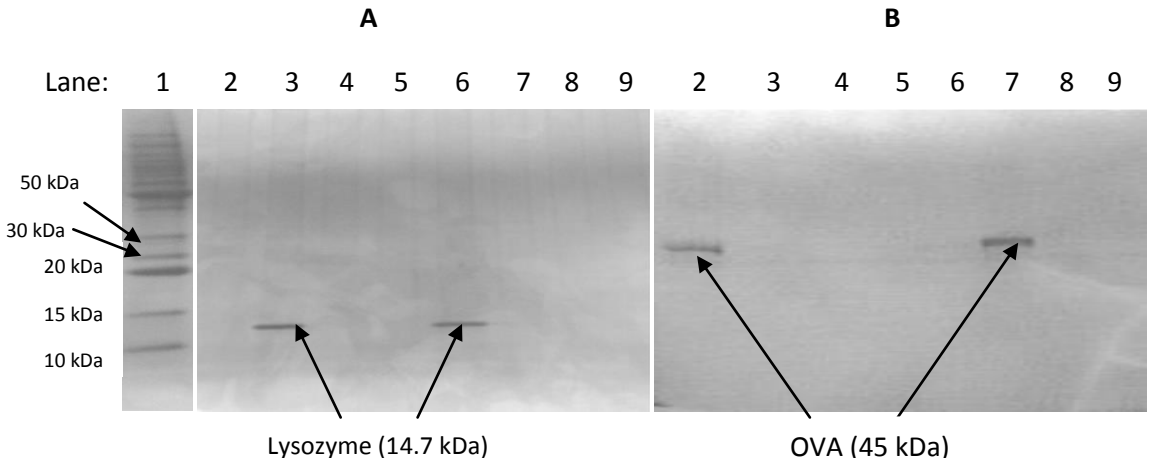


Figure 4-19. SDS-PAGE gel to show the presence of (A) lysozyme and (B) OVA in the supernatant or pellet fractions of DDA/TDB and DSPS/TDB liposomes (Lane 1: Prestained protein marker; Lane 2: DDA/TDB liposome pellet; Lane 3: DDA/TDB Supernatant 1; Lane 4: DDA/TDB Supernatant 2; Lane 5: DDA/TDB Supernatant 3; Lane 6: DSPS/TDB liposome pellet; Lane 7: DSPS/TDB Supernatant 1; Lane 8: DSPS/TDB Supernatant 2; Lane 9: DSPS/TDB Supernatant 3).

This gel (Figure 4.19A) showed that lysozyme was present in the initial supernatant fraction for the cationic DDA/TDB liposomes (Lane 3), and not in the pellet fraction (Lane 2). This backed up previous data from BCA total protein assays (Figure 4.18B) and antigen adsorption studies (Figure 4.2) which showed the importance of electrostatic interactions between oppositely charged liposome and antigen components. Also, lysozyme was present in the pellet fraction for DSPS/TDB liposomes (Lane 6), and there was no lysozyme present in the subsequent supernatant fractions (Lanes 7-9). This showed that this wash step was sufficient to eliminate the majority of the protein from the supernatant. Lysozyme was electrostatically binding to the surface of these anionic liposomes, whereas this protein lacks the ability to bind to DDA/TDB liposomes to any great extent as these components are similar in charge. A further SDS-PAGE gel was carried out to show the presence of OVA either in the pellet (liposome) or supernatant fractions, for both cationic DDA/TDB and anionic DSPS/TDB liposomes (Figure 4.19B).

Figure 4.19B showed that OVA was present in the pellet fraction for DDA/TDB liposomes (Lane 2), and no detectable protein was present in any of the subsequent supernatant fractions (Lanes 3-5). The opposite effect occurs for anionic DSPS/TDB liposomes with no protein being present in the pellet fraction (Lane 6), whilst ovalbumin (OVA) was present in the initial supernatant fraction (Lane 7). There was also no protein present in the subsequent supernatant fractions (Lanes 8 and 9) which showed that all of this protein is present in the initial supernatant fraction. This again showed the importance of electrostatic interaction in the binding between oppositely charged liposome and antigen components, with high adsorption of anionic OVA to cationic DDA/TDB liposomes. Whereas, none to minimal of the anionic OVA protein is present in the DSPS/TDB liposome pellet fraction due to the similar charge of these components therefore indicating a lack of electrostatic interactions. These results correlated with previous data from total protein assays (Figure 4.18A) and the displayed liposome characteristics in order to confirm the importance of antigen-liposome interaction at the surface of multilamellar vesicles.

4.3.3.2. Direct quantification of antigen adsorption through radiolabelling of vaccine antigen

Following the radiolabel of protein antigen, as described previously in Section 2.4.2, it was required to directly quantify antigen adsorption. Verification that antigen was not displaced from liposomes was carried out under simulated in vivo conditions and under control conditions. This simulated in vivo environment was made up of a solution containing 50 % Foetal Calf Serum (FCS) thereby aiming

to mimic the specific protein environment that the liposomes may encounter in vivo upon injection. For therapeutic applications, it is crucial that vaccine or drugs are retained within the liposome system for an appropriate amount of time and that the release kinetics were stable. The bilayer composition has been shown to be a key factor in the liposomal release of drug or protein antigen (Devaraj *et al.*, 2002) and therefore will be investigated in this study with the use of both cationic DDA/TDB and anionic DSPS/TDB formulations (prepared by the lipid-film hydration method).

Initial antigen loading studies demonstrated that using multilamellar vesicles (MLV) there was a higher adsorption of radiolabelled antigen to liposomes, when these components were opposing in charge (Figure 4.20A). Therefore, these charged liposomes (cationic DDA/TDB and anionic DSPS/TDB) are ideal components for antigen adsorption, with the specific antigen isoelectric point (pI) determining their adsorption to either anionic or cationic liposomes. Therefore the cationic protein lysozyme will favour high adsorption to the outer surface of negatively charged DSPS/TDB liposomes (80-85 % loading), whereas the anionic H56 TB antigen will become adsorbed (~ 90 % loading) to the positively charged outer surface of cationic DDA/TDB (Figure 4.20).

A short term (96 hour) study was carried out to measure the antigen loading and release kinetics of H56 and lysozyme when associated with DDA/TDB and DSPS/TDB liposomes placed in simulated in vivo conditions (FCS/Tris; 50:50 v/v). The initial levels of H56 antigen adsorption to DDA/TDB and DSPS/TDB liposomes were 90 % and 20 % respectively (Figure 4.20A). Upon placement of the liposomes in simulated in vivo conditions, there was an initial significant ($p < 0.001$) burst release (< 3 hours) of antigen from liposome formulations following which there were no further significant losses of H56 from both cationic DDA/TDB and anionic DSPS/TDB liposomes (Figure 4.20B). In previous studies, exposure of cationic liposomes to FCS leads to a dramatic increase in vesicle aggregation and decrease in zeta potential due to interaction with negatively-charged serum proteins, however FCS adds cumulatively to the vaccine antigen rather than competitively displacing this antigen from the liposome surface (Henriksen-Lacey *et al.*, 2010a).

The results obtained from lysozyme release from DDA/TDB and DSPS/TDB liposomes showed a very similar trend; after the initial burst release within 3 to 5 hours ($P < 0.001$), antigen release stabilises with no further significant loss of lysozyme from both formulations is noted (Figure 4.20B). A similar trend for antigen loading and release was noted in control conditions (10 mM, pH 7.4; 37 °C) with an initial significant ($P < 0.001$) burst release of antigen being measured in the initial 5 hours of the study. However antigen release stabilised for the remainder of the study period (Figure 4.21B).

These results in Figures 4.20 and 4.21 showed that liposomes were able to efficiently retain their adsorbed antigen. This builds on previous knowledge (Henriksen-Lacey *et al.*, 2010a) that has shown that electrostatic interaction can be exploited to load antigen onto liposomes. Liposomes composed of DDA/TDB and DSPS/TDB could load H56 antigen and lysozyme, respectively, therefore demonstrating electrostatic interaction between vaccine and delivery system. Furthermore the nature of the charge of liposomes is not relevant, with increasing impact coming from the ability of liposomes to electrostatically interact with antigen.

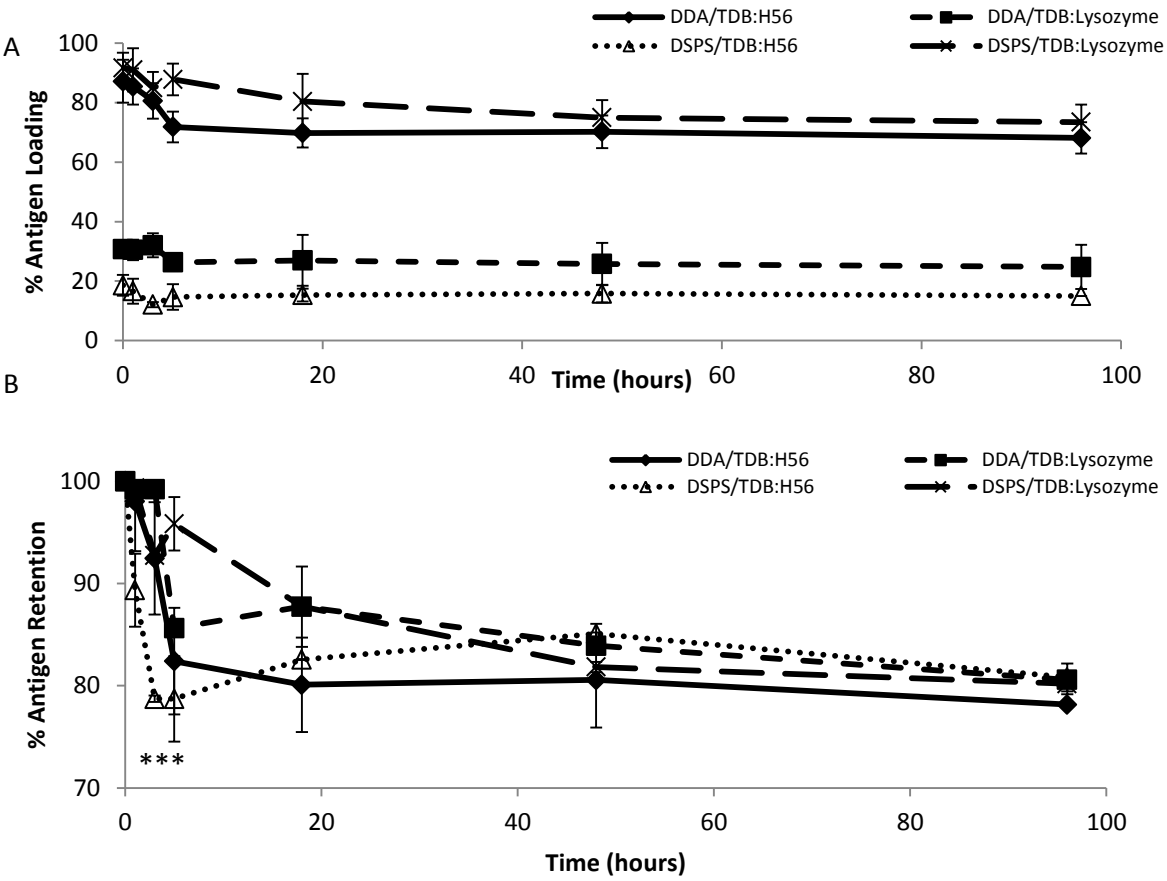


Figure 4-20. Antigen loading (A) and release profile (B) after addition of tested liposome formulations to a simulated in vivo environment (50 % FCS in Tris buffer 10 mM pH 7.4; 37 °C). Results represent the mean ± SD of triplicate experiments. Significance was measured by one-way ANOVA (*** $p<0.001$) in terms of antigen retention compared to the values at 0 h.

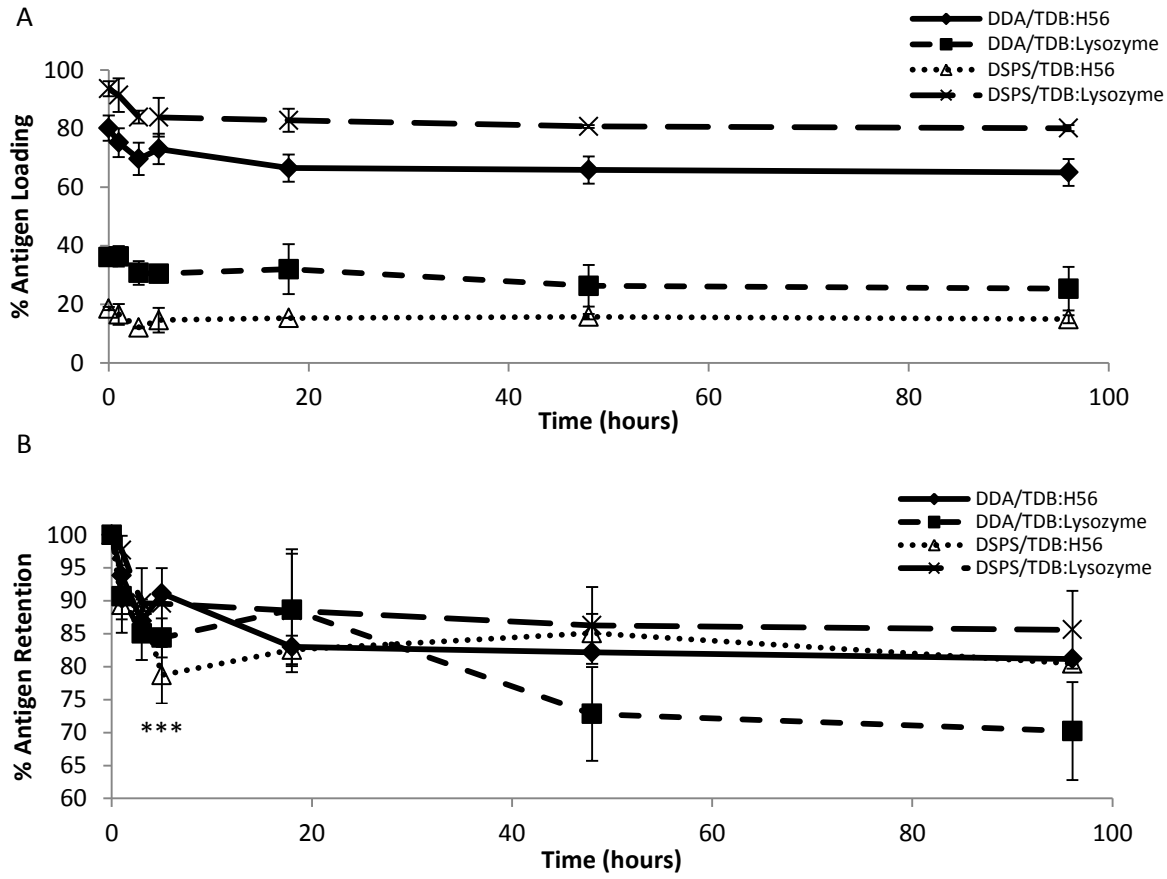


Figure 4-21. Antigen loading (A) and release profile (B) after addition of tested liposome formulations to a control environment (10 mM Tris buffer pH 7.4; 37 °C). Results represent the mean ± SD of triplicate experiments. Significance was measured by one-way ANOVA (*** $p<0.001$) in terms of antigen retention compared to the values at 0 h.

4.3.4. Investigating antigen protection offered by electrostatically binding antigens to liposomes.

Whilst the above studies demonstrate that electrostatic interactions can be used to load antigens to liposomes, the protection these liposomal carriers afford to the antigens was not clear. Therefore, to investigate this, liposomal-antigen systems were exposed to enzyme digestion, via incubation of antigen-adsorbing liposome formulations (DDA/TDB and DSPS/TDB) with increasing concentrations of trypsin. These results showed a significant reduction in % antigen loading ($P < 0.001$) by the liposome systems for all formulations tested (Figure 4.22). This confirmed that addition of antigen post-lipid hydration to the liposome system leads to surface-associated binding of protein antigen which is exposed to enzyme digestion. The significant reduction in antigen loading upon increasing the concentration of trypsin is due to this serine protease enzyme being unable to cross the liposome bilayer and thus only targeting and digesting surface-adsorbed protein antigen, thereby

confirming that protein antigen is directly surface-adsorbed to liposomes. The effect of trypsin on the digestion of surface-adsorbed protein antigen has also been confirmed by means of SDS-PAGE assay (Figure 4.23).

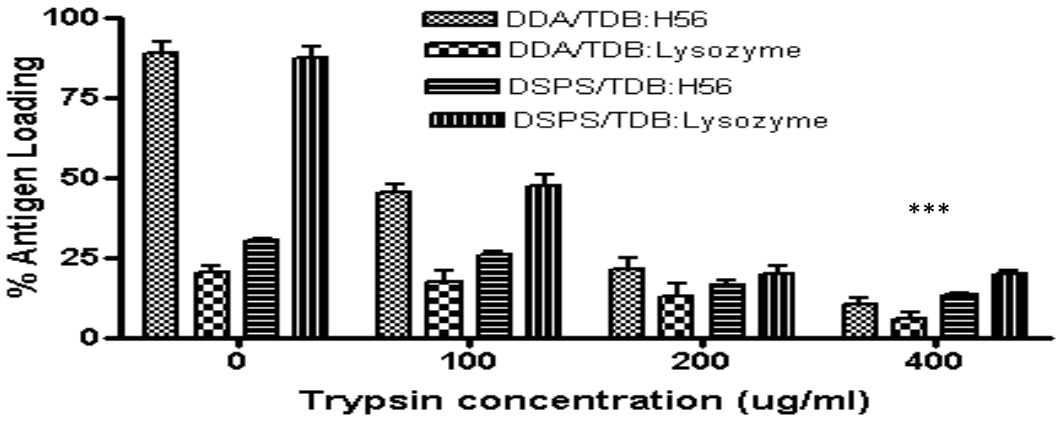


Figure 4-22. Initial antigen loading by liposome vaccine formulations and the effect of increased trypsin concentration on antigen loading. Results represent the mean ± SD of triplicate experiments. Significance is measured by one-way ANOVA, against the initial antigen loading values for each formulation. (*** $p<0.001$).

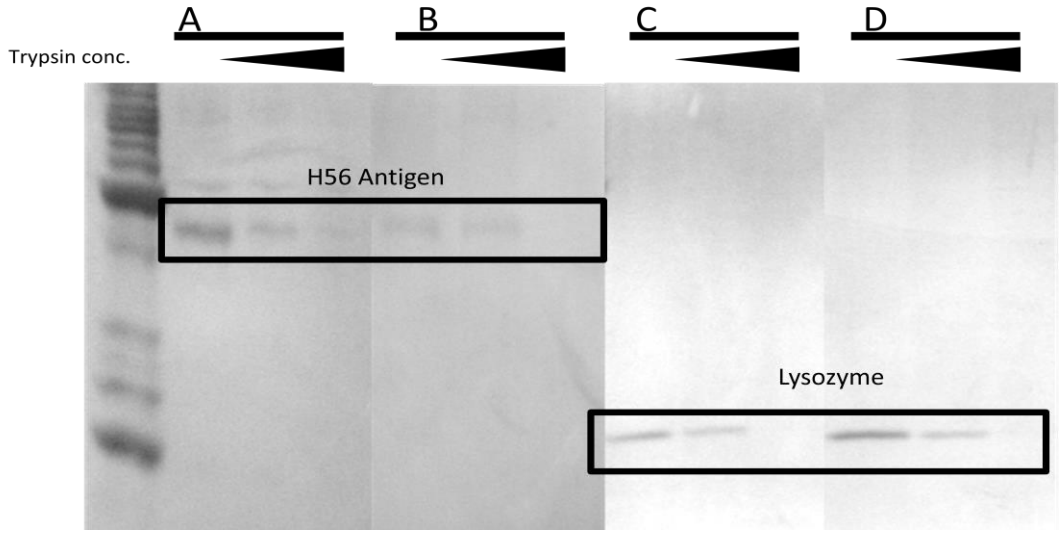


Figure 4-23. Liposomes associated with antigen were exposed to various concentrations of trypsin (0, 100 and 400 µg/mL) to free surface bound antigen. The pellets of washed formulations were analysed by SDS-PAGE using coomasie blue to stain gels. A: DDA/TDB:H56; B: DSPS/TDB:H56; C: DDA/TDB:Lysozyme; D: DSPS/TDB:Lysozyme.

The results in Figures 4.22 and 4.23 showed that upon exposure of antigen-adsorbing liposome vaccine formulations to increasing concentrations of trypsin this resulted in a significant reduction in antigen loading to the liposome delivery system. This correlates with previous knowledge (Kaur *et al.*, 2011) for OVA-adsorbing cationic DDA/TDB liposomes which showed that increasing trypsin

concentration significantly reduced the amount of protein associated with the formulation. Similar findings were also represented in the previous literature which showed that liposome-entrapped human interferon-gamma (IFN-γ), which was not exposed on the liposomal surface were protected from enzyme digestion by trypsin (van Slooten et al, 2001) The effect of liposome preparation method on the localisation of antigen within the delivery system will be further addressed in Chapter 5.

4.3.5. Stability of radioactive markers within liposome formulations

Liposomes (cationic DDA/TDB and anionic DSPS/TDB) were prepared by the lipid film-hydration method, with the incorporation of trace amounts of ^{3}H-Cholesterol (0.17 nM). Dialysis was used to study the retention of ^{3}H-Cholesterol within the liposome membrane in order to show that the radioactive markers used in the subsequent biodistribution study were stable and not lost from liposomes. At various time points (1h, 3h, 5h, 18h, 48h and 96h) over a 96 hour study period, 1 mL aliquots of dialysis media were removed and the amount of ^{3}H present were measured. These results have demonstrated that preparation has no significant effect on the loss of the radioactive marker (^{3}H-Cholesterol) from these liposome formulations. This was shown by minimal release of ^{3}H-labelled Cholesterol from the delivery system over the time course of this study. For LH-prepared formulations, there is a maximum ^{3}H-Cholesterol release of ~ 3 % from the liposome bilayer (Figure 4.24) over the 96 hour period of the study.

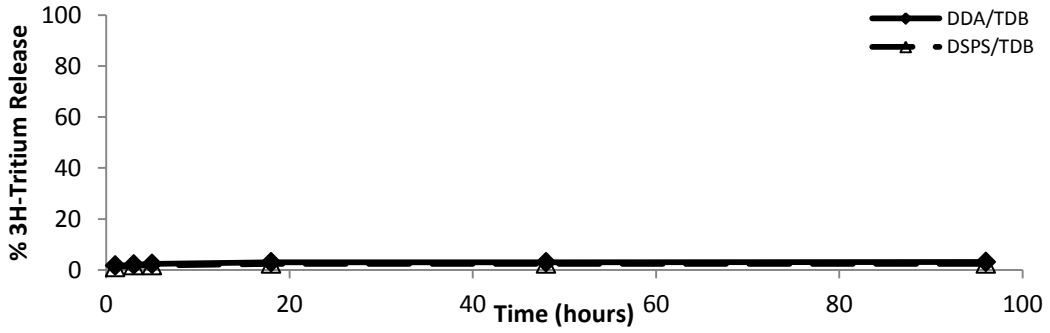


Figure 4-24. Membrane stability of DDA/TDB and DSPS/TDB liposomes (prepared by the lipid film hydration method) was studied by the addition of a trace amount of ^{3}H-Cholesterol within the liposome formulation. Results express the % of the original ^{3}H-Cholesterol dose added to liposomes detected in the dialysis buffer over a 96 hr time period. The samples were stored at 37 °C in 50 % FCS in Tris buffer (10 mM; pH 7.4). Results denote the mean of triplicate experiments ± SD.

4.3.6. Biodistribution studies: the effect of liposome surface charge and antigen adsorption

To determine the role of liposome surface charge on the antigen (and liposome) depot-effect at the SOI, DDA/TDB and DSPS/TDB liposomes were produced with the inclusion of ^{3}H-Cholesterol within the bilayer membrane and ^{125}I-labelled Ag85B-ESAT6-Rv2660c (H56) antigen or lysozyme adsorbed to the delivery system as previously outlined. The SOI and various other tissues were collected and the percentage of the administered dose (% dose) quantified.

The application of dual radiolabelled vaccines provides an ideal method for measuring the pharmacokinetic properties and tissue distribution of both the liposome and antigen (Henriksen-Lacey *et al.*, 2010b). However to overcome the problem of ^{125}I counting using the scintillation counter, a standard curve can be used to factor out the presence of ^{125}I counts. A plot of the cpm values derived from the γ-counter (x-axis) and the cpm values derived from the scintillation counter (γ-axis) was made. The line of best fit and equation for samples below 50,000 cpm (2 % of the dose or less), and samples above 50,000 cpm (2 % of the dose or more) were derived (Figures 4.25 A and B). For both standard curves, the efficiency of ^{125}I was about half of that seen by the γ-counter. Therefore, uncorrected values for scintillation counts for both liposome formulations were applied into the specific equation in order for actual scintillation counts to be measured (without ^{125}I overspill) as will be required for measuring the liposome dose retention.

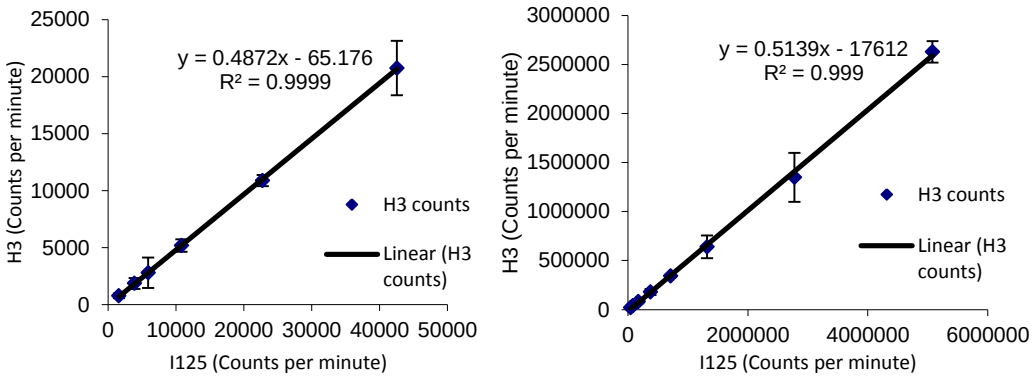


Figure 4-25. Standard curve for ^{125}I values (A) below 50,000 cpm and above 50,000 cpm (x-axis) plotted against their corresponding ^{3}H count values. The equation and R^2 values represent the line of best fit. Results are the mean of 3 measurements ± SD.

4.3.6.1. Vaccine retention at the injection site

The biodistribution of radiolabelled liposome and antigen components was investigated at days 1, 4 and 14 post-injection. Mice were vaccinated i.m. with ^{125}I-labelled antigen (either H56 or lysozyme) with and without ^{3}H-labelled liposomes (cationic DDA/TDB or anionic DSPS/TDB) in order to analyse the fate of injected antigen both at the vaccination site (left quadricep) and the draining popliteal lymph node (PLN). These were the only organs investigated as previous studies have shown negligible presence of liposome and antigen in the lung, heart, kidney, liver, brain and small intestine (Henriksen-Lacey *et al.*, 2010a).

As previously reported for DDA/TDB (Henriksen-Lacey *et al.*, 2010a), high levels of retention at the injection site was noted for these liposomes with around 80, 45 and 25 % of the liposome dose detected on days 1, 4 and 14 p.i (post-injection) respectively (Figure 4.26A). In contrast, anionic DSPS/TDB liposomes drained rapidly from the SOI and by day 4 p.i only 25 % of the injected dose was detected, decreasing to ~ 8 % on day 14 (Figure 4.26A). Therefore the liposome dose retention at the SOI was significantly higher for cationic systems compared to their anionic counterparts ($P < 0.001$).

When H56 antigen was delivered in combination with cationic DDA/TDB liposomes an 'antigen depot' at the SOI was observed with similar drainage kinetics observed (80 %, 45 % and 10 % of the dose detected at days 1, 4 and 14 p.i respectively (Figure 4.26B)). In contrast, lysozyme delivered in combination with DDA/TDB drained rapidly with ~ 20 % retention at day 1 p.i and less than 1 % of the injected dose detected at the SOI on day 14 p.i. Delivery of lysozyme with the anionic liposome DSPS/TDB significantly improved the early retention of lysozyme with around 40 % dose retention at the SOI ($p < 0.001$), however by day 14 p.i the levels present were low for all formulations tested (Figure 4.26B). However, when anionic H56 was delivered in combination with the DSPS/TDB delivery system this resulted in minimal levels of antigen retention at the vaccination site with only 18 % antigen remaining 1 day p.i with a further significant reduction ($P < 0.001$) at later time points (Figure 4.26B).

Therefore, these studies have demonstrated the importance of cationic liposome charge for increased retention of the delivery system at the SOI and also that H56 antigen adsorption to the liposome surface (via electrostatic interactions) results in an increased antigen depot, due to charge-related electrostatics between antigen and delivery system. Anionic DSPS/TDB liposomes have also been formulated which showed less retention at the SOI compared to their cationic counterparts. This is due to the increased ability of cationic DDA/TDB liposomes to interact with negatively

charged interstitial proteins at the SOI (Henriksen-Lacey *et al.*, 2010a). Cationic liposomes, whilst retained at the injection site are not able to retain lysozyme due to their inability of these components to exploit electrostatic interaction. In contrast, whilst DSPS/TDB liposomes are lesser retained at the injection site they can electrostatically interact with lysozyme, and not anionic H56 antigen. Henriksen-Lacey et al have previously shown the importance of charge-mediated electrostatic interactions for the efficient delivery of antigen and subsequently induce enhanced immune responses (Henriksen-Lacey *et al.*, 2010c).

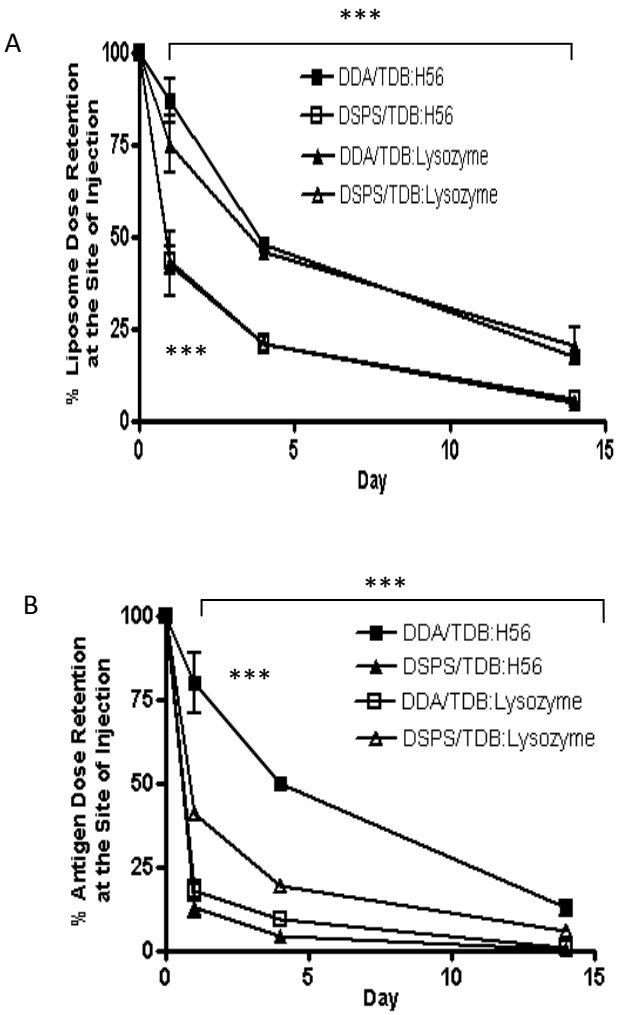


Figure 4-26. Biodistribution of vaccine components, liposome (A) and antigen (B) at the site of injection (SOI) following intramuscular (i.m.) injection of liposome vaccine formulations. Results represent the mean ± SD of 4 mice per group. Significance was measured by one-way ANOVA (*** $p<0.001$).

4.3.6.2. Movement of vaccine to the draining lymph nodes

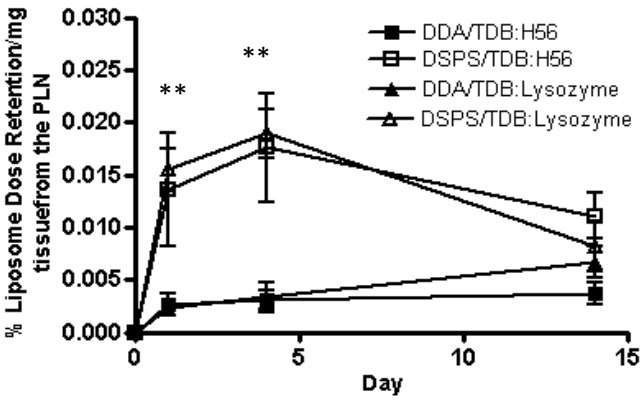
The percentage dose of antigen and liposome at the draining popliteal lymph node (PLN) was also calculated, as this is the local lymphoid tissue to which antigen detected at the SOI (left quadricep) drains. These lymph nodes will therefore be the primary site at which the antigen will be presented to T-cells.

Liposomal draining to the PLN followed two distinct patterns (Figure 4.27A); anionic DSPS/TDB liposomes were found in significantly higher amounts at the earlier time points of 1 and 4 day p.i ($P < 0.01$) whilst cationic DDA/TDB liposomes were detected in low amounts (< 0.005 % dose/mg tissue at all 3 time points. The levels of liposome and antigen were also measured in the non-draining popliteal lymph node (PLN) of the non-injected leg. This gave rise to negligible levels of these vaccine components, which were even lower than that in the draining PLN of the injected leg (results not shown).

In terms of antigen retention at the draining PLN (Figure 4.27B) an antigen depot of either H56 or lysozyme was present at days 1 (~ 0.015 % dose/mg tissue) and 4 p.i (~ 0.01 % dose/mg tissue) respectively when co-administered with DSPS/TDB rather than DDA/TDB (Figure 4.28B), with similar levels of antigen retention displayed for all formulations at day 14 p.i. ($\leq$ 0.005 % dose/mg tissue). These results suggested that antigen as well as liposomes drain rapidly to the PLN when a depot of antigen at the injection site is not obtained (Figure 4.27B). This is due to the general trend that DSPS/TDB are less likely to be retained at the SOI and so are more likely to drain to the PLN. This pattern and the absolute values of liposome drainage are very similar to that observed previously (Henriksen-Lacey *et al.*, 2010c) when DDA/TDB liposomes were compared with 'neutral' DSPC/TDB liposomes.

The prolonged retention of antigen at the SOI mediated by DDA/TDB liposomes as compared to DSPS/TDB could result in a prolonged uptake of electrostatically-bound antigen by APCs, which has been shown previously when cationic liposomes were compared against neutral liposomes (Foged *et al.*, 2004). The rapid drainage of anionic liposomal adjuvants from the injection site was shown to correlate with increased early presence in the draining PLN, which has previously been shown to be disadvantageous in the induction of Th1 immune responses (Henriksen-Lacey *et al.*, 2010c), however this will be further investigated in Section 4.3.7.

A



B

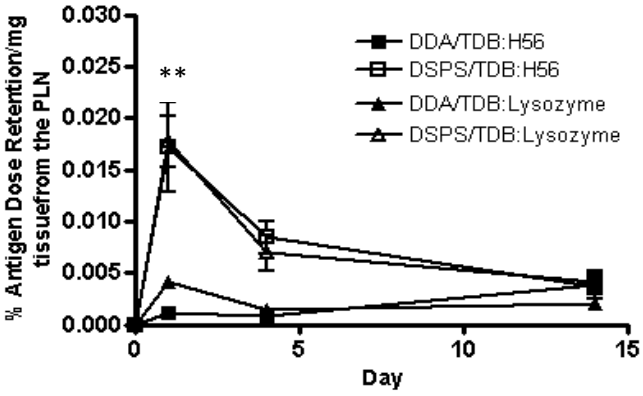


Figure 4-27. Biodistribution of vaccine components, liposome (A) and antigen (B) at the popliteal lymph node (PLN) following intramuscular (i.m.) injection of liposome vaccine formulations. Results represent the mean ± SD of 4 mice per group. Significance was measured by one-way ANOVA (** $p<0.01$).

4.3.6.3. Pontamine Blue as a marker for innate immune responses

Similarly to previous studies, pontamine blue was used as a marker for influx of monocytes at the SOI (Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2011a). The results shown in Figure 4.28 are in correlation with previous studies which have shown increased pontamine blue staining at the SOI after injection of cationic DDA/TDB liposomes. However, following i.m. immunisation with anionic DSPS/TDB this resulted in low or minimal staining at the SOI. These results suggest that when cationic liposomes are involved, regardless of the antigen associated, a higher infiltration of macrophages to the SOI is observed. This may provide the transport mechanism of liposomes (and hence adsorbed antigen) from the vaccination site to the draining lymph nodes (Henriksen-Lacey *et al.*, 2010a). This is due to slower drainage of DDA/TDB liposomes when compared to DSPS/TDB liposomes, which suggests that the immune response to DDA/TDB is significantly enhanced due to the increased induction of proinflammatory responses at this site including tissue damage which is

due to increased toxicity of cationic liposomes (Filion and Phillips, 1997). This will lead to the infiltration of APCs to such areas which actively undergo endocytosis which is important for subsequent antigen presentation. This reported influx of innate immune cells to the SOI also suggests that DDA/TDB liposomes induce a "danger effect", as originally described by Matzinger (Matzinger, 1994). This danger signal can be initiated upon parenteral delivery of vaccines which causes localised tissue damage, which may lead to the rupture of cells causing the release of alarmins (Bianchi et al, 2007) at the injection site, therefore leading to monocyte recruitment at this site.

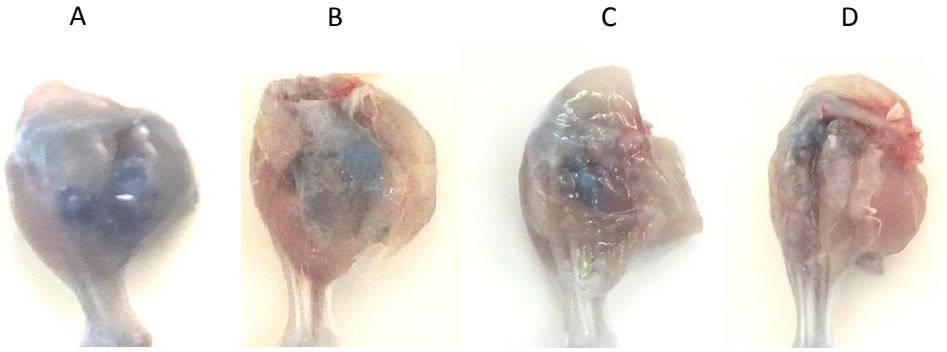


Figure 4-28. Pontamine Blue Staining at the site of injection (SOI) following i.m. injection of DDA/TDB (A and B) or DSPS/TDB (C and D), either adsorbing H56 (A,C) or lysozyme (B,D). These photos are taken at 1 day p.i.

4.3.7. The ability of liposomal vaccines to induce immune responses

In order to investigate the role of surface charge on the immunogenicity of liposome vaccine formulations, we will carry out a number of vaccine studies in order to show the immune response generated (Th1 response Vs Th2 response). The H56 TB antigen was formulated within cationic DDA/TDB and anionic DSPS/TDB liposomes, respectively, before intramuscular (i.m.) injection of these vaccine formulations into the left quadriceps. During this study, a triple homologous vaccination regimen was maintained in which each mouse was injected three times at two-weekly intervals (day 1, 15 and 29).

4.3.7.1. Generation of antigen-specific antibody responses

The ability of cationic DDA/TDB liposomes and anionic DSPS/TDB liposomes (in combination with the anionic H56 antigen) to induce IgG (total), IgG1 and IgG2b antibody isotypes was investigated using antibody ELISAs. Blood was collected on days 0, 14, 28, 36 and 49 and mice were injected with

liposome vaccine formulations on days 1, 15 and 29. These results in Figures 4.29 A to C showed that DDA/TDB:H56 promoted higher antibody responses than DSPS/TDB:H56 and antigen alone.

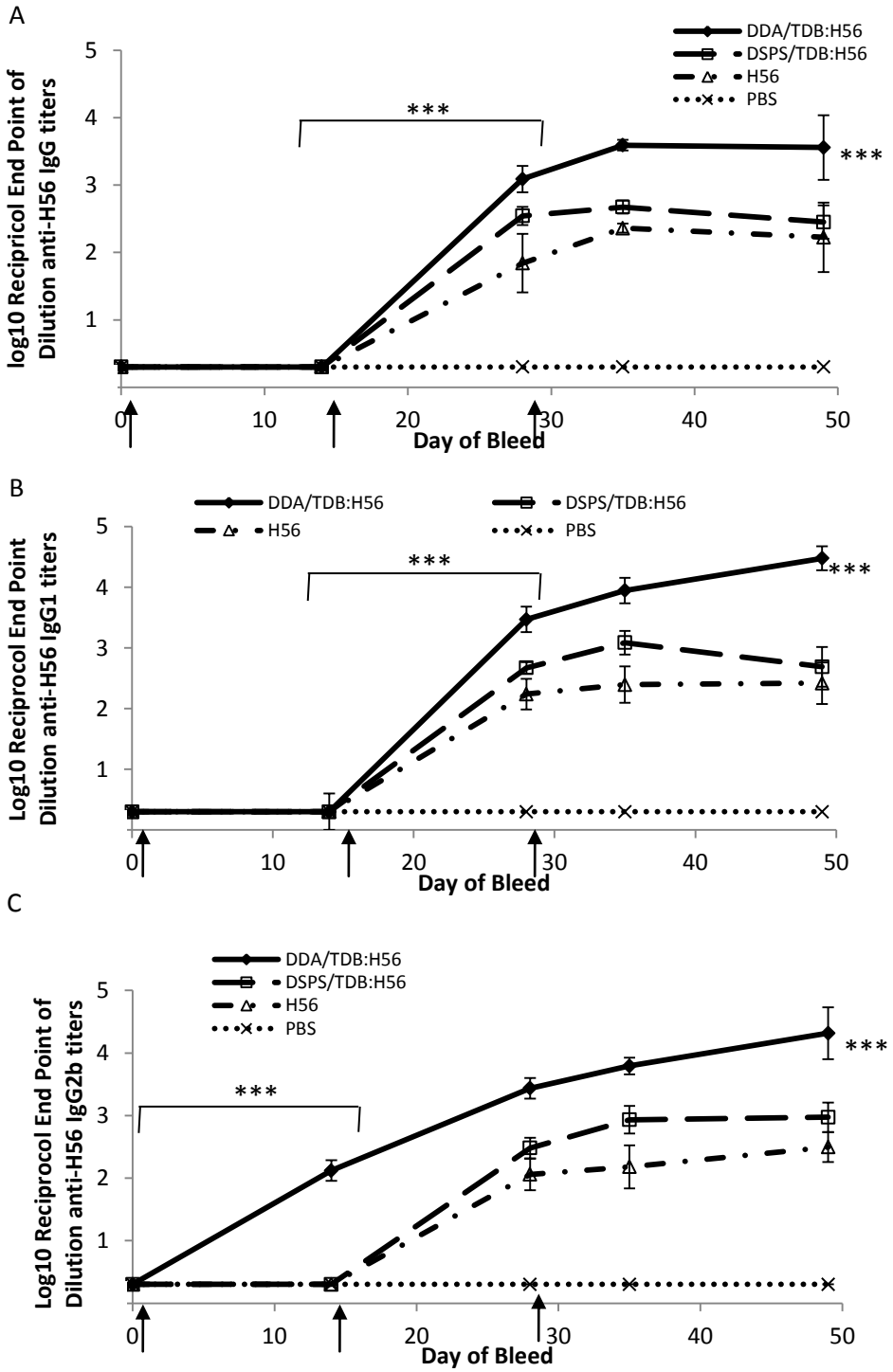


Figure 4-29. Ag85B-ESAT6-Rv2660c (H56) specific antibody titres; IgG (A), IgG1 (B) and IgG2b (C). Groups of five female C57/BL6 mice, approximately six to eight weeks old, received doses of vaccine formulations containing 5 µg of Ag85B-ESAT6-Rv2660c (H56) in a 50 µl volume. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks at days 1, 15 and 29 (as represented by arrows). Serum samples were taken at days 0, 14, 28, 35 and 49. Results represent the mean of 5 mice per group ± SD. Significance was measured by one-way ANOVA between time points and between formulations (*** $p<0.001$).

For IgG and IgG1 responses, for all formulations tested the antibody titres increased from background levels by day 28 (following 2 x immunisations) thus showing the requirement for a memory response. So upon reencounter of the H56 antigen (following immunisation 2) this resulted to the body responding by raising antibody titres in the blood sera which is important in initiating humoural and cell-mediated immune responses. Interestingly, in terms of the IgG2b antibody response, H56 coadministered with DDA/TDB liposomes was able to generate antibody responses at day 14 (following one immunisation) which are further boosted following further immunisation (Figure 4.29C). This has not been noted before in the previous literature (Henriksen-Lacey et al., 2011b, Kaur et al., 2011) however these studies used the H1 antigen in combination with cationic DDA/TDB liposomes, whilst this study uses the latent TB subunit vaccine H56 in combination with adjuvant delivery system which has been to shown to offer significantly better protection and control over late stages in TB, partly due to improved T-cell responses (Aagaard *et al.*, 2011).

Results in Figure 4.29 suggested that DDA/TDB liposome induced significantly higher antibody titres than their anionic DSPS/TDB counterparts. This could be due to increased adsorption of H56 antigen to cationic DDA/TDB liposomes, as well as better retention of these liposomes at the SOI which has previously been shown due to interaction with anionic serum proteins (Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2010c). This higher retention of H56 antigen by DDA/TDB liposomes allows for higher bioavailability thus allowing production of higher antigen-specific antibody titres in the blood sera. Previous literature has also suggested that antigen adsorbed to cationic DDA/TDB liposomes resulted in the eliciting of strong antibody responses and cell-mediated immunity (Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2011c).

4.3.7.2. Splenocyte proliferation in response to vaccine antigen

During this study, the ability of the TB antigen H56, either alone or in combination with cationic DDA/TDB or anionic DSPS/TDB liposomes, to be delivered to antigen presenting cells (APCs) in the spleen was investigated by means of a splenocyte proliferation assay. Figure 4.30 showed the proliferative ability of splenocytes in response to the positive control stimuli, ConA. All mice responded to ConA and the average ^{3}H-thymidine uptake was between 20,000 – 60,000 counts per minute (CPM). Splenocytes derived from unimmunised mice or those immunised with PBS, free H56, anionic DSPS/TDB:H56 and cationic DDA/TDB:H56 responded to nearly equal proliferative levels (P = n/s). In addition to inducing splenocyte proliferation with the non-specific stimulator ConA,

splenocytes were also exposed to previously encountered H56 (Ag85B-ESAT6-Rv2660c) antigen. Figure 4.31 gives an overview of the proliferative ability for all liposomal vaccine formulations, assessing the proliferative ability of splenocytes following reencounter with increasing concentrations of H56 antigen.

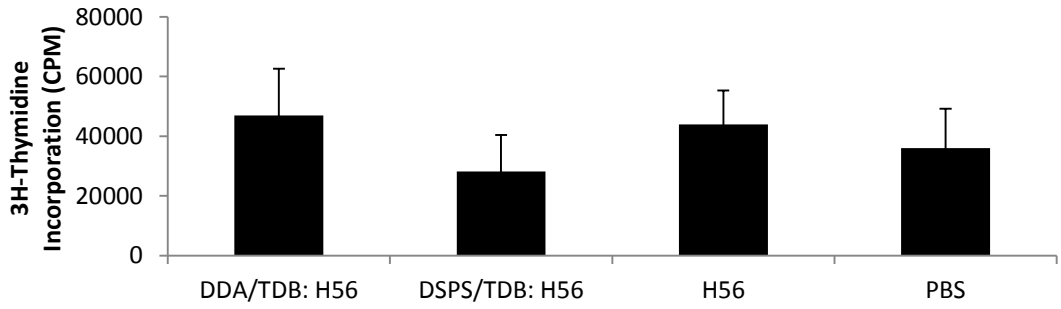


Figure 4–30. Splenocyte proliferation in response to stimulation or restimulation with ConA (at a concentration of 2 µg/mL), as measured by ^{3}H-thymidine incorporation. Results are the mean of triplicate wells ± SD.

The results in Figure 4.31 demonstrated a charge dependent trend in terms of splenocyte proliferation upon restimulation using increasing concentrations of H56 antigen (0, 0.05, 0.5 and 5 µg/mL respectively). Upon restimulation of splenocytes with H56, those derived from mice immunised with DDA/TDB:H56 gave rise to the highest levels of splenocyte proliferation. Compared to free antigen, or antigen administered with DSPS/TDB liposomes these levels of proliferation were significantly higher ($P < 0.001$) at all H56 restimulation conditions (Figure 4.31).

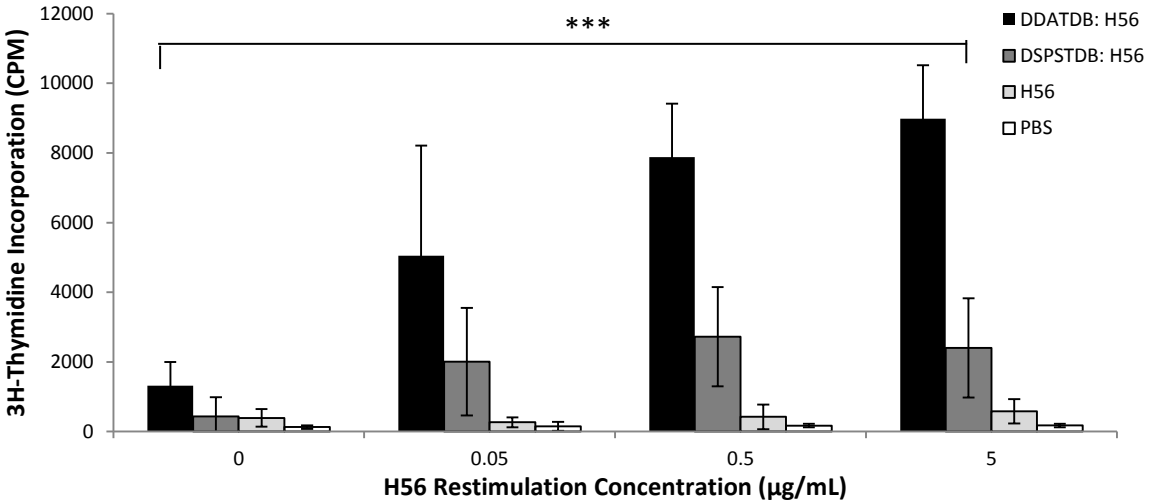


Figure 4-31. Splenocyte proliferation in response to stimulation or restimulation with the H56 antigen, as measured by ^{3}H-thymidine incorporation. Results are the mean of triplicate wells ± SD. Significance was measured by one-way ANOVA (*** p<0.001).

These results suggested that liposome charge has an important role to play in terms of the ability of liposome vaccine formulations to deliver antigen to APCs in the spleen with DDA/TDB liposomes being able to induce a population of T-cells that could respond upon re-exposure to the disease antigen. The inability of DSPS/TDB:H56 immunised mice to mount increased levels of splenocyte proliferation is partly due to the lesser levels of antigen adsorption between these vaccine components, as described previously, therefore upon re-exposure to antigen, a smaller population of memory T-cells are initiated. However, these levels of proliferation are higher than mice immunised with H56 alone ($P < 0.001$; Figure 4.31). Therefore the ability of the spleen cells to efficiently proliferate upon response to H56 TB antigen leads to their ability to induce spleen-specific immune responses which will be further discussed in Section 4.3.7.3. H56-adsorbing cationic DDA/TDB liposomes have the ability to produce a strong cell-mediated immune responses as well as high levels of humoural immune response, due to their ability to form a strong antigen depot (Perrie *et al.*, 2013) as well as being released from this site around the body in order to stimulate a strong antibody response.

4.3.7.3. Cytokine response from *ex vivo* restimulated splenocytes

The supernatants of restimulated splenocytes were assayed for the presence of cytokines IFN-γ, IL-2, IL-5, IL-6, IL-10. Figures 4.32 to 4.34 showed cytokine production for splenocytes derived from individual formulations following splenocyte restimulation with ConA, media only (negative control) and H56 antigen respectively. During this investigation, the presence of cytokines which were associated with generating a Th1-biased immune response, namely interferon-gamma (IFN-γ) and interleukin-2 (IL-2) were measured. Secondly, the presence of cytokines which were associated with generating a Th2-biased immune response namely interleukins 5, 6 and 10 (IL-5, IL-6 and IL-10) were measured.

The results presented in this study showed the importance of liposome surface charge for the effective delivery of H56 TB antigen and, hence, the generation and production of cytokines associated with Th1 and Th2-immunity. In terms of splenocyte cells restimulated with the positive control stimuli, ConA, this led to higher production of all cytokines tested in the study with liposome vaccine formulations (in association with H56) initiating slightly higher levels of all these cytokines (Figure 4.32). In terms of our negative control, in which splenocyte cells were restimulated with medium only this resulted in negligible production of all cytokines tested for in this study, which was fully due to the fact that these restimulation conditions do not therefore allow the enhanced

activation of a population of memory T-cells that have previously encountered the disease antigen so leading to a lack of cytokine production (Figure 4.33).

Splenocytes restimulated with H56 antigen (at a concentration of 5 μg/mL) resulted in greater cytokine release compared to the negative control for all cytokines tested for in this study. Splenocytes derived from DDA/TDB:H56 immunised mice were able to show higher levels of the Th1 cytokines IFN-γ and IL-2 with values of ~ 2300 pg/mL and 1100 pg/mL being measured respectively (Figure 4.34). In contrast there was a liposome charge-dependent trend in which DSPS/TDB:H56, free H56 and PBS-immunised mice respectively produced significantly less ($P < 0.001$) amounts of these cytokines [IFN-γ and IL-2]. This trend was also evident in the production of interleukin-6 (IL-6) in which mice immunised with cationic DDA/TDB:H56 gave rise to production of this cytokine at levels of 500-600 pg/mL (Figure 4.34). In comparison, a two-fold decrease ($P < 0.001$) was noted upon i.m. injection of anionic DSPS/TDB:H56 with lower values being recorded for H56 alone- and PBS-immunised mice respectively (Figure 4.34). In contrast in terms of the production of Th2-cytokines, interleukins 5 and 10 (upon splenocyte restimulation with H56 antigen), the opposite effect was observed with slightly higher production noted upon immunisation with the anionic DSPS/TDB:H56 liposome vaccine formulation with concentrations of ~ 300 pg/mL and ~ 700 pg/mL being measured respectively (Figure 4.34). This was in comparison to mice immunised with DDA/TDB:H56, H56-alone and PBS respectively which led to lower levels of these cytokines being produced. However the difference between cationic DDA/TDB:H56 and anionic DSPS/TDB:H56 liposomes was not significant.

Therefore these studies showed that immunisation of mice with H56 (in combination with cationic DDA/TDB liposomes) led to a bias towards a Th1 immune response, as characterised by high production of IFN-γ and IL-2. This has been shown previously following the delivery of TB subunit vaccine antigen (such as Ag85B-ESAT6 or H1) within cationic DDA/TDB liposomes (Davidsen et al., 2005, Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2011b). Replacing the cationic lipid, with the anionic lipid DSPS, within the formulation led to a significant reduction in Th1 immune responses which are slightly higher than H56 alone-immunised mice. Also, these studies have clearly demonstrated that the function of TDB within the formulation is highly dependent on the composition of the delivery system as shown by significant reduction in the measured Th1 responses after replacing cationic DDA with anionic DSPS within the formulation. However, the generated Th2 responses obtained are not dependent on the liposome surface charge. However, these liposome adjuvants in general induced low levels of IL-5 as compared to such adjuvants as aluminium hydroxide which are able to induce strong Th2 responses (Davidsen *et al.*, 2005).

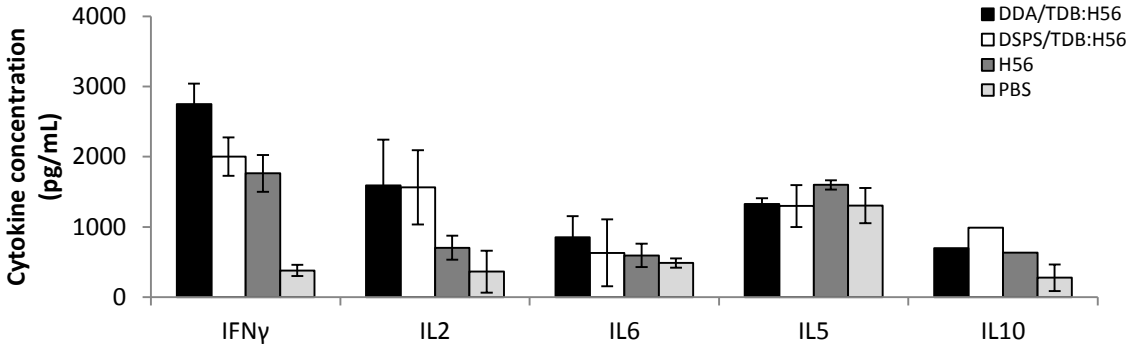


Figure 4-32. Cytokine production from splenocytes restimulated with ConA at 2 µg/mL. Results are shown for mice immunised with PBS (negative control) and H56 either administered alone or in combination with cationic DDA/TDB or anionic DSPS/TDB liposomes. Mice received 3 injections with 2-week intervals and splenocytes were obtained 3 weeks post the final immunisation. Cytokines were detected from splenocyte supernatants, following 48h restimulation, and measured using sandwich ELISAs. Results are the mean of 5 mice per group ± SD. Significance between experimental groups was measured by one-way ANOVA (* p<0.001; ** p<0.01; *** p < 0.05) as compared to PBS-immunised mice.

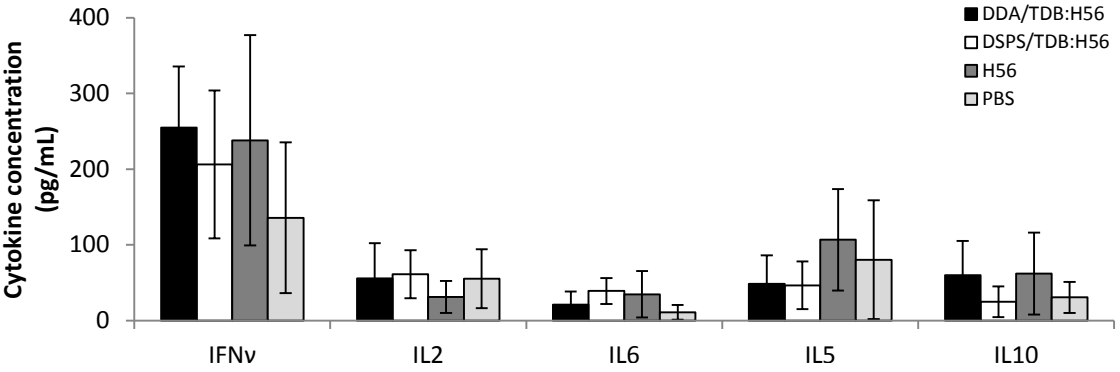


Figure 4-33. Cytokine production from splenocytes restimulated with media only (negative control). Results are shown for mice immunised with PBS (negative control) and H56 either administered alone or in combination with cationic DDA/TDB or anionic DSPS/TDB liposomes using the same experimental method described above.

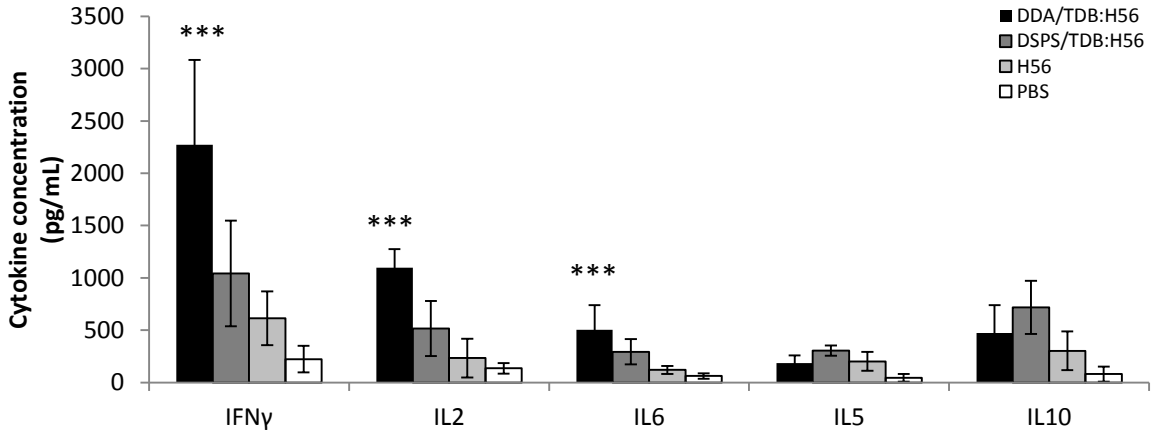


Figure 4-34. Cytokine production from splenocytes restimulated with H56 at 5 µg/mL. Results are shown for mice immunised with PBS (negative control) and H56 either administered alone or in combination with cationic DDA/TDB or anionic DSPS/TDB liposomes using the same experimental method described above. Significance was measured by one-way ANOVA (*** p<0.001) in comparison to anionic DSPS/TDB:H56.

4.3.7.4. Cytokine analysis at the injection site

During this investigation there was a need to monitor and study 'leg immune responses' at the site of injection (SOI). This was in order to monitor the immune response in regards to cytokines associated with the inflammasome complex such as interleukin1-beta (IL-1β) as well as interleukin-18 (IL-18) and interleukin-33 (IL-33). Results from this study demonstrated a significant ($P < 0.001$) liposome charge-dependent relationship in the cytokine response at the vaccination site. IL-1β and IL-18 levels from mice immunised with DDA/TDB:H56 were in the region of 16 ng/g tissue and 7 ng/g tissue respectively (Figure 4.35). However, for mice immunised with DSPS/TDB:H56, free H56 and PBS these gave IL-1β levels of 4, 2 and 0.2 ng/g tissue respectively. There was also a similar correlation in terms of IL-18 levels at the vaccination site with concentration of 1.8, 1 and 0.4 ng/g tissue determined for DSPS/TDB:H56, free H56 and PBS control immunised mice respectively (Figure 4.35). For DDA/TDB:H56 immunised mice, these IL-18 and IL-1β levels (at the vaccination site) are in correlation with increased production of IFN-γ and IL-2 (from H56 restimulated splenocytes) therefore this showed a Th1-bias in cytokine production for this cationic liposome formulation (DDA/TDB:H56) at both the injection site and from restimulated splenocytes.

The production of IL-33 (Figure 4.35) was also monitored at the vaccination site following this 7-week immunisation study (mice terminated at day 49). DSPS/TDB:H56 immunised mice are able to produce slightly higher levels of IL-33 (12 ng/g tissue) in comparison to DDA/TDB:H56, free H56 and PBS-control immunised mice which gave levels of 10, 1.8 and 0.5 ng/g tissue respectively (Figure 4.35). The levels of IL-33 at the vaccination site correlated with IL-5 and IL-10 production from H56 restimulated splenocytes, thus representing a slight change in bias from Th1 to Th2 immunity upon immunisation with anionic DSPS/TDB:H56 liposome vaccine formulation. Upon intramuscular injection of DDA/TDB:H56, a moderate amount of Th2 cytokines (IL-5, IL-10 and IL-33) are still produced, which suggests that a balanced Th1/Th2 immune response is generated, however this balanced response is strongly skewed towards Th1 cell-mediated immunity.

The presence of the pro-inflammatory cytokines IL-1β, IL-18 and IL-33 suggested a role for the NALP3 inflammasome complex in the generation of the desired immune response (Dinarello, 1999, Gracie et al., 2003, Schmitz et al., 2005, Sharp et al., 2009). This multi-molecular complex controls the activity of the interleukin (IL) converting enzyme, caspase-1. The presence of endogenous signals such as pathogen-associated stimuli (by Toll-like receptors) and high energy (in the form of ATP), have been hypothesised to lead to the activation of the NALP3 inflammasome which results in the conversion of inactive pro-caspase1 to form the active caspase1 (Sutterwala et al., 2006a, Sutterwala

et al., 2006b, Petrilli et al., 2007, Muruve et al., 2008, Stutz et al., 2009). This enzyme is able to proteolytically cleave pro-forms of IL1β, IL-18 and IL-33 in order to form these bio-active cytokines. Both IL-1β and IL-18 are highly potent proinflammatory cytokines (Dinarello, 1999, 2000) which are involved in the induction of interferon-gamma (IFN-γ) expression in order to promote the differentiation of type 1 T-helper cells (Th1). However, a classical Th2 cytokine IL-33, which is a member of the same family of cytokines as IL-1β and IL-18, is able to promote immune responses by type 2 helper T-cells (Th2) as shown by Schmitz and colleagues in 2005 (Schmitz *et al.*, 2005). This evidence and results from these studies therefore suggested that caspase-1 and the NALP3 inflammasome is critical as part of the immune response generated by these liposome vaccine formulations.

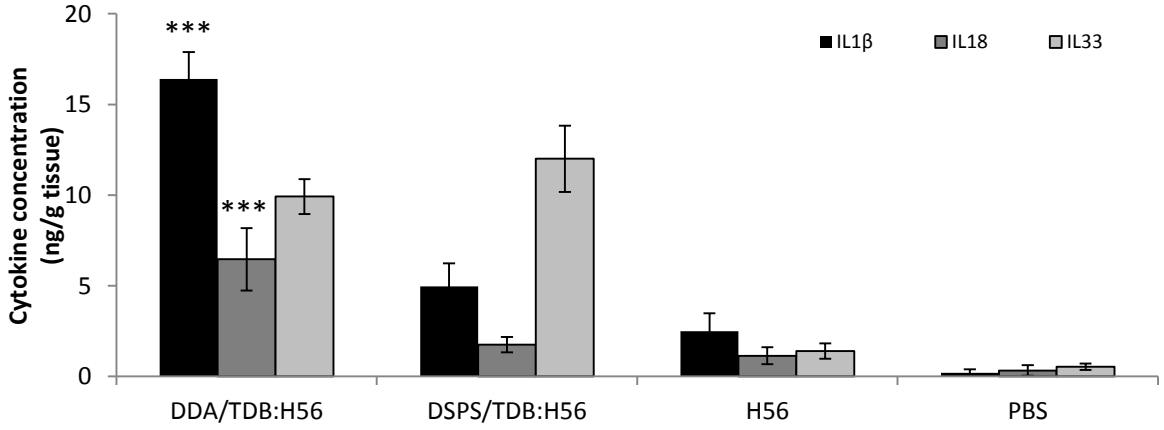


Figure 4-35. IL-1β (A), IL18 (B) and IL33 (C) production from excised leg muscle from the SOI derived from mice immunised with Ag85B-ESAT6-Rv2660c (H56) in combination with either DDA/TDB or DSPS/TDB, as well as negative control groups (H56 and PBS respectively). Mice received 3 injections with 2-week intervals; muscle from the SOI was collected at day 49 upon termination of the study. Leg muscle was excised, digested and homogenised. Cytokines were measured from muscle supernatants using sandwich ELISAs. Results represent the mean of 5 mice per group ± SD. Significance between experimental groups was measured by one-way ANOVA (*** $p<0.001$) as compared to DSPS/TDB:H56 immunised mice.

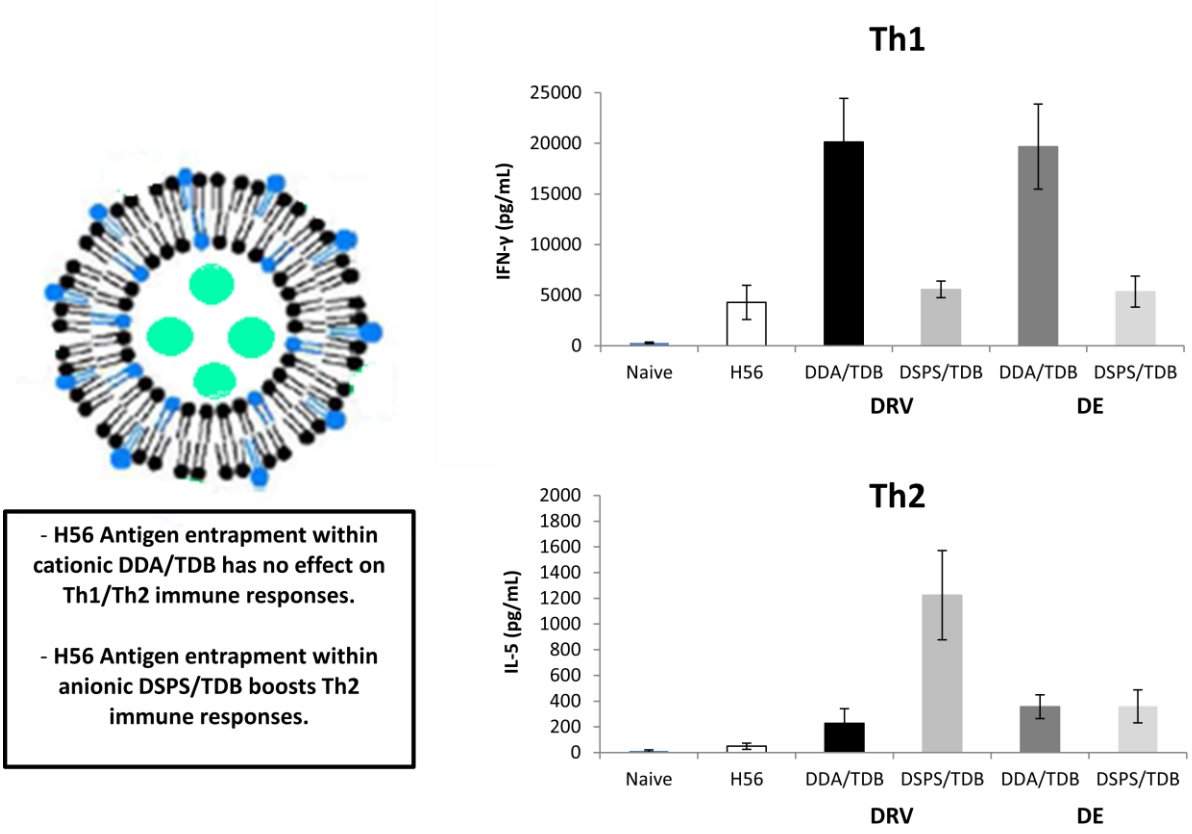
4.4. Conclusions

The results in this chapter show that by changing the main lipid constituent within the formulation to DSPS instead of DDA this led to liposomes with slight reduction in vesicle size and reversal in surface charge to highly negative (anionic) respectively. Upon addition of protein antigen, post lipid-film hydration (LH), to cationic DDA/TDB and anionic DSPS/TDB liposomes, this resulted in antigen loading when both vaccine components are opposing in charge, which suggests electrostatic binding. This antigen loading was reflected by subsequent changes in vesicle characteristics, with increasing vesicle size and neutralisation of the surface charge being measured. This binding at the liposome

surface was confirmed by means of trypsin digestion, with increasing trypsin concentration leading to significant reduction in antigen loading. In simulated *in vivo* conditions these formulations were able to efficiently retain antigen therefore confirming that these formulations remain stable. Further short term stability studies have also confirmed that these liposomes maintain constant vesicle size and surface charge characteristics over a 28-day period at storage temperatures of 4 °C and 25 °C. The biodistribution profile of these vaccine formulations was investigated with cationic liposome surface charge resulting in greater retention at the SOI, and hence controlled drainage to the local popliteal lymph node (PLN). Whereas anionic liposomes are non-depot forming and hence drain more rapidly to the PLN; which was disadvantageous in the induction of Th1 immune responses. Antigen, when opposing in charge to the delivery system, was efficiently retained by liposomes therefore causing deposition of antigen at the SOI and, hence, subsequent drainage to the PLN. Liposome retention was confirmed by increased pontamine blue staining at the SOI, which is a measure for influx of macrophages and monocytes to this site. Therefore upon the formation of a liposome 'depot at the SOI this subsequently resulted in the influx of monocytes and innate immune cells to this site.

These results correlated with immunisation studies in which depot-forming cationic DDA/TDB:H56 induce strong cell-mediated Th1 responses (Perrie *et al.*, 2013) both at the spleen and SOI, as well as inducing low levels of humoural immunity. However, non- depot forming anionic DSPS/TDB:H56 liposomes resulted in a reduction in Th1 cytokine production as well as a slight increase in Th2 immune responses. Overall these studies have demonstrated a lack of interaction between delivery system and antigen, in combination with a reduction in the 'depot effect' (for DSPS/TDB liposomes) leads to less efficient antigen delivery and consequently a reduced T-cell response with these levels representing very low T-cell activation in general. As a result, DSPS/TDB is not able to induce the pro-inflammatory signals, which therefore supported TDB in modulating the antigen presenting cells to induce T-cell responses.

Chapter 5: The role of liposome preparation method in vaccine delivery and formulation



Some of the results presented in this chapter have been published in the paper:

Wilkinson A., Kaur R., Korsholm K.S., Christensen D., Mohammed A.R., Perrie Y. "Investigating the role of liposome preparation method on ability of vaccine formulations to form an antigen 'depot' at the site of injection and the subsequent effect on the generated immune response" (Manuscript in preparation).

5.1. Introduction

Since the initial discovery of liposomes by Bangham and co-workers in 1965 (Bangham *et al.*, 1965), a variety of different methods have been developed for the preparation and formulation of liposomes. Due to the flexibility and versatility of liposomes, drug or vaccine can become adsorbed to the liposome surface, reconstituted within the membrane bilayer, encapsulated in the aqueous interior of the vesicle or covalently conjugated to the liposome surface (Watson *et al.*, 2012). This apparent versatility of these vesicles allows proteins, nucleic acid and antigens of all types to be incorporated within liposome formulations; however, the ability to deliver these moieties can be dictated by a number of factors including antigen size and liposome surface charge (as studied during Chapter 4). In relation to this investigation, the literature is very mixed in terms of whether localisation of a vaccine within the liposomal system impacts on the subsequent immune response. Many initial studies in this field have shown that both antigen surface adsorption to, and encapsulation within liposomes induce T-cell and antibody responses to associated protein antigens (Gregoriadis et al., 1987, Shahum and Therien, 1995, Fortin et al., 1996). More recent studies have shown that protein antigens adsorbed to the surface of cationic DDA/TDB liposomes led to the induction of strong cell-mediated and enhanced antibody responses (Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2011b). Initial studies have been carried out to investigate the role of antigen entrapment, compared to surface adsorption, in association with cationic DDA/TDB liposomes (Kaur *et al.*, 2011). However, in order to further understand the effect of antigen localisation more further investigations need to be carried out.

Therefore to study the impact of antigen entrapment within liposomes, a modified double emulsion solvent evaporation method was investigated for the formulation of liposomes with protein antigen added to the inner water phase during the preparation process (Nordly *et al.*, 2011c). The premise of this present study was to formulate a stable adjuvant composed of protein antigen (H56 and lysozyme) and the promising adjuvant system termed DDA/TDB (or CAF01) as well as the anionic liposome system, DSPS/TDB. This method is advantageous as heating above the phase transition temperature (for the main lipid in the formulation) is avoided (Nordly *et al.*, 2011c). The use of ethanol in the outer water phase of the preparation process has been hypothesised to destabilise the lipid membrane and help in the formation of liposomes (Maurer *et al.*, 2001) with associated antigen or immunostimulatory Toll-like Receptor (TLR) agonists, such as polyI:C (Nordly *et al.*, 2011c). Therefore, the aim was to formulate antigen within liposome adjuvants, prepared as double emulsion (DE) vesicles.

In comparison, the previously established dehydration-rehydration vesicle (DRV) method (Gregoriadis *et al.*, 1987) was investigated for the same liposomal adjuvants with the aim of entrapping protein antigen within the delivery system. The advantage of this method is its relative simplicity combined with the fact that a range of moieties (protein, nucleic acid and antigens) can become efficiently incorporated within the delivery system (Gregoriadis et al., 1987, Perrie and Gregoriadis, 2000, Kaur et al., 2011).

5.2. Aim and Objectives

The aims of the work reported within this chapter was to compare liposomal adjuvants prepared by different preparation methods which can impact on the location of antigen within the liposomal system, and thus potentially influencing their ability to co-deliver the antigen and liposomes to appropriate antigen presenting cells. Therefore the objectives of this work were to:

- physicochemically characterise cationic DDA/TDB and anionic DSPS/TDB liposomes prepared by DRV and DE methods,
- compare their antigen release kinetics in simulated *in vivo* conditions and their shelf-life stability at 4 °C and 25 °C temperature conditions,
- measure the ability of these liposome formulations in combination with various antigens (cationic lysozyme or anionic H56 TB antigen (Ag85B-ESAT6-Rv2660c)) to form an antigen depot during *in vivo* biodistribution studies,
- compare the immunological activity of these adjuvant systems and thus consider the impact of antigen location in their efficacy.

5.3. Results and Discussion

In this chapter, both cationic DDA/TDB and anionic DSPS/TDB liposome delivery systems were prepared using two different methods: the dehydration-rehydration vesicle method and the double emulsion solvent evaporation method, in order to understand how antigen-liposome interactions and localisation of antigen with the delivery system (surface adsorption against entrapment) contributed to the measured liposome characteristics, antigen loading and antigen release kinetics.

5.3.1. Investigating the effect of liposome preparation method on the physicochemical characteristics of liposomal adjuvants

As described in Chapter 4, the lipids DDA and DSPS were combined in an 8:1 molar ratio with TDB in order to formulate DDA/TDB and DSPS/TDB liposomes respectively.

Dehydration-rehydration vesicles for both cationic DDA/TDB and anionic DSPS/TDB liposomes were prepared and various liposome characteristics were obtained, both without antigen and with the anionic H56 or cationic lysozyme antigen (at the *in vivo* dose of 0.1 mg/mL). The addition of H56 or lysozyme to cationic DDA/TDB liposomes at the concentration used, resulted in no significant difference in vesicle size, polydispersity or zeta potential (Figure 5.1). For DSPS/TDB liposomes, again there was no significant increase in vesicle size upon addition of protein to these delivery systems; however, there was a significant ($P < .0.05$) reduction in zeta potential (from ~ -60 mV to -44 mV; Figure 5.1). When comparing these DRV systems with the previously formulated MLV liposomes (prepared by the lipid-film hydration method, where the antigen was adsorbed to the liposomal surface), at equivalent concentrations, these MLV were also shown to be similar in size (Chapter 4; Section 4.3.2.1).

DDA/TDB and DSPS/TDB liposomes prepared by the double emulsion method were also characterised, both as 'empty' liposomes and with the addition of H56 antigen or lysozyme (at an *in vivo* dose of 5 µg or 0.1 mg/mL respectively; Figure 5.1). For cationic DDA/TDB, upon addition of anionic H56 antigen there was a significant effect ($P < 0.05$) on liposome characteristics between empty vesicles and antigen-loaded vesicles with an increase vesicle size from 300-350 nm to around 490 nm and a reduction in zeta potential from 40-50 mV to around 30 mV (Figure 5.1). However DSPS/TDB liposomes formulated by the double emulsion method displayed no significant change in size on addition of H56; 'empty' vesicles were in the region of 240 nm and a zeta potential of around – 50 mV (Figure 5.1) and upon addition of H56 antigen at an *in vivo* dose of 5 µg), there was no significant change. Whereas upon addition of a lysozyme to anionic DSPS/TDB double emulsion liposomes there was a significant increase ($P < 0.05$) in the size of these vesicles (compared to empty liposomes) with their sizes increasing from ~ 240 nm to ~ 440 nm (Figure 5.1A). Considering, the zeta potential of these anionic DSPS/TDB double emulsion liposomes, the anionic zeta potential of these systems was masked by the addition of antigen and increased to -25.4 ± 0.9 mV (Figure 5.1B).

When comparing empty DDA/TDB and DSPS/TDB liposomes formulated by the DE method (~350 nm and 240 nm respectively), these vesicles were significantly smaller in size ($P < 0.001$) compared to their DRV counterparts and previously characterised MLVs (Chapter 4). This suggested that liposome

preparation method played a significant role in the assembly of these liposomal adjuvants as all vesicles were prepared at the same lipid/TDB ratio.

During the DRV preparation method, the rehydration of the freeze-dried lipid/protein cake resulted in the protein antigen becoming part-associated within the liposome delivery system (within the membrane bilayer) rather than just adsorbing at the liposome surface. Therefore, charge-related electrostatics can still play a role in the association between antigen and liposome. This method began with the preparation of empty small unilamellar vesicles (SUV) before mixing of the protein antigen which was required to be entrapped. Post freeze-drying of the vesicles, they were rehydrated using water. As the vesicles become increasingly concentrated during the dehydration (freeze-drying) procedure, this leads to their flattening and fusion leading to the formation of multilamellar planes (Gregoriadis *et al.*, 1987) in which protein antigen can become bound or attached (Kaur *et al.*, 2011). Therefore upon hydration this results in the formation of larger vesicles, with entrapped and bound antigen. The results in Figure 5.1 suggested that as the liposomes were able to retain their size, following addition of protein antigen at the same concentration, this may be reflected with entrapment of antigen within the delivery system. There were also no resulting changes in vesicle characteristics following surface adsorption of antigen to the delivery system (at these low protein concentrations). However upon addition of higher concentrations of protein, measured changes in vesicle characteristics were observed such as the increase in vesicle size which was due to higher antigen association at the liposome surface (as described previously in Chapter 4) which could also cause 'masking' of the cationic surface charge as described in the previous literature following association of nucleic acids with the liposome delivery system (Moghaddam *et al.*, 2011). However at these low concentrations used in Figure 5.1, there were no significant changes in vesicle characteristics possibly due to the high cationic lipid/protein ratios used, therefore no significant highlighted differences were noted for these formulations.

It was hypothesised that the changes in vesicle characteristics were more notable for DE formulations, upon addition of oppositely-charged antigen, (at this lower protein concentration) due to the initial smaller size of these vesicles noted (Figure 5.1A) as has been shown also in the previous literature (Milicic *et al.*, 2012). So upon addition of antigen to the formulation there was a smaller surface area of the delivery system to associate onto, therefore resultant changes in vesicle characteristics were measured. Therefore, these smaller vesicles were less able to incorporate antigens of the opposite charge to the vesicles without some aggregation effects being noted.

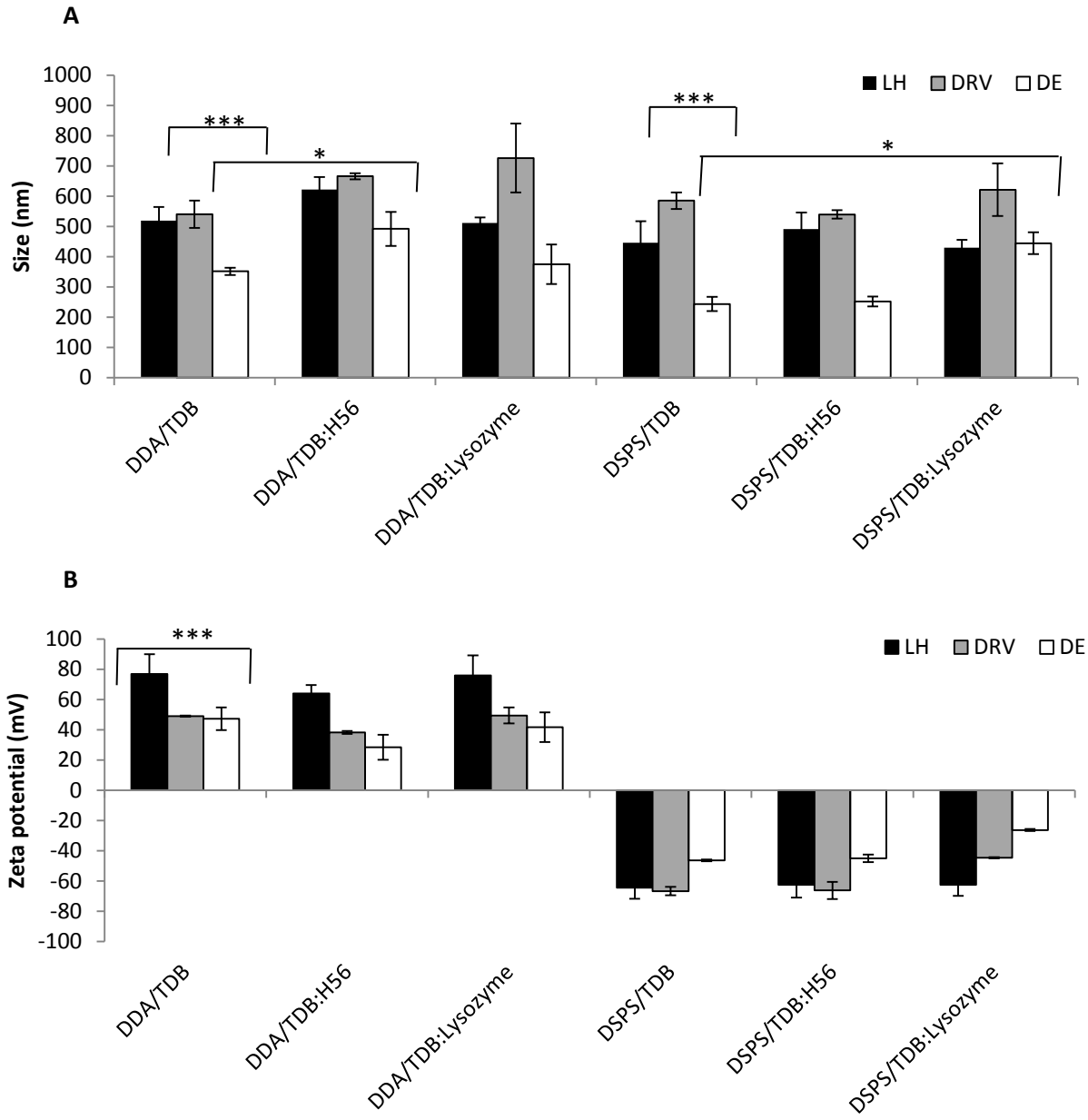


Figure 5-1. Vesicle size (A) and zeta potential (B) for cationic DDA/TDB and anionic DSPS/TDB liposomes with or without the addition of protein antigen at a concentration of 0.1 mg/ml (H56 or lysozyme). Results are the mean of triplicate experiments ± SD. Significance between various liposome characteristics was measured by one-way ANOVA (*** $p<0.001$; * $p<0.05$).

To consider the ability of these systems to carry and deliver antigen, their loading capacity was compared using the above outlined antigen doses. These results showed that the antigen loading was relatively low for all DE formulations tested (Table 5.1), with oppositely charged liposome and antigen displaying higher levels of initial antigen association (DDA/TDB:H56 and DSPS/TDB:Lysozyme: ~ 50 %; DDA/TDB:Lysozyme and DSPS/TDB:H56: ~ 18 %). In contrast, all DRV formulations showed enhanced loading in comparison to their DE-counterparts (Table 5.1) with

oppositely charged systems showing around 90 % antigen loading and similarly charged systems showing loading levels of ~ 50 %.

The results in Table 5.1 suggest that the choice of liposome preparation method has a notable effect on the subsequent ability to load protein antigen and is not only controlled by electrostatic interactions. Also, the higher levels of loading (~ 50 %) of antigen (which was similar in charge to delivery system) suggested that antigen may become entrapped within the delivery system as has been described previously for the delivery of DNA within liposomes of varying composition (Perrie and Gregoriadis, 2000, Perrie et al., 2001). The DE method was suggested to give lower levels of antigen loading for all formulations tested as a result of this method of preparation, in which sonication was used during liposome formulation therefore this may have affected antigen which was not entrapped within the delivery system (Nordly *et al.*, 2011c). During previous studies using DE-formulated liposomes the antigen was merely surface-associated to preformed liposomes which simplified the preparation process as otherwise care needs to be taken to circumvent denaturation and degradation of the antigen during the liposome preparation process (Nordly *et al.*, 2011c).

Table 5-1. Antigen loading values for double emulsion and dehydration-rehydration vesicles for DDA/TDB and DSPS/TDB liposomes. Antigen (H56 or lysozyme) was added at a concentration of 0.1 mg/mL (or 5 μg per dose) during the preparation process. Significance, in comparison to LH-prepared counterparts, was measured by one-way ANOVA (*** $p<0.001$).

	Antigen loading (% of initial amount used)		
Formulation	**DE**	**DRV**	**LH**
DDA/TDB:H56	49.7 ± 10.6 (***)	88.7 ± 1.6	77.3 ± 4.6
DDA/TDB:Lysozyme	18.2 ± 7.9	44.8 ± 0.7 (***)	20.3 ± 5.2
DSPS/TDB:H56	17.0 ± 10.7	47.8 ± 6.1 (***)	27.9 ± 7.1
DSPS/TDB:Lysozyme	55.6 ± 3.6 (***)	90.3 ± 0.5	82.6 ± 3.5

To consider if the antigen loading recorded in Table 5.1 represented intact protein, or if degradation was occurring, the stability of protein associated with vesicles produced by the double emulsion solvent evaporation method and the dehydration-rehydration vesicle method was assessed by SDS-PAGE (Figure 5.2). These results suggest potentially lower levels of protein noted for the DE liposomal system (Table 5.1) which may be a result of sonication used during this procedure, indeed subjecting 'free' antigen to sonication resulted in complete destruction of the protein (Figure 5.2; Lane 5) and this may be the reason for the reduced antigen loading noted for the DE formulations compared to their DRV counterparts. However at these protein concentrations used in this study, the intensity of protein bands were similar upon detection using SDS-PAGE (Figure 5.2).

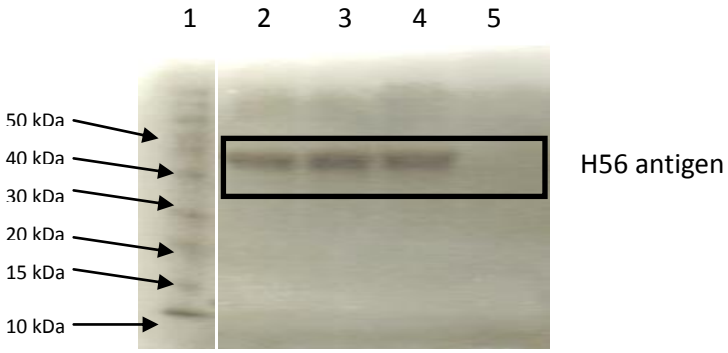


Figure 5-2. SDS-PAGE gel to represent the stability of liposome-associated protein for vesicles produced by the double emulsion method and DRV method. Lane 1; Pre-stained protein marker; Lane 2 - DE DDA/TDB:H56 post-formulation; Lane 3 – DRV DDA/TDB: H56 Liposome post-formulation; Lane 4 – Free H56; Lane 5 – H56 after sonication.

5.3.2. Stability of DRV and DE-prepared formulations, with and without entrapped antigen

The stability of liposomes is a crucial factor in the development and formulation of novel vaccine delivery systems. With this in mind, both cationic DDA/TDB and anionic DSPS/TDB liposome vaccine delivery systems were placed into stability trials in order to assess the time development of characteristics (size and zeta potential) over a 28-day period. These liposome delivery systems were prepared by either the DRV or the DE method, and were stored at 4 °C or 25 °C temperature storage conditions in an aqueous format.

Cationic DDA/TDB liposomes prepared by the DRV method displayed an initial vesicle size of ~ 550-600 nm and a zeta potential of between 50-60 mV, both with and without H56 antigen (Figure 5.3A). Throughout the time period of this study there was a general trend of increasing size over time noted for all formulations, however these changes were not deemed significant. For the vesicle surface charge (as represented by the zeta potential) there was no obvious trend between formulations, but fluctuations were noted (Figure 5.3B). DSPS/TDB liposomes prepared by the DRV method, vesicles had an initial size of ~ 500 nm (with and without H56 antigen; Figure 5.3C) a zeta potential of -70 mV and -50 mV with and without H56 antigen respectively (Figure 5.3D). There were no significant increases in vesicle size (over the 28 day period) with empty DRV DSPS/TDB vesicles showing a vesicle size of ~ 600 nm and 700 nm (at storage temperatures of 4 °C and 25 °C respectively) by day 28. However, antigen-loaded DRV vesicles maintained a fairly constant size throughout this study. The vesicle charge (as represented by zeta potential) also remained constant up until day 28, with no significant differences between the initial vesicle characteristics for all formulations tested.

Cationic DDA/TDB liposomes prepared by the DE method were similarly studied. At the initial time point (day 0), these vesicles were either 230 nm or 480 nm in size, with and without H56 antigen respectively (Figure 5.3E). Antigen-loaded DE vesicles were significantly larger in size than their 'empty' counterparts ($P < 0.001$), which was primarily due to antigen association with the liposome delivery system (as described in previous characterisation studies in Section 5.3.1). During the time period of this study, antigen-loaded DE vesicles did not show a significant change in size; however, empty DE vesicles significantly increased ($P < 0.01$) in vesicle size to ~ 400 nm (by day 28; Figure 5.3E). Considering their zeta potential, cationic DDA/TDB DE vesicles gave initial values 50-60 mV with no significant difference noted if antigen was present or not within the formulation. Throughout the time period of this study, antigen-loaded cationic DE vesicles showed a significant ($p < 0.001$) reduction in zeta potential with values of ~ 30 mV being measured after day 28 (Figure 5.3F). So these liposome adjuvants showed reduced zeta potential following storage. The zeta potential for empty DE vesicles did not significantly change following storage at 4 °C or 25 °C over this time period (Figure 5.3F). Anionic DSPS/TDB liposomes prepared by the DE method displayed an initial vesicle size of ~ 250 nm, both with and without H56 antigen and did not significantly change over the period of the study (Figure 5.3G). At the initial time point (day 0) in terms of zeta potential, the anionic DE DSPS/TDB liposomes displayed values of -40 mV and -50 mV (with and without H56 antigen respectively) with no significant difference noted if antigen was present or not within the formulation and throughout the time period of this study (between day 0 and day 28), antigen-loaded DSPS/TDB vesicles showed no significant changes with a zeta potential of around – 40 mV being measured both at storage temperatures of 4 °C and 25 °C. However empty DSPS/TDB vesicles showed a reduction in zeta potential, following storage at 25 °C and 4 °C, with values of -26 mV ($P < 0.01$; compared to day 0) and -35 mV being measured respectively (Figure 5.3H).

The stability of TDB-containing liposomal adjuvants was previously shown in Chapters 3 and 4. However during this section the effect of preparation method on the stability of liposome formulations was further investigated. These stability studies have showed that DRV formulations were more suitable, in comparison to their DE-counterparts, for storage in an aqueous format over an initial 28 day time period both at 4 °C and 25 °C temperature conditions. This was further demonstrated in Figure 5.4, as DRV formulations remained homogenous and exhibited minimal phase separation even after a 28-day storage period (Figure 5.4). Following storage over a 28-day period, DE-formulations are more prone to changes in vesicle characteristics therefore long term storage in the aqueous phase would not be suitable for these formulations. Liposome storage in the aqueous format may thus have an effect on the inherent instability of these formulations as has been suggested previously (Davidsen *et al.*, 2005). Therefore a number of liposome vaccine

formulations used in the clinic are in a freeze-dried format (Mohammed *et al.*, 2006). In order to enhance vesicle stability during the freeze-drying process a number of cryoprotectants including saccharides (such as sucrose, trehalose, and lactose) and their derivatives can be employed (Mohammed et al., 2006, Christensen et al., 2008, Mohammed et al., 2010).

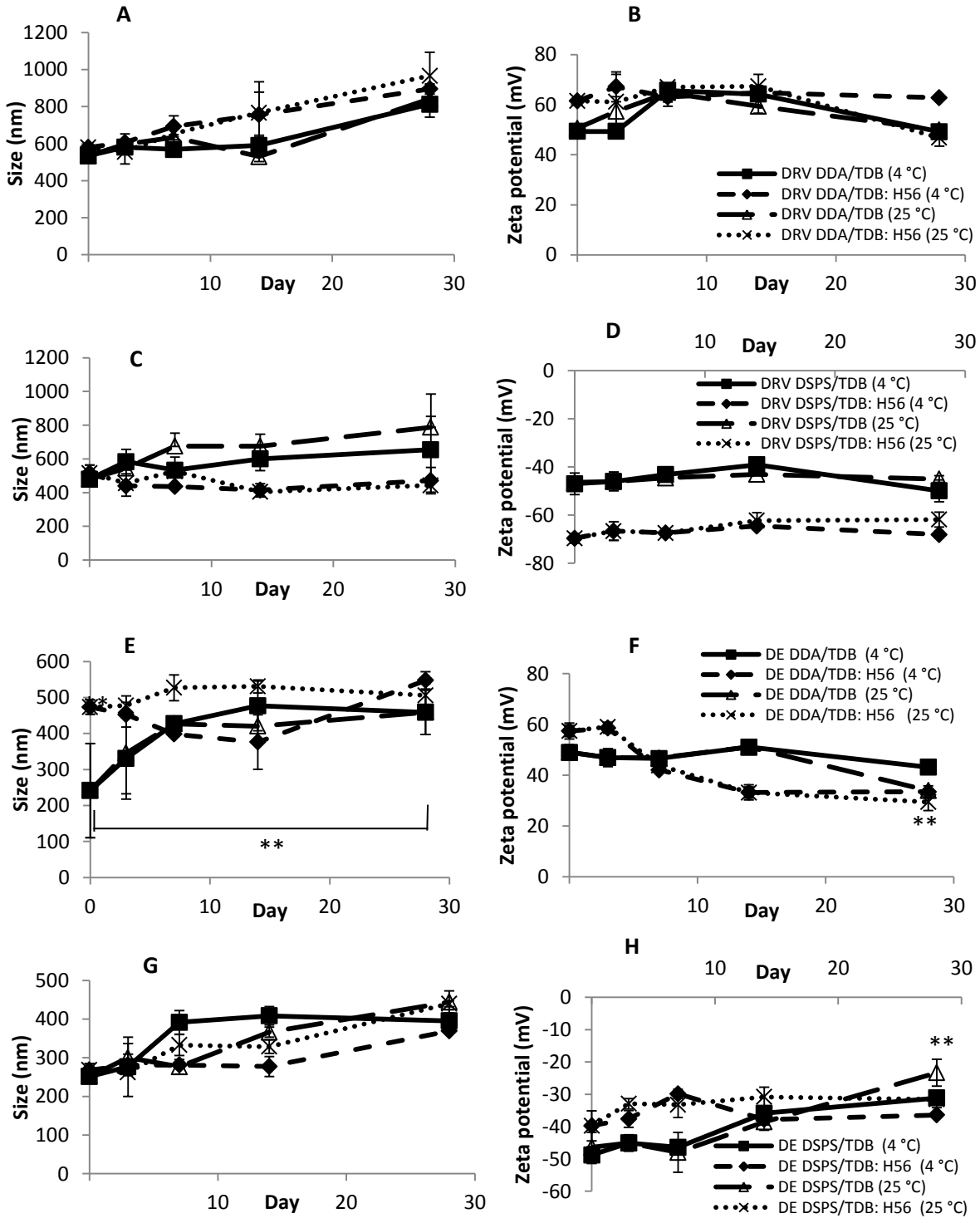


Figure 5-3. Time development of the average size (A, C, E and G) and zeta potential (B, D, F and G) of DDA/TDB and DSPS/TDB liposomes prepared by either the DRV or DE methods (both with and without H56 antigen at *in vivo* dose of 5 µg) following storage at 4 ˚C and 25 ˚C. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (** p<0.01).

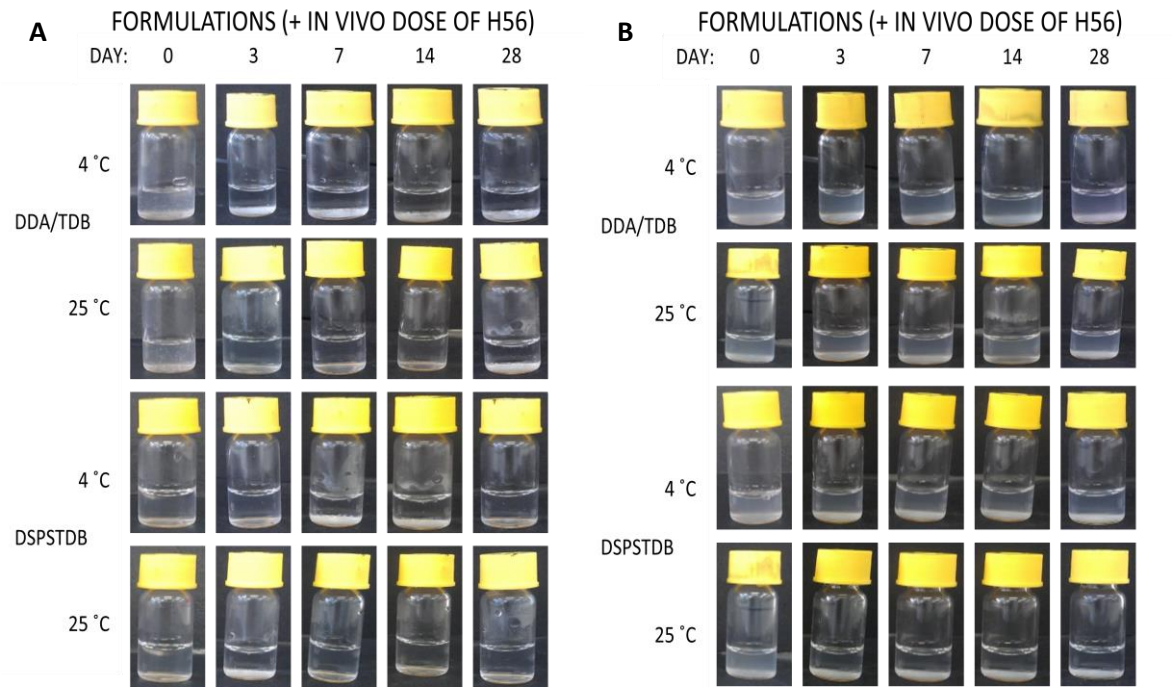


Figure 5-4. DDA/TDB and DSPS/TDB liposome samples stored into glass vials were prepared by the (A) double emulsion (DE) solvent evaporation method or the (B) dehydration-rehydration vesicle (DRV) method and were stored for 28 days at 4 ˚C and 25 ˚C.

5.3.3. Antigen release from liposomal formulations in simulated *in vivo* conditions

During initial experiments, the antigen loading and release of both H56 and lysozyme from double emulsion vesicles for DDA/TDB and DSPS/TDB liposomes was quantified directly both in simulated *in vivo* conditions (FCS/Tris; 50/50 v/v) and control conditions (Tris buffer; 10 mM, pH 7.4). In order to quantify loading, antigen (H56 or lysozyme) was radiolabelled with ^{125}I before being formulated with or within the delivery system. At a number of time points, following centrifugation, the antigen loading was determined by calculating the percentage of radiolabelled antigen found within the liposome pellet fraction.

These results showed that the antigen loading was low for all DE formulations tested (as described previously in Table 5.1), with oppositely charged liposome and antigen displaying significantly higher levels ($P < 0.001$) of initial antigen association (Figure 5.5). As shown previously for MLV formulations (Chapter 4) there was an initial release of antigen from all liposome formulations (irrespective of antigen charge), before the stabilisation of antigen release after around 5 hours (Figure 5.5).

During further studies, the antigen loading and release kinetics of the anionic H56 TB antigen and the cationic protein lysozyme from dehydration-rehydration vesicles for cationic DDA/TDB and

anionic DSPS/TDB liposomes were quantified directly both in simulated *in vivo* conditions (FCS/Tris: 50/50 v/v; 37 °C) and control conditions respectively (Tris 10 mM; pH 7.4; 37 °C). These initial results showed that the levels of antigen loading were increased upon binding between oppositely charged liposome and antigen components [DSPS/TDB:Lysozyme and DDA/TDB:H56 respectively) with levels of antigen loading measured between 85-90 % (Figure 5.6). So, antigen becomes bound with the liposome delivery system upon the rehydration of the freeze-dried lipid/protein mixture. These results showed similar levels of antigen release from all formulations tested in simulated *in vivo* conditions compared to control conditions.

However a general trend showed that antigen release for DRV formulations was significantly lower compared to their DE-prepared counterparts ($P < 0.01$), which may be ascribed to more efficient entrapment of antigen within DRV-formulations, compared to lower levels of loading by DE-formulations. However as described previously, following the initial burst release of antigen from the formulation this H56 antigen release stabilised over time with the remaining antigen staying with the delivery system.

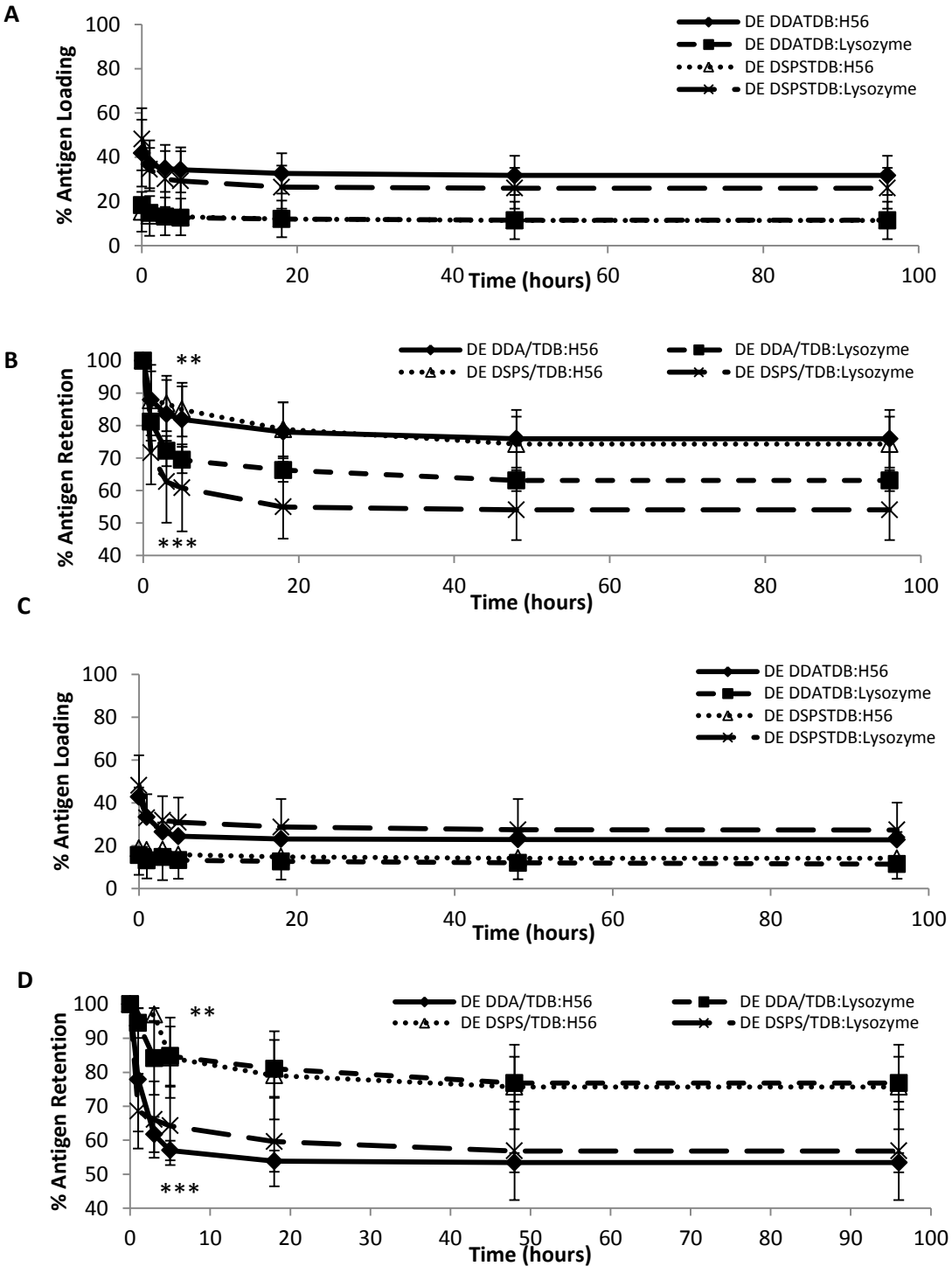


Figure 5-5. Antigen loading (A, C) and release profile (B, D) from double emulsion vesicles (DEs) for DDA/TDB and DSPS/TDB liposomes when stored under simulated *in vivo* conditions (A and B - 50 % FCS in Tris Buffer, 10 mM pH 7.4; 37 ˚C) and control conditions (C and D - 10 mM Tris buffer pH 7.4; 37 ˚C). Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$) compared to the initial antigen loading values at 0 h

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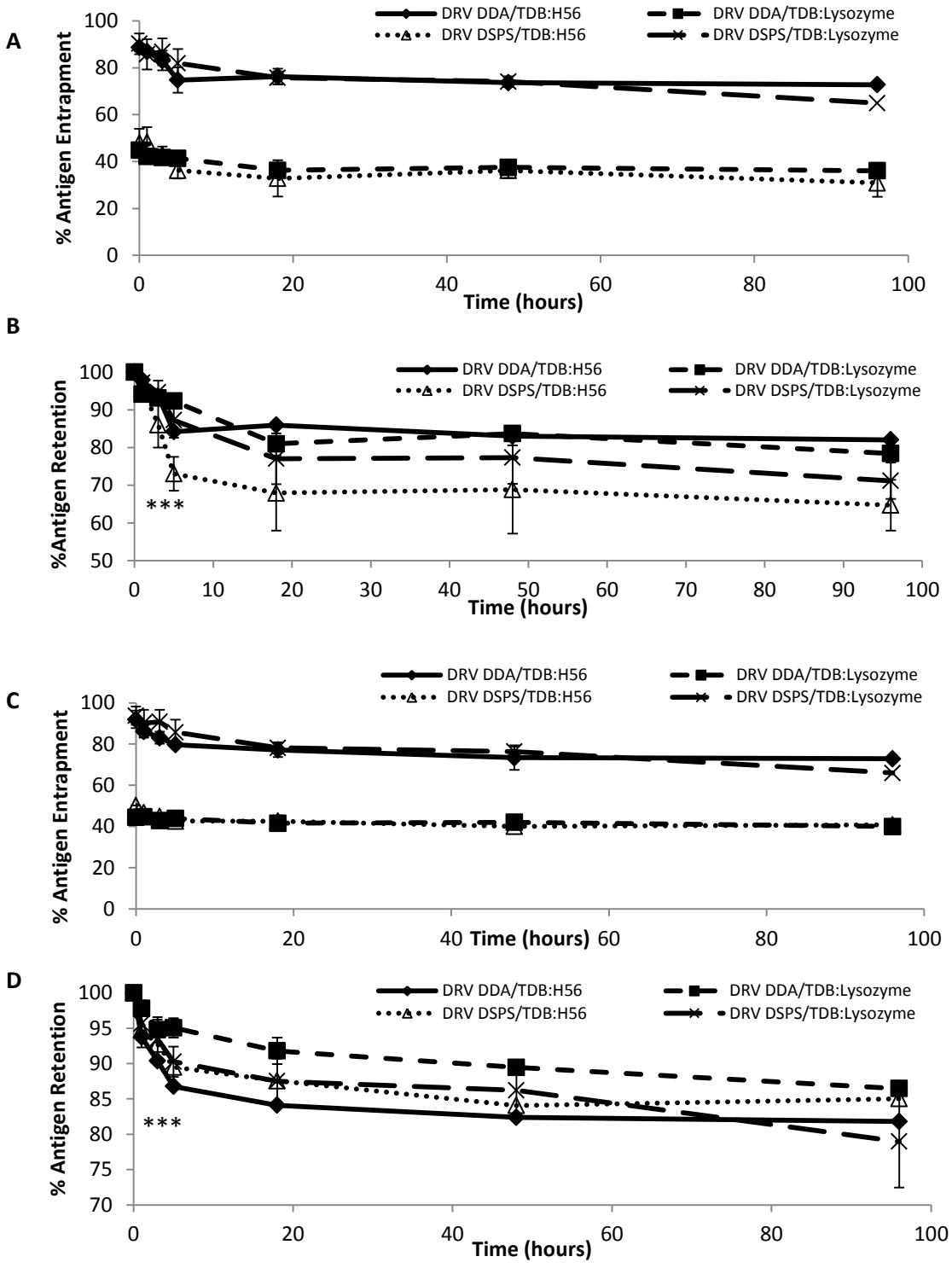


Figure 5-6. Antigen loading (A, C) and release profile (B, D) from dehydration-rehydration vesicles (DRV) for DDA/TDB and DSPS/TDB liposomes when stored under simulated *in vivo* conditions (A and B - 50 % FCS in Tris Buffer, 10 mM pH 7.4; 37 ˚C) and control conditions (C and D - 10 mM Tris buffer pH 7.4; 37 ˚C). Results are the mean of 3 experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$) compared to the initial antigen loading values at 0 h.

The antigen release from liposome formulations was studied in order to consider if antigen remained associated with the liposome delivery system, especially under simulated *in vivo* conditions. For the use of liposomes as effective vaccine delivery systems it is a prerequisite for antigen to remain associated or bound to these vesicles. With this in mind, Foetal Calf Serum (FCS) was used to simulate the *in vivo* environment (Henriksen-Lacey *et al.*, 2010a), in order to promote to conditions more similar to what liposomes may encounter upon administration *in vivo*. In general terms, an initial burst release of antigen was observed under this simulated environment before antigen release stabilised over the time period of these studies (Figures 5.5 and 5.6).

For both DRV and DE vesicles; when opposing in charge with protein antigen this led to higher levels of initial antigen association to the liposome delivery system. However when opposing in charge, MLV (Chapter 4) and DRV liposomes (for cationic DDA/TDB and anionic DSPS/TDB) have been shown to display higher levels of antigen adsorption compared to their double emulsion (DE) vesicle counterparts. The mechanism of action for antigen association with liposome delivery systems therefore varied depending on the method of liposome preparation. During this chapter and previous studies for multilamellar vesicles (Chapter 4), adsorption of antigen to the liposome surface has been proposed to be the mechanism of action for this antigen association (Henriksen-Lacey et al., 2010c, McNeil et al., 2011). For DRV, antigen can associate both at the liposome surface and can bind the cationic charges within the membrane bilayer itself as discussed previously (Perrie and Gregoriadis, 2000), rather than solely at the liposome surface for LH-prepared formulations (Henriksen-Lacey *et al.*, 2010c).

The use of the DE method (Nordly *et al.*, 2011c) has proven to be the least effective in terms of antigen-liposome association. For this preparation method, electrostatic interactions between oppositely charged liposome and antigen still played a role (Henriksen-Lacey *et al.*, 2010c) in this association with higher entrapment values being associated with oppositely charged liposome and antigen (DDA/TDB:H56 and DSPS/TDB:Lysozyme).

5.3.4. Investigating antigen localisation within the liposomal adjuvants

In terms of DDA/TDB and DSPS/TDB, given the respective cationic and anionic nature of these vesicles it is likely that antigen may become attached at the liposome surface as well as incorporated within the delivery system. In order to establish the localisation of antigen in regards to the delivery system both DE and DRV liposome vaccine formulations (in combination with either anionic H56 or cationic lysozyme) were subjected to increasing concentrations of trypsin and subsequently the antigen retention was measured.

Using the anionic H56 antigen, for cationic DRV vesicles this resulted in similar antigen loading values irrespective of the concentration of trypsin (Figure 5.7) as has been shown by SDS-PAGE (Figure 5.8) and loading of radiolabelled antigen which demonstrated that antigen was becoming entrapped within the delivery system due to this preparation method. A similar trend was also noted for all other formulations in this study. However for cationic DE liposome formulations, SDS-PAGE and antigen retention data has shown that increasing the concentration of trypsin resulted in a significant reduction in antigen loading ($P < 0.01$) therefore this showed that antigen was localised at the surface of the liposome delivery system (Figure 5.7) as well as entrapped within the formulations, albeit at lower levels.

Whilst using the cationic protein, lysozyme in combination with anionic DSPS/TDB this led to the same findings in which increasing the concentration of trypsin had a greater effect on DE-liposome vaccine formulations ($P < 0.001$) when compared to their DRV counterparts as again was confirmed by SDS-PAGE (Figure 5.8) and tracking of radiolabelled antigen with the formulation (Figure 5.7). This showed that antigen was located at the liposome surface, as well as entrapped within the delivery system depending on the method of liposome preparation.

When analysing the data for DDA/TDB:Lysozyme (+/+) and DSPS/TDB:H56 (-/-), these formulations offered lower levels of initial antigen loading when prepared by both DRV and DE methods (Figures 5.7). However, the same trend was present as described previously, in the fact that upon exposure to increasing concentrations of trypsin, DRV formulations retained more antigen than their respective DE counterparts.

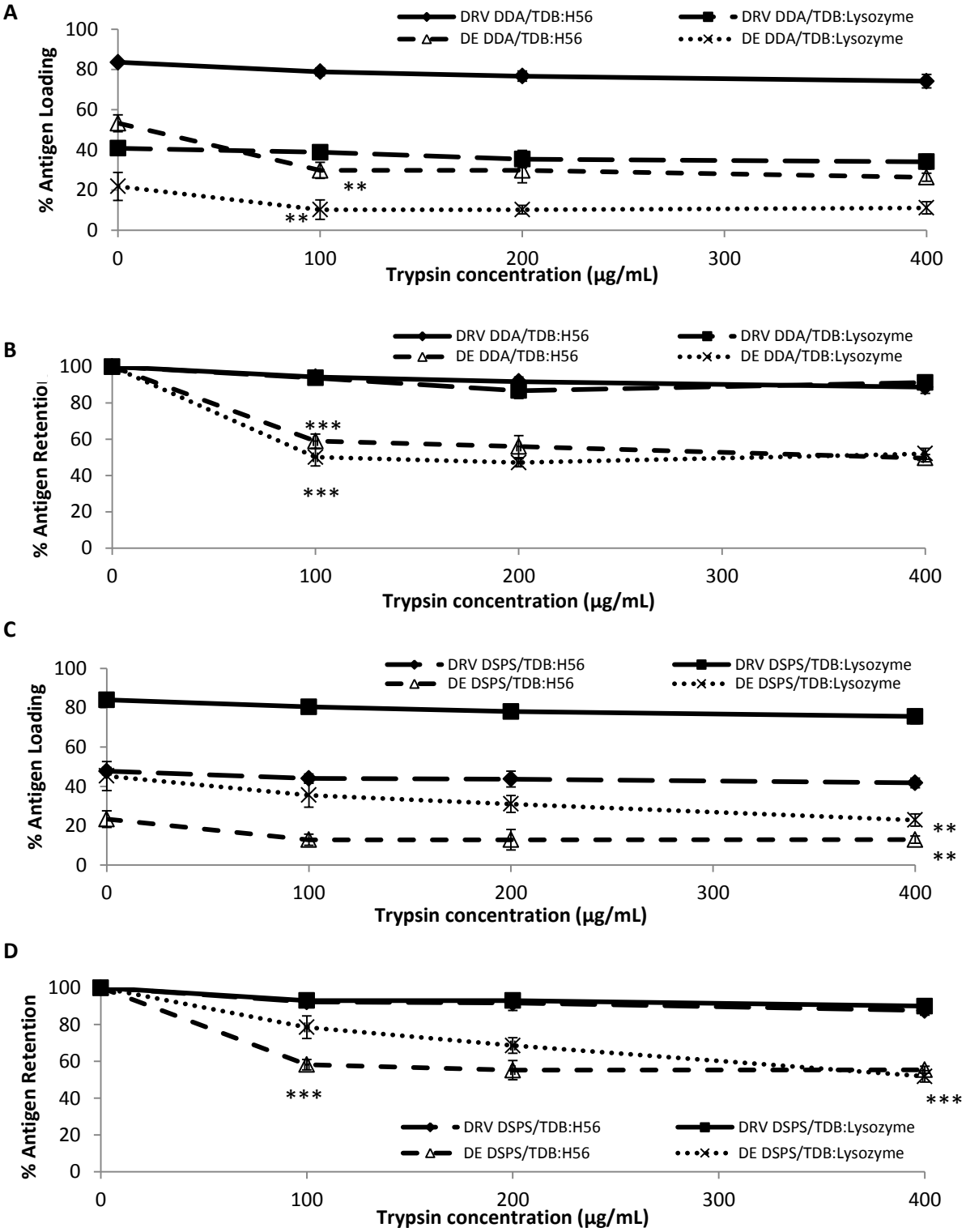


Figure 5-7 Trypsinisation Studies showing antigen loading (A and C) and retention (B and D) for cationic DDA/TDB (A and B) and anionic DSPS/TDB formulations. Antigen (H56 or lysozyme) is radiolabelled and added at a dose concentration of 5 µg (0.1 mg/mL). Vaccine formulations were subjected to increasing concentrations of trypsin (0, 100, 200 and 400 µg/mL) and % antigen loading was determined. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$) compared to the initial antigen loading values at 0 h.

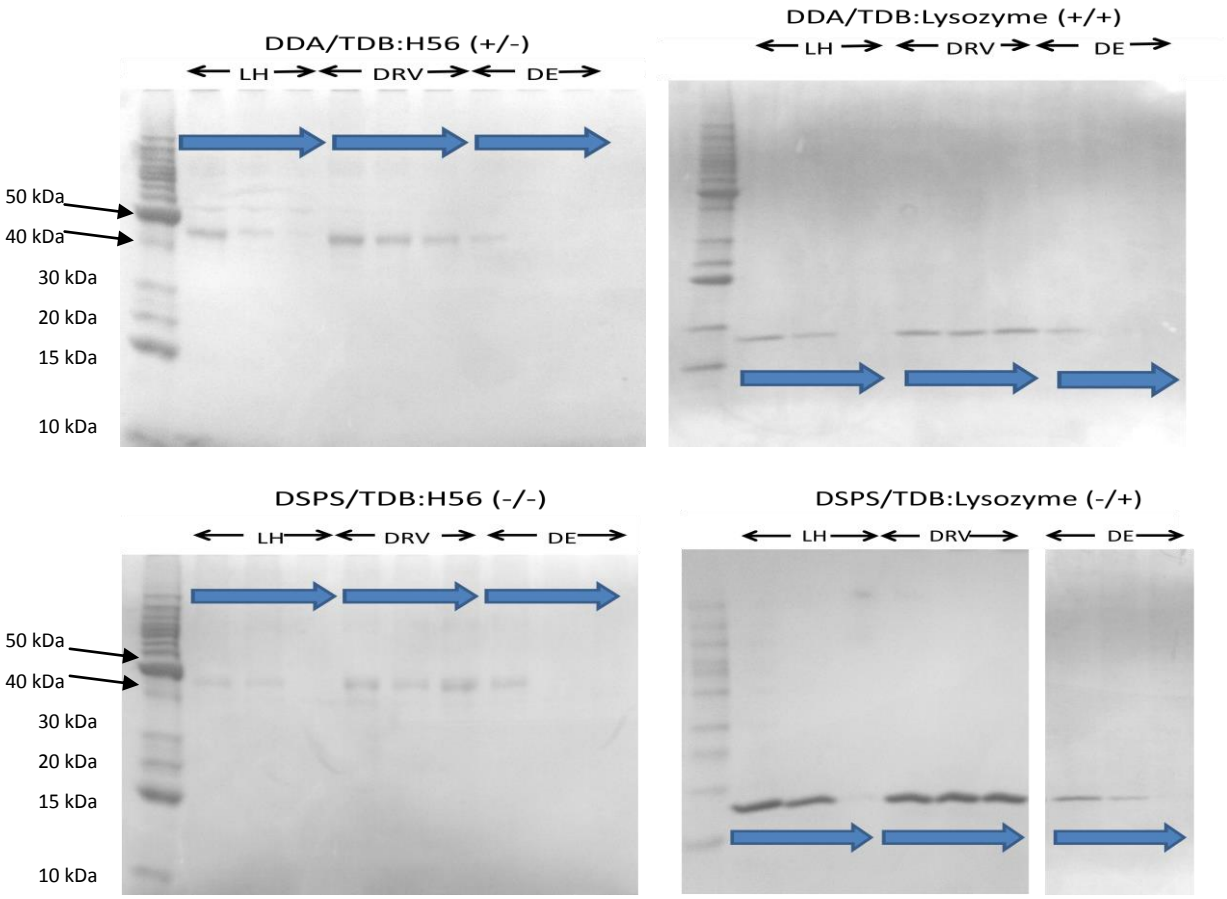


Figure 5-8. SDS-PAGE analysis in order to investigate the effect of trypsinisation on antigen loading and localisation in regards to the delivery system. Blue arrows indicate increasing concentrations of trypsin (0, 100 and 400 µg/mL).

5.3.5. Stability of radioactive markers within liposome formulations in order to investigate membrane stability

Liposomes (cationic DDA/TDB and anionic DSPS/TDB) were prepared by either the dehydration-rehydration vesicle method or the double emulsion solvent evaporation method, with the incorporation of trace amounts of ^{3}H-Cholesterol (0.17 nM). Dialysis was used to study the retention of ^{3}H-Cholesterol within the liposome membrane. At various time points (1h, 3h, 5h, 18h, 48h and 96h) over a 96 hour study period, 1 mL aliquots of dialysis media were removed and the amount of ^{3}H present were measured. As described previously with the lipid-film hydration method, only very low levels of loss of ^{3}H-Cholesterol were noted (Figure 5.9). These results suggested that liposomes prepared by the DRV and DE methods respectively were stable and these radiolabelled liposome formulations were taken forward into *in vivo* biodistribution studies.

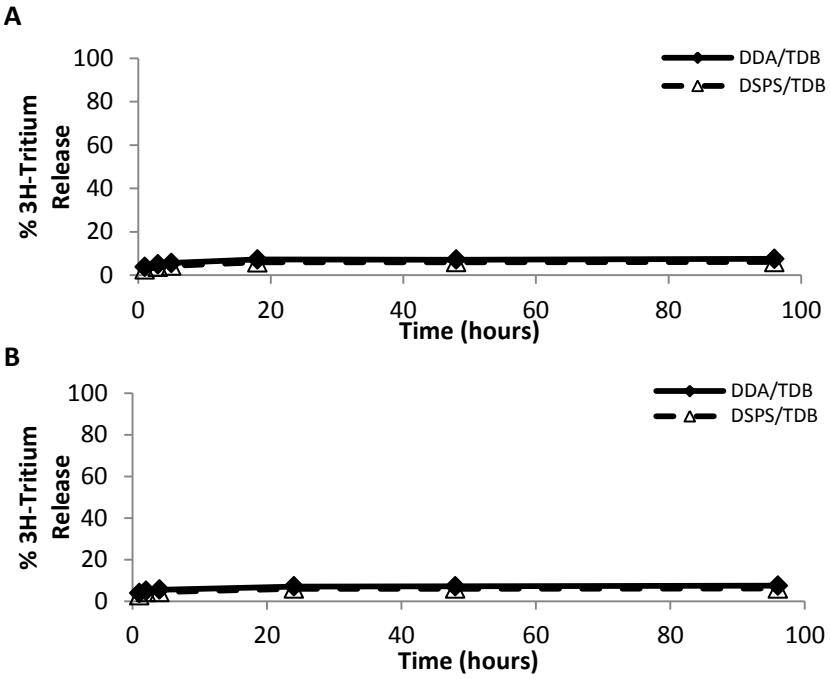


Figure 5-9. Membrane stability of DDA/TDB and DSPS/TDB liposomes prepared by the dehydration-rehydration vesicle method (A) and double emulsion method (B) were studied using a trace amount of ^{3}H-Cholesterol. Results express the % of the original ^{3}H-Cholesterol dose added to liposomes that was detected in the dialysis buffer over a 96 hr time period. The samples were stored at 37 °C in 50 % FCS. Results denote the mean of triplicate experiments ± SD.

5.3.6. Biodistribution studies: the effect of preparation method

5.3.6.1. Determination and quantification of vaccine retention at the injection site

To determine the role of liposome preparation method on the antigen (and liposome) depot-effect at the SOI, DDA/TDB liposomes were produced with the inclusion of ^{3}H-Cholesterol in the bilayer membrane and ^{125}I-labelled antigen (H56 or lysozyme) as previously outlined (Chapter 4). The SOI and various other tissues were collected and the percentage of the administered dose (% dose) quantified.

These results (Figure 5.10) showed that irrespective of preparation method (DE or DRV), cationic liposomes have a notable advantage in terms of liposome retention at the vaccination site with around 85 % (at day 1 p.i) of the liposome dose recovered at the SOI (when combined with anionic H56) or 55-60 % (when combined with cationic lysozyme) at day 1. This difference may be attributed to the antigen which is associated with the delivery system. At subsequent time points, days 4 and 14 p.i, there was a decrease in recovery of the injected dose; however, around 20 % of the injected dose was still recovered at day 14 p.i. This strong retention of cationic liposomes at the SOI is primarily due to interactions between cationic liposomes with negatively-charged serum proteins at the SOI through electrostatic-mediated interactions, therefore cationic DDA/TDB form a ‘depot’ at

the vaccination site (Henriksen-Lacey *et al.*, 2010a). In comparison, irrespective of the preparation method, there was significantly lower retention ($P < 0.01$) of anionic DSPS/TDB liposome vaccine formulations (around 40 %, 15 % and 5 % at days 1, 4 and 14 p.i respectively) which showed that these liposomes are removed/drain faster from the vaccination site which is due to their anionic nature meaning they do not interact with interstitial proteins present at the injection site. Therefore, this will lead to reduced retention at the SOI, therefore a liposome 'depot' was not formed. These results suggested that for liposome formulations, the method of preparation had no significant effect on the subsequent ability to form a depot at the injection site.

When considering the retention of antigen at the SOI this was not significantly changed due to the method of liposome preparation (Figure 5.10). However electrostatic interactions between oppositely-charged liposome and vaccine antigen components resulted in increased antigen dose retention at the SOI as has been shown in previous studies (Henriksen-Lacey et al. 2010a) and in Chapter 4 of this thesis. Delivery of H56 antigen in combination with cationic DDA/TDB liposomes (either prepared as DRV or DE) resulted in 60 %, 20% and 4 % recovery of the antigen dose at days 1, 4 and 14 post-injection (p.i) respectively. Whilst delivery of lysozyme in combination with anionic DSPS/TDB liposomes (DE or DRV) gave antigen recovery of 35 % and around 10 % at days 1 and 4 p.i respectively, with minimal recovery by day 14 p.i.

Irrespective of the method of liposome preparation, the co-administration of similarly charged liposome and antigen as is the case with DSPS/TDB liposomes in combination with H56, and DDA/TDB liposomes in combination with lysozyme, resulted in a significant reduction ($P < 0.001$) in initial antigen retention with around 10-15 % antigen remaining with the delivery system at day 1 p.i (Figure 5.10). At days 4 and 14 p.i there was subsequent reduction ($P < 0.001$) in antigen dose retention due to drainage of the dose from the SOI.

As a negative control, free H56 antigen was delivered i.m into the left quadricep (as for the other formulations). Upon recovery, very low levels of antigen retention were measured at the injection site at all time points, which therefore indicated the rapid drainage of free antigen from the SOI. This data also indicated the importance for a liposome delivery system in order to deliver antigen, as antigen can be strongly retained at the SOI when delivered within liposomes.

Therefore in terms of liposome preparation method, there was no significant effect noted between formulations thereby suggesting that antigen localisation does not significantly enhance antigen retention at this injection site.

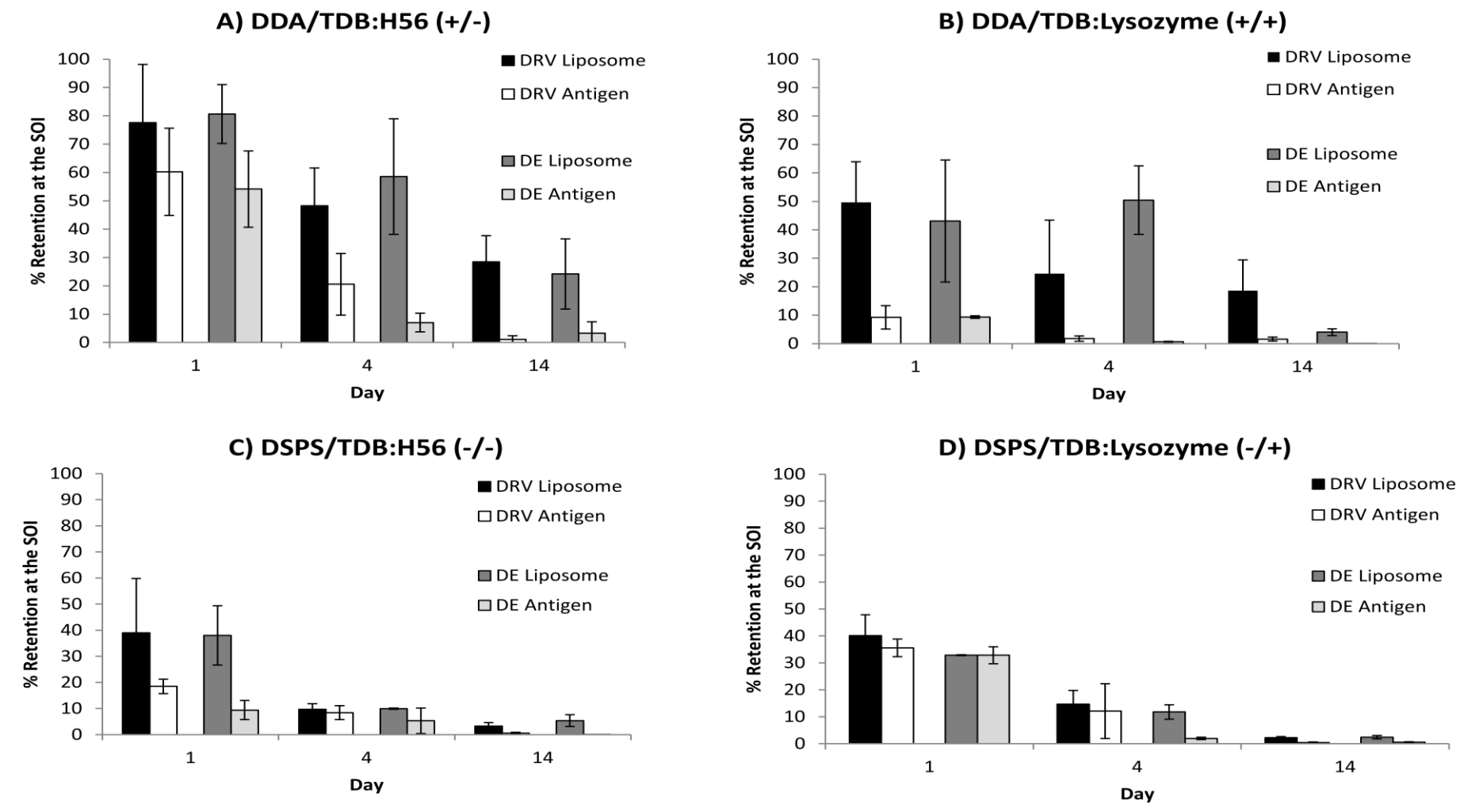


Figure 5-10. Biodistribution of vaccine components, liposome and antigen, at the SOI following intramuscular (i.m.) injection of liposome vaccine formulations. Results are shown as % dose recovered at the SOI and represent the mean ± SD of 4 mice per group.

5.3.6.2. Determination of vaccine drainage to the popliteal lymph node (PLN)

In addition to investigating the ability of cationic DDA/TDB and anionic DSPS/TDB liposomes to retain antigen at the SOI, various tissues were assayed and the percentage dose of both liposome and antigen components determined. The left popliteal lymph node (PLN) is the local lymph tissue to which liposome (and associated antigen) drained to following intramuscular injection into the left quadricep. Therefore, this was the primary site at which antigen will be presented to members of the T-cell family in order to initiate the immune response.

The results showed that vesicle charge plays a role in the clearance of liposomes to the local popliteal lymph node (PLN) with anionic DSPS/TDB appearing in the PLN faster than cationic DDA/TDB liposomes (as described previously in Chapter 4). However by days 4 and 14 p.i, the presence of anionic DSPS/TDB liposomes was significantly reduced ($P < 0.001$) at the PLN. In contrast, cationic DDA/TDB liposomes were slowly drained to the PLN at day 1 p.i (which corresponds to high liposome retention at the SOI), however by days 4 and 14 p.i the levels of these cationic liposomes were increased therefore indicating controlled release and drainage of these formulations to the draining lymph node (Figure 5.11) which was significant over the time period of this study ($P < 0.05$). In terms of liposome preparation method (DE vs DRV), there was no significant effect on the drainage of either cationic or anionic liposomal adjuvants to the draining PLN over this study

When observing the drainage of antigen to the popliteal lymph node (PLN), in regards to the effect of liposome preparation method, there were no significant differences between tested liposome vaccine formulations (Figure 5.11). Antigen, when associated with anionic DSPS/TDB, was rapidly drained and present in the PLN at day 1 p.i. However, as with liposome dose drainage, this antigen was present in significantly lower amounts ($P < 0.001$) by days 4 and 14 p.i. In contrast, antigen when associated with cationic DDA/TDB liposomes was lowly retained at at all three time points however H56-adsorbing cationic DDA/TDB liposomes (DRV- and DE-formulated) exhibited controlled release with higher amounts of antigen present at day 14 p.i when compared to day 1 p.i. As a control, liposome and antigen drainage to the distally located popliteal lymph node (of the uninjected leg) were measured, with even lower drainage observed than the PLN, therefore showing that upon i.m. injection vaccine is drained to the associated lymph nodes (results not shown).

Therefore these studies have suggested that antigen entrapment with the delivery system (as for DRV formulations) had no significant effect on the delivery of these liposomal vaccines to the draining lymph node.

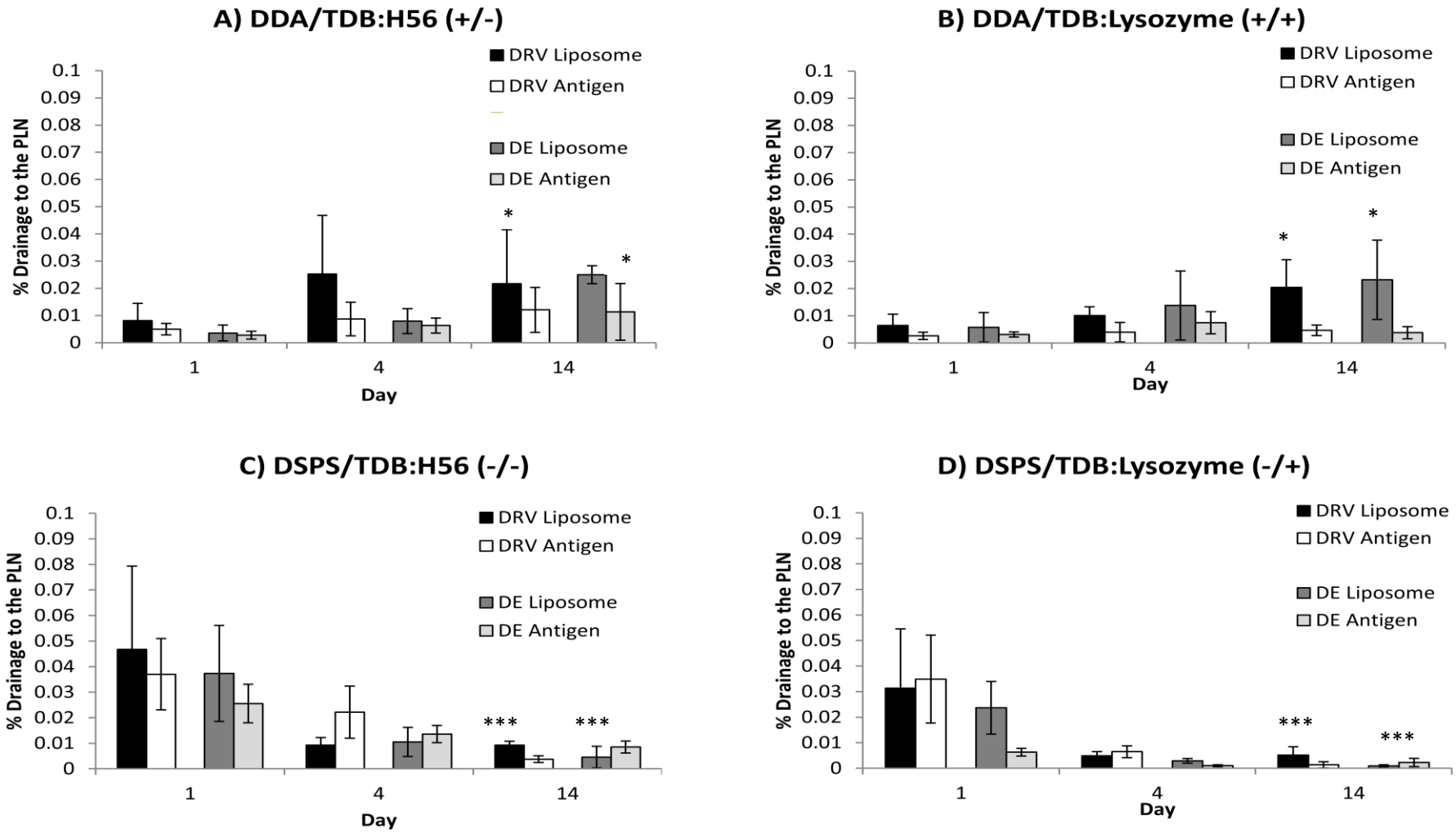


Figure 5-11. Biodistribution of vaccine components, liposome and antigen, at the PLN following intramuscular (i.m.) injection of liposome vaccine formulations. Results are shown as % dose per mg tissue from the PLN and represent the mean ± SD of 4 mice per group. Significance was measured by one-way ANOVA (*** $p<0.001$; * $p<0.05$) compared drainage levels at day 1 p.i

This data demonstrated the movement and drainage of liposome (and associated antigen) from the SOI to the draining PLN. However antigen entrapment (as for DRV) did not significantly enhance vaccine retention at the SOI, as well as delivery to the PLN. The formation of a strong 'depot' at the injection site (as shown for cationic DDA/TDB formulations), coupled with controlled release of both liposome and associated antigen was previously shown to be advantageous in the induction of Th1 immune responses, as characterised by IFN-γ production and the promotion of strong antibody responses (Henriksen-Lacey et al., 2010a, Christensen et al., 2012, Perrie et al., 2013).

5.3.6.3. Monocyte influx at the injection site

Similarly to previous studies carried out within our laboratory, pontamine blue was used as a marker for influx of monocytes at the SOI (Henriksen-Lacey *et al.*, 2010a). All liposome vaccine formulations induced monocyte infiltration to the vaccination site; however, the kinetics and intensity were varied depending on the liposome surface charge and preparation method (Figure 5.12).

Irrespective of the method of liposome preparation (DE or DRV), post-i.m. injection of H56 antigen or lysozyme in combination with cationic liposome formulation DDA/TDB resulted in monocyte influx (as shown by blue staining) at the vaccination site (Figure 5.12), which was due to higher deposition of the cationic liposomes at the injection site (Figure 5.10). However, irrespective of preparation method there was a reduction in infiltration of monocytes, at the SOI after injection of lysozyme or H56 antigen co-administered with the anionic DSPS/TDB formulation (Figure 5.12).

Also irrespective of the method of liposome preparation and antigen location within the vesicles, the charge of the liposomes is the key driver for monocyte influx which was shown to play a role in the transport mechanism of liposomes (and hence adsorbed antigen) from the vaccination site to the draining popliteal lymph node (PLN) due to increased antigen presentation on MHC, thus making antigen available to interact with T-cells in order to initiate the immune response (Henriksen-Lacey *et al.*, 2010a).

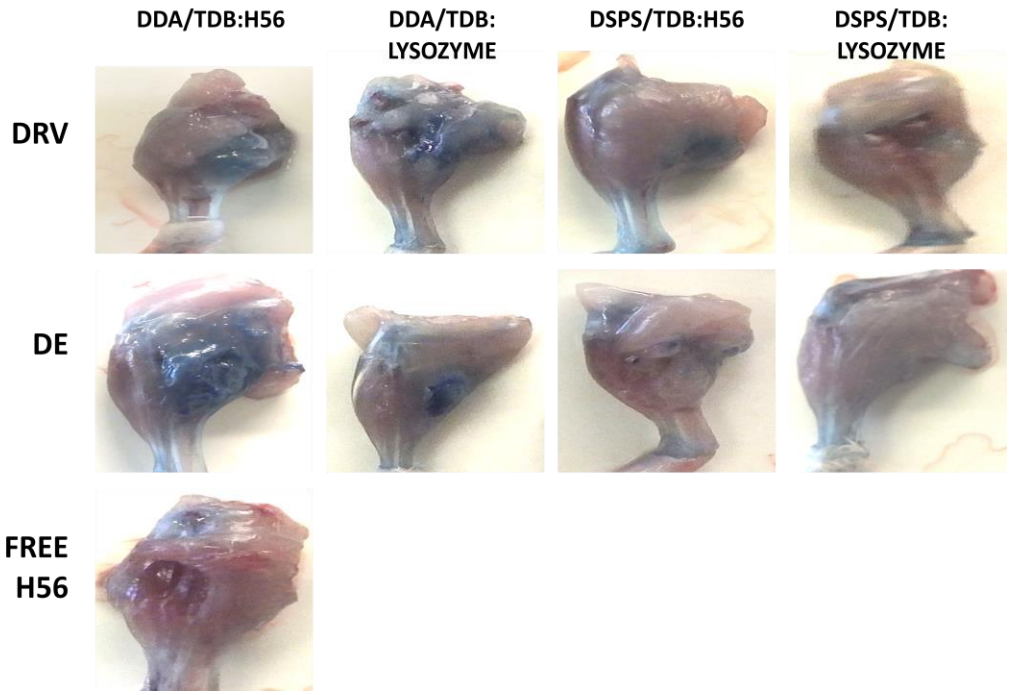


Figure 5-12. Liposome preparation method has no dramatic effect of the intensity of blue staining seen at the site of injection. However as described previously, following administration of cationic liposomes this leads to enhanced staining at this site compared to their anionic counterparts which is due to enhanced monocyte influx. Pictures are representative of 4 mice/group and show the quadriceps muscle from the vaccine injected leg at day 1 post-injection (p.i).

5.3.7. Immunogenicity of liposomal vaccines-adsorbing H56 antigen

In order to investigate the role of liposome preparation method on the immunogenicity of liposome vaccine formulations, a number of vaccine studies were carried out (at Statens Serum Institute) in order to show the immune response generated (Th1 response Vs Th2 response). The H56 TB antigen was formulated within cationic DDA/TDB and anionic DSPS/TDB liposomes respectively (formulated by LH, DRV and DE methods) before intramuscular (i.m.) injection of these vaccine formulations into the left quadriceps. During this study a triple homologous vaccination regimen was maintained in which each mouse was injected three times at two-weekly intervals (day 1, 15 and 29).

5.3.7.1. Generation of H56-specific antibody responses

In terms of all antibody responses (IgG1, Ig2a and IgG2b), naive (unimmunised) mice gave no responses as would be expected, both at days 13 and 46 (Figures 5.13 to 5.18). Also for H56-immunised mice, only minimal responses to all antibody isotypes were measured with the general trend that liposome-formulated H56 vaccines gave enhanced responses compared to H56 antigen alone (Figures 5.13 to 5.18).

In terms of liposome preparation method, there was no significant difference in IgG1 antibody production between the different preparation types. However, in terms of the production of IgG2a and IgG2b antibody isotypes (associated with the Th1 immune response (Mosmann et al., 1986, Martin and Lew, 1998)), the antigen location within the vesicles had an impact; either entrapped or surface-adsorbed, had an effect on the generated IgG2a and IgG2b antibody responses in the blood sera at day 46 p.i (Figures 5.16 and 5.18), with higher initial levels of H56 antigen loading by these liposome formulations leading to increased production of these antibodies as has been noted for DRV (antigen-entrapped) and LH-prepared (surface-adsorbed antigen) formulations.

These results also demonstrated a general 'liposome-charge dependent' trend in the production of all antibody isotypes (IgG1, IgG2a and IgG2b) with cationic DDA/TDB:H56 vaccine formulations displaying enhanced antibody titres compared to their anionic DSPS/TDB:H56 counterparts at day 46 ($p < 0.001$). This was described previously in Chapter 4 as well as the previous literature that cationic formulations showed an advantage in terms of generating increased antibody responses in the blood sera (Henriksen-Lacey *et al.*, 2010c) which was ascribed to the higher bioavailability of antigen, thus allowing higher antigen-specific antibody titres. Also as would be expected, all antibody titres displayed at day 46 were significantly higher ($P < 0.001$) than at day 13, upon delivery of liposome-formulated antigen. This was due to these mice having received their three immunisations of the liposomal vaccine formulations, therefore they are able to recognise antigen and boost the immune response in terms of antibody production.

Therefore the method of antigen association within the liposomal adjuvant can be a critical parameter influencing the immunogenicity of liposomal vaccines as has been reviewed previously by Watson and colleagues (Watson *et al.*, 2012) who noted mixed responses noted in terms of whether liposome surface-adsorbed antigen or liposome-entrapped antigen displayed higher levels of immune responses. The results presented within this section have showed that for H56-adsorbing DDA/TDB formulations, DRV and LH-prepared formulations gave higher IgG2a and IgG2b responses than their respective DE-counterparts. However, for H56-mixed with DSPS/TDB formulations, preparation method seemed to have no impact on the generation of increased antibody responses, which was hypothesised to be due to the low antigen-loading ability coupled with the fact that these liposomes are unable to form a depot at the SOI (as described previously within this chapter; Section 5.3.6). In contrast for DE-formulations, there was a general trend for the delivery of H56 vaccine antigen with cationic DDA/TDB and anionic DSPS/TDB to have no significant effect on the production of all antibody isotypes studied at the day 46 time point (following 3 x i.m. injections).

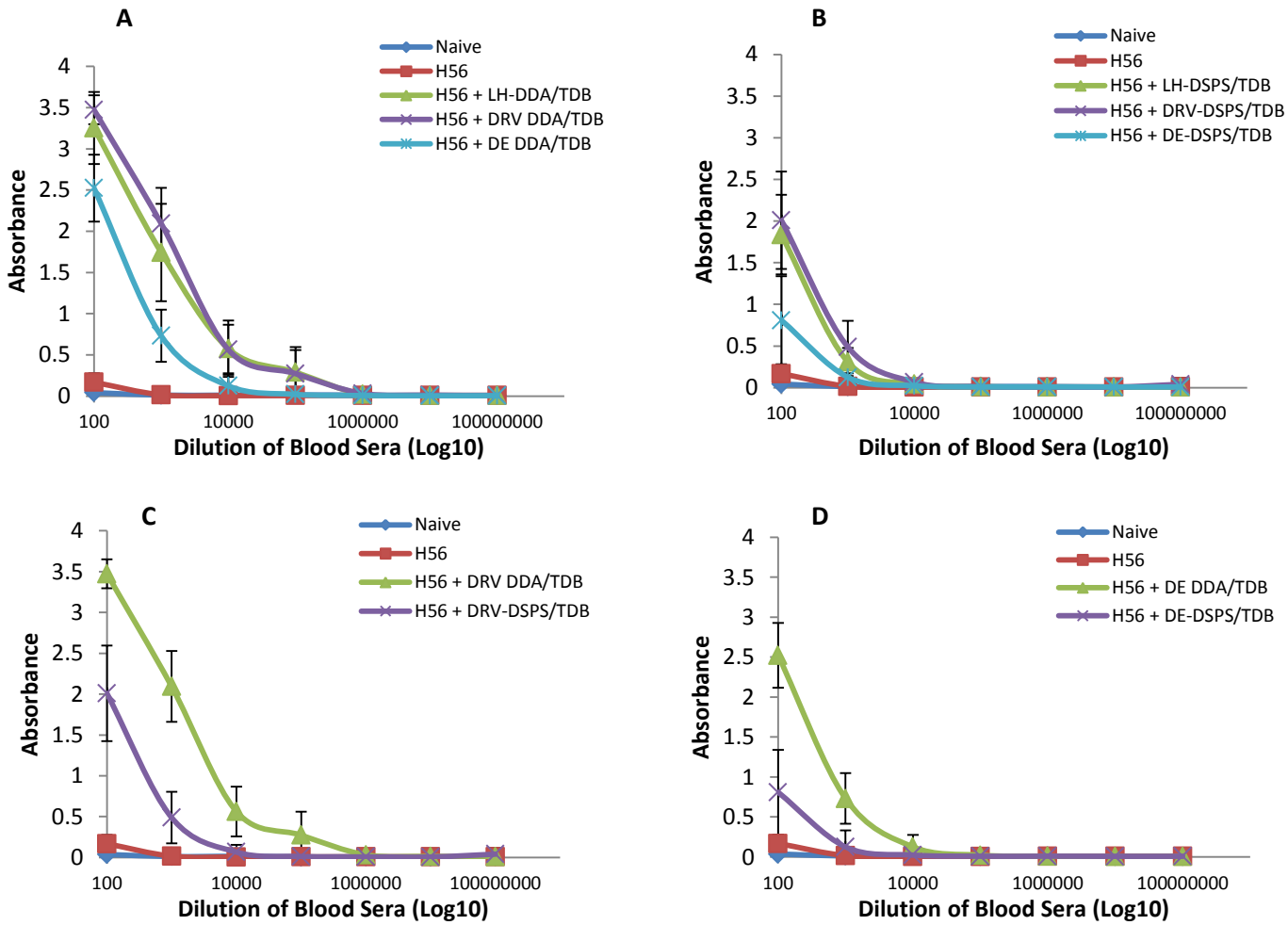


Figure 5-13. Antibody response curves. Blood sera was collected at day 13 and tested for the presence of IgG1 antibodies. (A) shows the effect of preparation method for cationic DDA/TDB formulations. (B) shows the effect of preparation method for anionic DSPS/TDB formulations. (C) and (D) show the effect of liposome surface charge for DRV and DE formulations respectively. Results are the mean ± SD of 6 mice per experimental group.

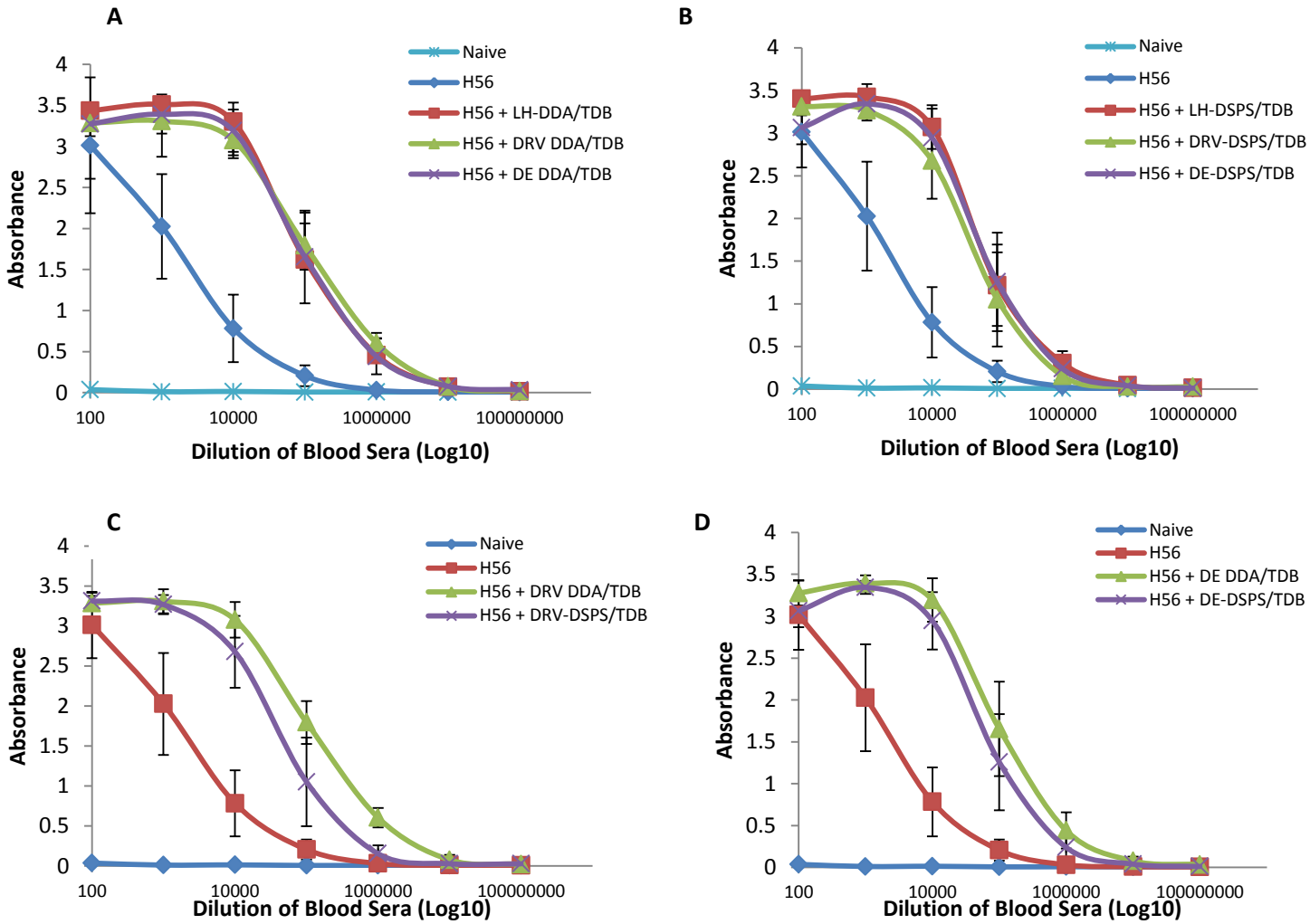


Figure 5-14. Antibody response curves. Blood sera was collected at day 46 and tested for the presence of IgG1 antibodies. (A) shows the effect of preparation method for cationic DDA/TDB formulations. (B) shows the effect of preparation method for anionic DSPS/TDB formulations. (C) and (D) show the effect of liposome surface charge for DRV and DE formulations respectively. Results are the mean ± SD of 6 mice per experimental group.

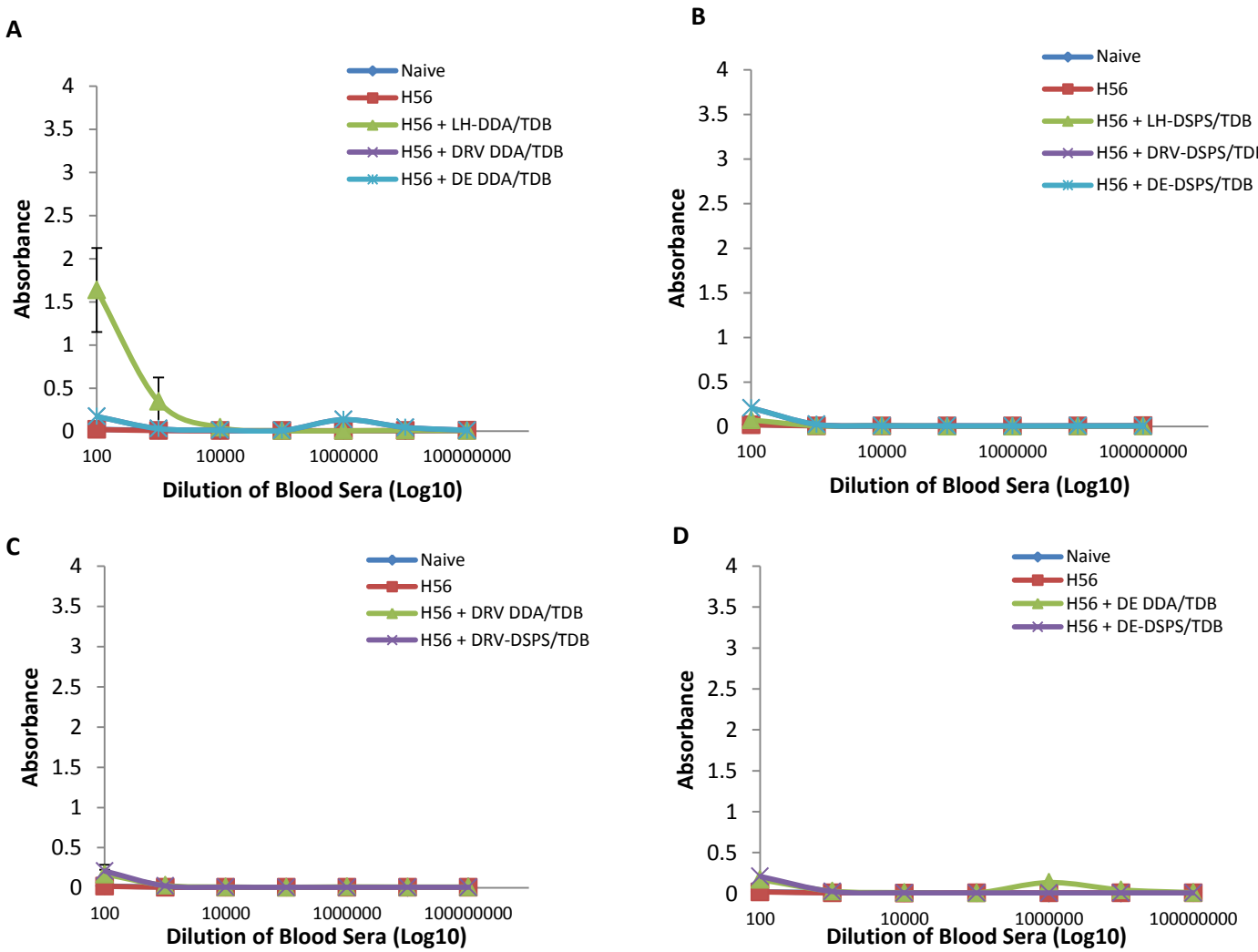


Figure 5-15. Antibody response curves. Blood sera was collected at day 13 and tested for the presence of IgG2a antibodies. (A) shows the effect of preparation method for cationic DDA/TDB formulations. (B) shows the effect of preparation method for anionic DSPS/TDB formulations. (C) and (D) show the effect of liposome surface charge for DRV and DE formulations respectively. Results are the mean ± SD of 6 mice per experimental group.

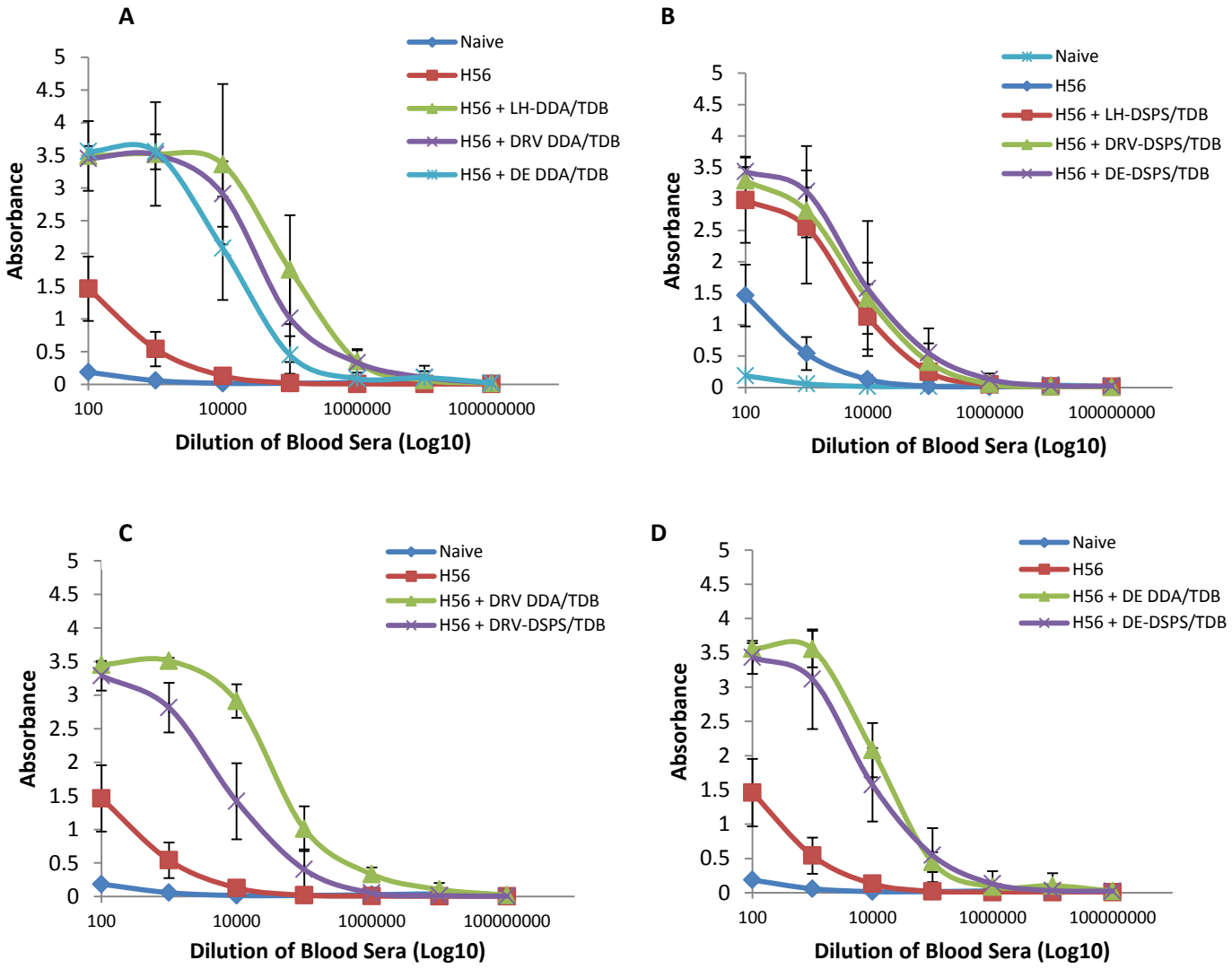


Figure 5.16. Antibody response curves. Blood sera was collected at day 46 and tested for the presence of IgG2a antibodies. (A) shows the effect of preparation method for cationic DDA/TDB formulations. (B) shows the effect of preparation method for anionic DSPS/TDB formulations. (C) and (D) show the effect of liposome surface charge for DRV and DE formulations respectively. Results are the mean ± SD of 6 mice per experimental group.

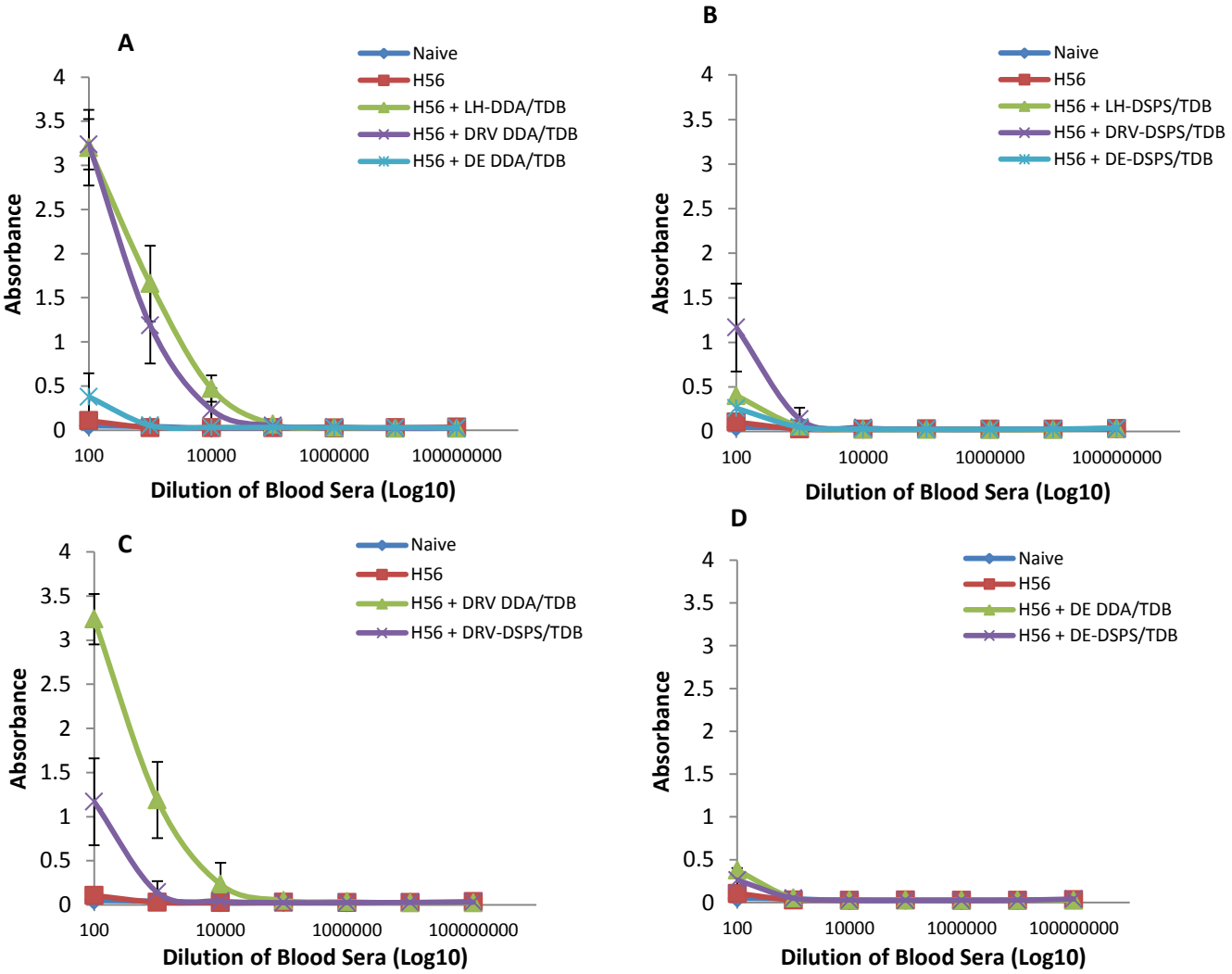


Figure 5-17. Antibody response curves. Blood sera was collected at day 13 and tested for the presence of IgG2b antibodies. (A) shows the effect of preparation method for cationic DDA/TDB formulations. (B) shows the effect of preparation method for anionic DSPS/TDB formulations. (C) and (D) show the effect of liposome surface charge for DRV and DE formulations respectively. Results are the mean ± SD of 6 mice per experimental group.

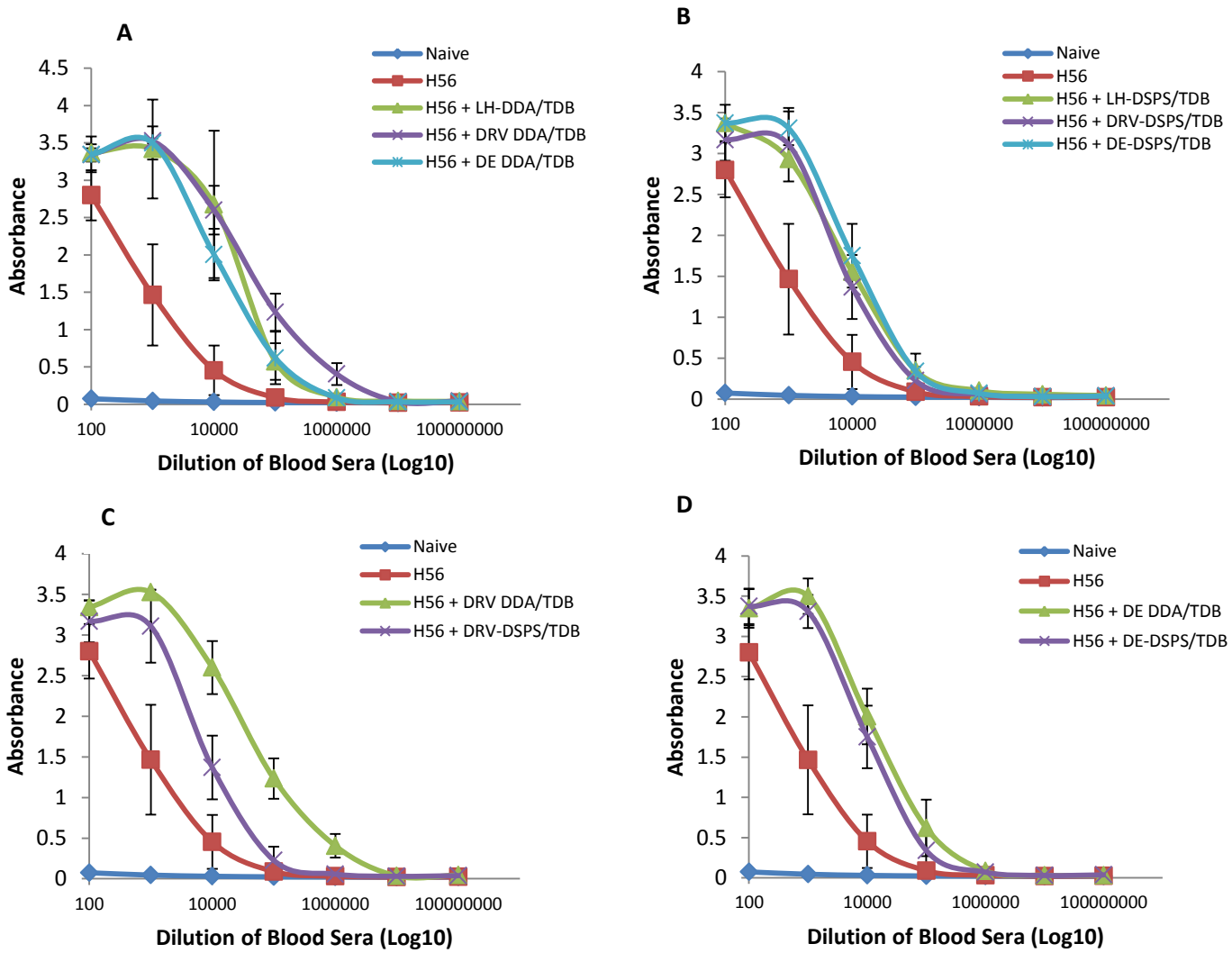


Figure 5.18. Antibody response curves. Blood sera was collected at day 46 and tested for the presence of IgG2b antibodies. (A) shows the effect of preparation method for cationic DDA/TDB formulations. (B) shows the effect of preparation method for anionic DSPS/TDB formulations. (C) and (D) show the effect of liposome surface charge for DRV and DE formulations respectively. Results are the mean ± SD of 6 mice per experimental group

5.3.7.2. Cytokine analysis from *ex vivo* restimulated splenocytes and lymph nodes

Following the determination of the ability of H56 vaccine antigen, when formulated with cationic DDA/TDB and anionic DSPS/TDB liposomes, to induce antibody responses in the blood sera it was required to analyse the production of Th1 and Th2-related cytokines from restimulated splenocytes and popliteal lymph node cell upon restimulation with previously re-encountered H56 vaccine antigen. Upon termination of the experiment, spleens and popliteal lymph nodes (PLN) were dissected and collected according to formulation. Following the mashing of these organs, splenocytes and lymph node cells were restimulated with PMA/Ionomycin (positive control), media only (negative control) or H56 antigen (at concentrations of 0.05, 0.5 and 5 μg/mL respectively). Following 72 h incubation at 37 °C, the supernatant was then decanted for use in cytokine ELISA assays.

In order to serve as a positive control, splenocytes and lymph node cells were restimulated with PMA/Ionomycin and the cytokine release from the cells was measured using ELISA. These results are displayed in Figures 5.19 to 5.21 in terms of cytokine release for IFN-γ, IL-17 and IL-5 respectively. Ionomycin is an ionophore produced by the bacterium *Streptomyces conglobatus*. In research, ionomycin was used to raise the intracellular level of calcium (Ca^{2+}) therefore upon cell restimulation these enhanced levels of calcium can stimulate T-cell activation, proliferation and the intracellular production of cytokines, usually in conjunction with phorbol 12-myristate 13-acetate (PMA).

These results showed that upon restimulation of splenocytes and PLN with the positive control stimuli, PMA/Ionomycin, this resulted in high cytokine production with no significant trends observed between the cells derived from tested formulations. This shows that our positive control worked successfully in that these cells, upon restimulation, respond with observed cytokine production.

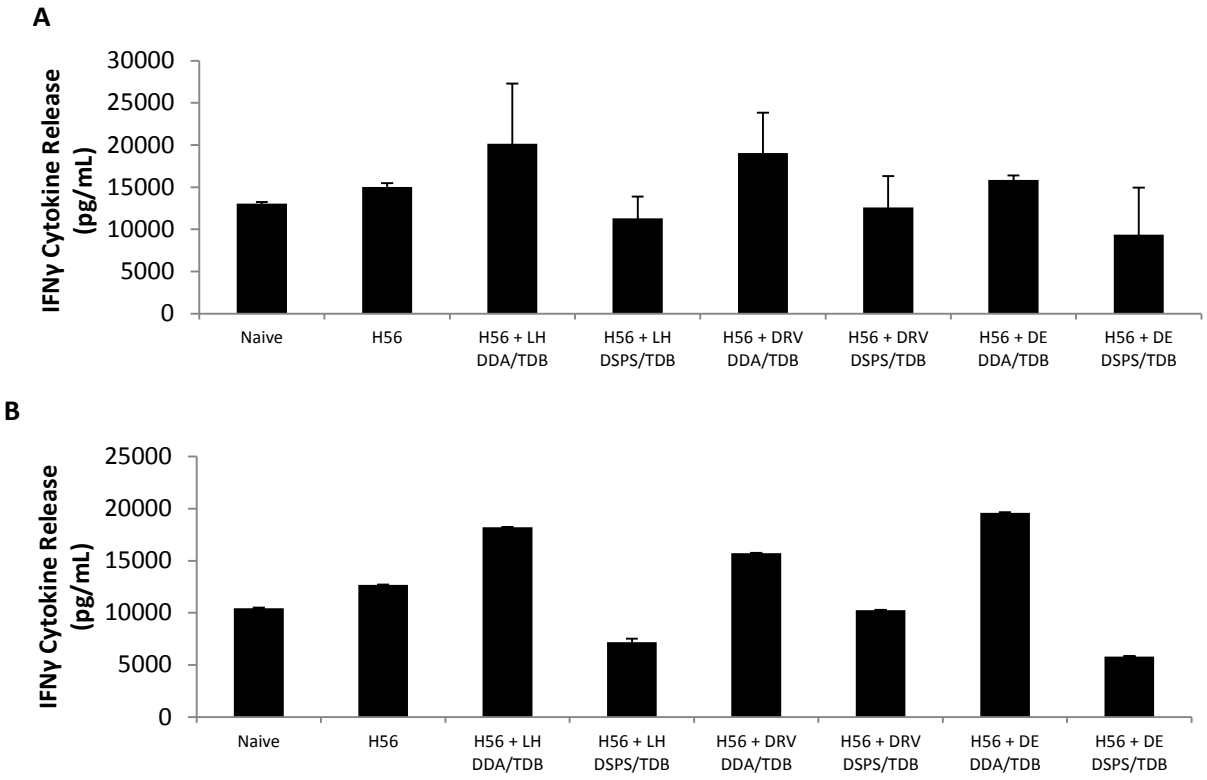


Figure 5-19. IFN-γ cytokine analysis from PMA/Ionomycin restimulated splenocytes (A) and popliteal lymph nodes (B). Results are the mean of 6 mice per experimental group ± SD.

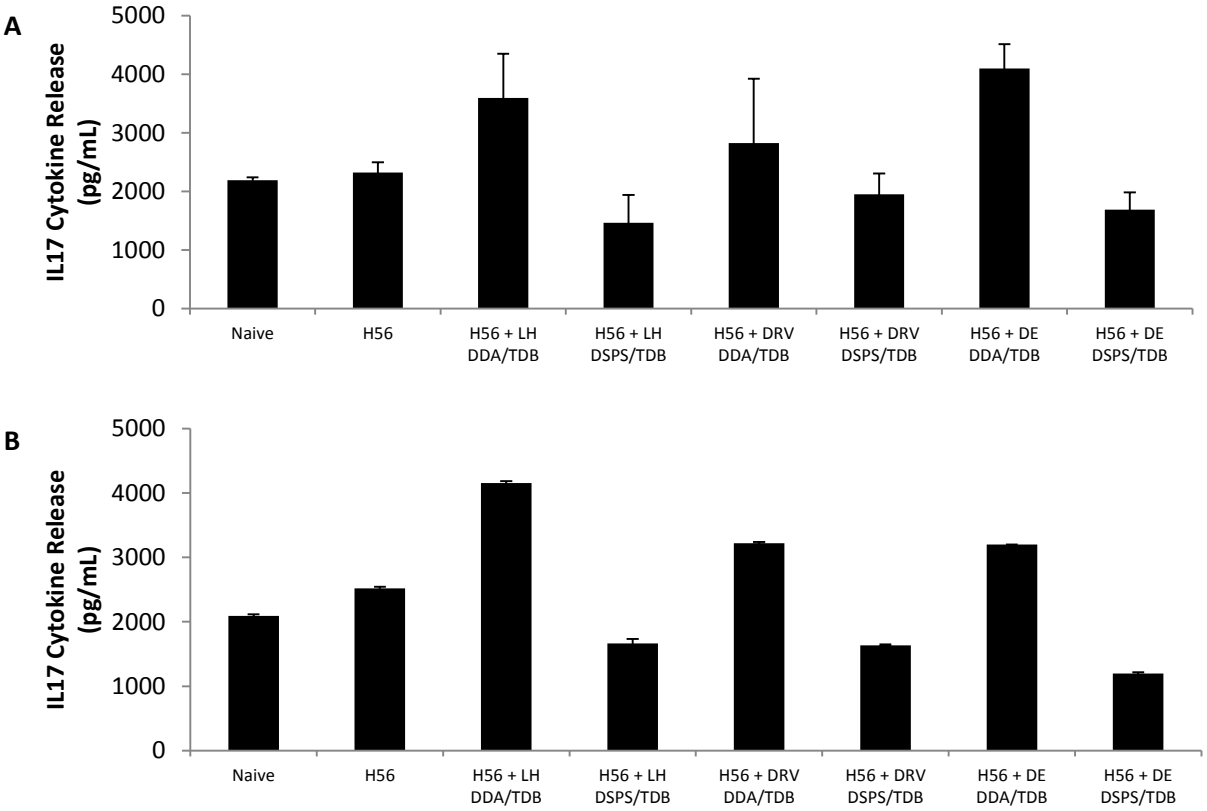


Figure 5-20. IL-17 cytokine analysis from PMA/Ionomycin restimulated splenocytes (A) and popliteal lymph nodes (B). Results are the mean of 6 mice per experimental group ± SD.

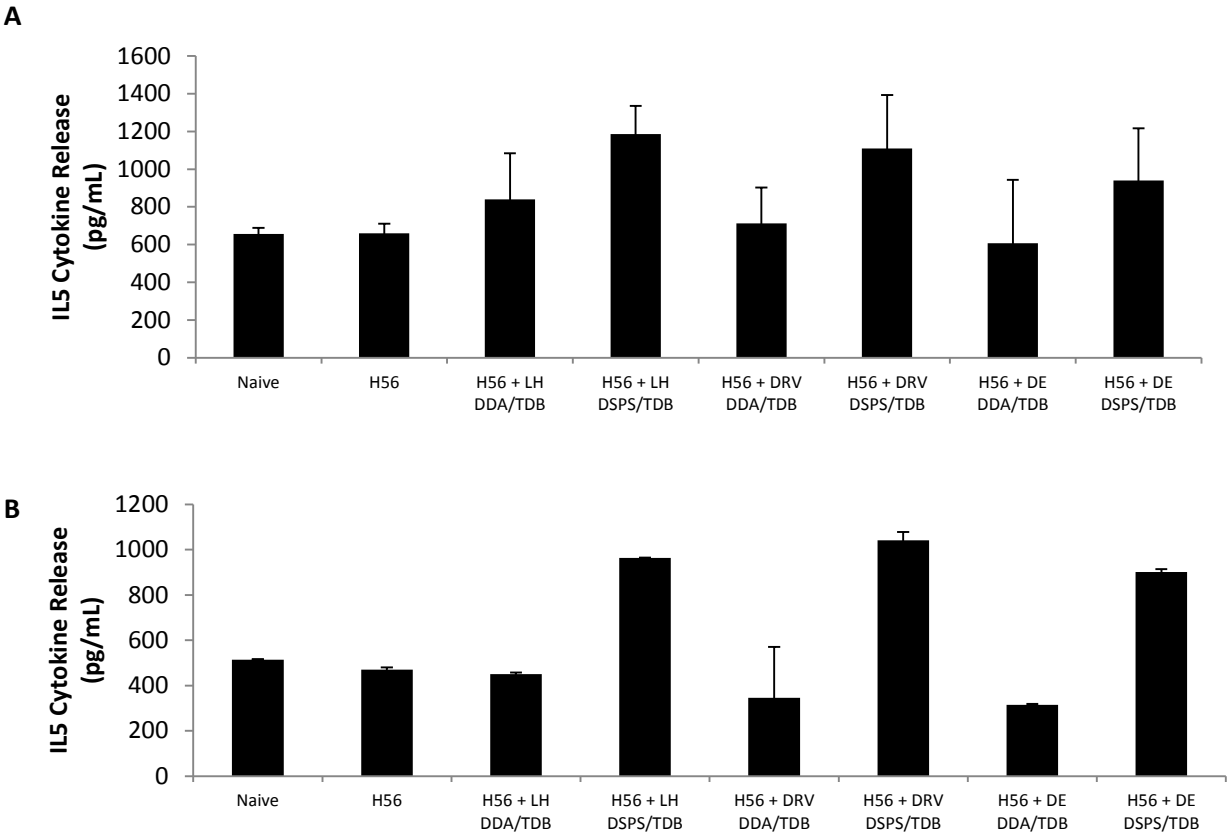


Figure 5-21. IL-5 cytokine analysis from PMA/Ionomycin restimulated splenocytes (A) and popliteal lymph nodes (B). Results are the mean of 6 mice per experimental group ± SD.

During this study, general trends were found in that in terms of the negative control (cell restimulated with media only), only background levels of cytokine production were measured which were significantly lower ($P < 0.001$) than the corresponding cells restimulated with H56 vaccine antigen, Therefore this demonstrated that these cells require stimulation with previously encountered antigen in order for the immune response to be initiated as characterised by cytokine production at the spleen and lymph nodes (Figures 5.22 to 5.24). Also as a control experimental group, splenocytes and PLN were derived from naive unimmunised mice. Upon restimulation, these cells did not respond and therefore only background levels of all cytokines were produced (Figures 5.22 to 5.24).

The first cytokine investigated was IFN-γ which is a commonly used marker to determine the efficacy of tuberculosis vaccines (Agger and Andersen, 2001). In terms of the generated IFN-γ responses at both the spleen and PLN, antigen entrapment within cationic DDA/TDB liposomes (as for DRV formulations) did not enhance Th1 responses, as compared to liposome-surface adsorbed antigen (Figures 5.22), however DDA/TDB liposomes with surface-adsorbed antigen (LH-prepared) seemed to generate higher IFN-γ responses at the PLN than their DE- and DRV counterparts (Figure 5.22). In

terms of the IFN-γ immune response generated by anionic DSPS/TDB formulations, similarly low levels of production were noted irrespective of the liposome preparation method (Figure 5.22). This may be due to lesser loading of H56 antigen by these formulations, and also anionic formulations have previously been shown to reduce and inhibit Th1 immune responses as has been described in Chapter 4 and in the previous literature (Foged et al., 2004, Parker et al., 2008). Also this correlation between IFN-γ production, both at the splenocytes and PLN (Figure 5.22), showed that cationic liposomes are promoting immune responses at both of these sites.

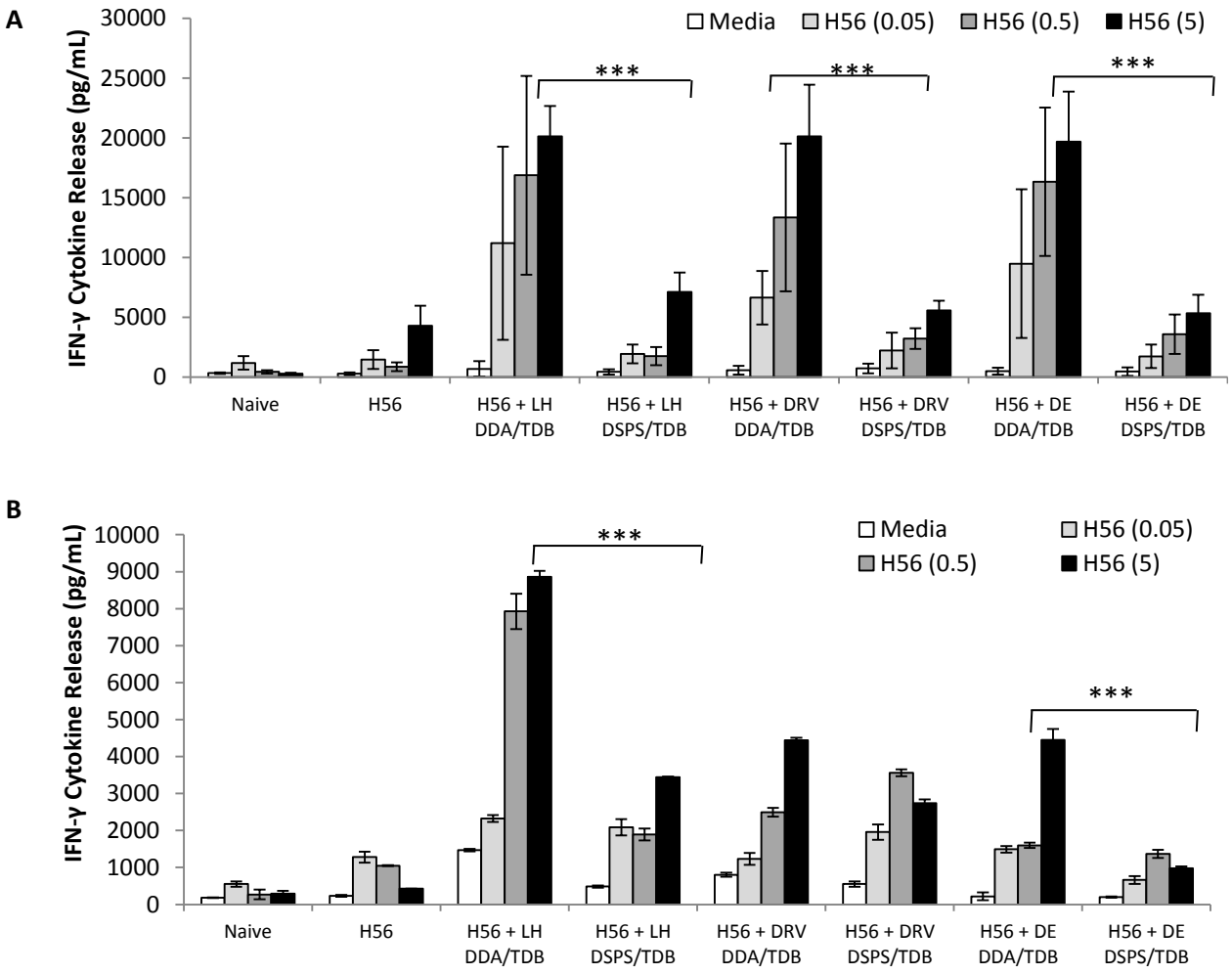


Figure 5-22. IFN-γ cytokine analysis from H56 antigen restimulated splenocytes (A) and popliteal lymph nodes (B). Results are the mean of 6 mice per experimental group ± SD. Significance was measured between experimental groups by one-way ANOVA (*** $p<0.001$).

During the recent decade, the Th17 subset of T-cells have gained unprecedented attention, with this T-cell subset being shown to play a role in antimicrobial responses against viral and bacterial infections, as well as the polarising conditions for Th17 immunity having been firmly established (Dong, 2008, Lindenstrom et al., 2012). *In vivo* generated Th17 cells have been shown to stably

express the cytokine interleukin-17 (IL-17) and be completely separate to Th1 and Th2 polarising signals *ex vivo* (Lexberg *et al.*, 2008). The results for IL-17 cytokine release at both the splenocytes and PLN were shown in Figure 5.23.

In terms of the effect of antigen location with cationic DDA/TDB liposome formulations on the generated immune response, antigen entrapment (for DRV formulations) gave rise to no significant effect on the production of IL-17 (following splenocyte and PLN restimulation with previously re-encountered H56 antigen), when compared to DE and LH-formulations (increased bias towards surface adsorbing antigen) with similarly high levels of IL-17 production noted. Also a similar trend was noted for anionic DSPS/TDB formulations, with similarly low levels of IL-17 being produced (irrespective of the liposome preparation method). This demonstrated that antigen, whether surface-adsorbed or entrapped within the formulation has the ability to be taken up by macrophages at the injection site, and lead to similar subsequent generated immune responses.

These results also demonstrated a general liposome charge-dependent trend, in that immunisation of mice with H56 (in combination with cationic DDA/TDB) gave rise to the higher levels of IL-17 production ($P < 0.001$), both for restimulated splenocyte and PLN (Figure 5.23). However immunisation of the H56 vaccine (in combination with DSPS/TDB) gave rise to lower IL-17 cytokine production for both sets of restimulated cells. This may be due to lesser initial loading of H56 antigen by these formulations therefore less antigen will be available for antigen presentation upon drainage of the vaccines to T-cell rich sites such as the spleen and lymph node.

These results correlated with the previous literature in which DDA/TDB have been reported to drive IL-17 responses, through the interaction of TDB with its C-Lectin type receptor Mincle leading to downstream activation of the innate immune response through polarisation of Th17 cells through proinflammatory cytokine production (Werninghaus et al., 2009, Schoenen et al., 2010).

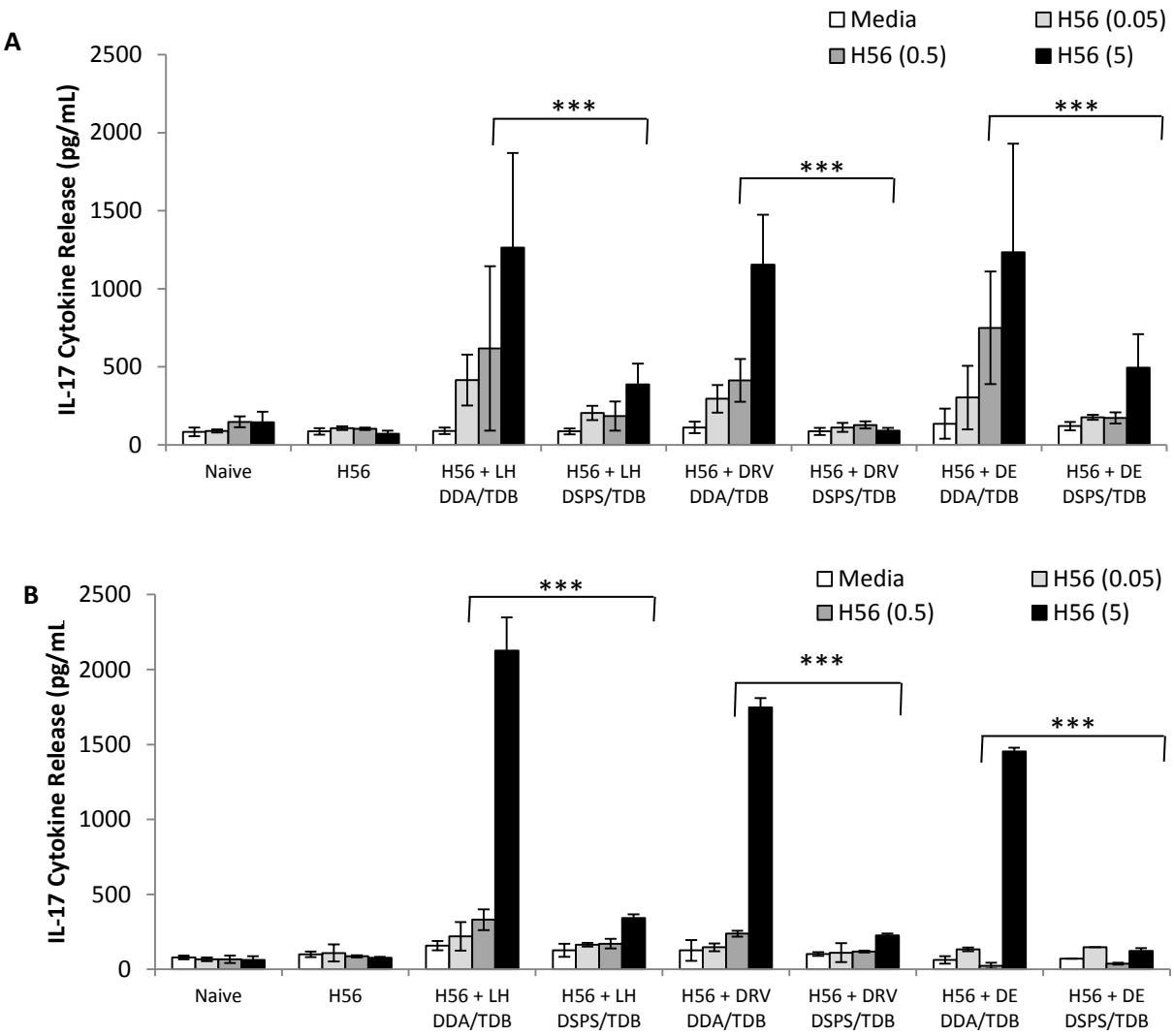


Figure 5-23. IL-17 cytokine analysis from H56 antigen restimulated splenocytes (A) and popliteal lymph nodes (B). Results are the mean of 6 mice per experimental group ± SD. Significance was measured between experimental groups by one-way ANOVA (** p<0.001).

The final cytokine to be investigated during this immunisation study was interleukin-5 (IL-5). This cytokine was shown to play a crucial role in activating naïve T cells towards a Th2 phenotype. The results for IL-5 cytokine release at both the splenocytes and PLN were shown in Figure 5.24.

These results suggested that antigen entrapment within liposomal adjuvants did not result in improvement in Th1 responses, however for cationic DDA/TDB with surface-adsorbed antigen (LH-prepared) this resulted in increased levels ($P < 0.01$) of IL-5 production from restimulated splenocytes (Figure 5.24A) however no trends were noticed between cationic DDA/TDB formulation in terms of IL-5 production from restimulated lymph node cells. However these studies showed that liposome preparation method had a significant impact on the immunogenicity of non-depot forming

anionic DSPS/TDB:H56 vaccine formulations, with LH- and DRV-prepared formulations giving rise to significantly higher ($P < 0.001$) IL-5 responses in comparison to their DE-counterparts from restimulated splenocytes and PLN (Figure 5.24). However liposomal vaccines prepared by the double emulsion (DE) method, irrespective of surface charge, gave rise to low levels of IL-5 responses which was possibly due to the lower level of antigen adsorption by both of these liposomal adjuvants (cationic DDA/TDB and anionic DSPS/TDB).

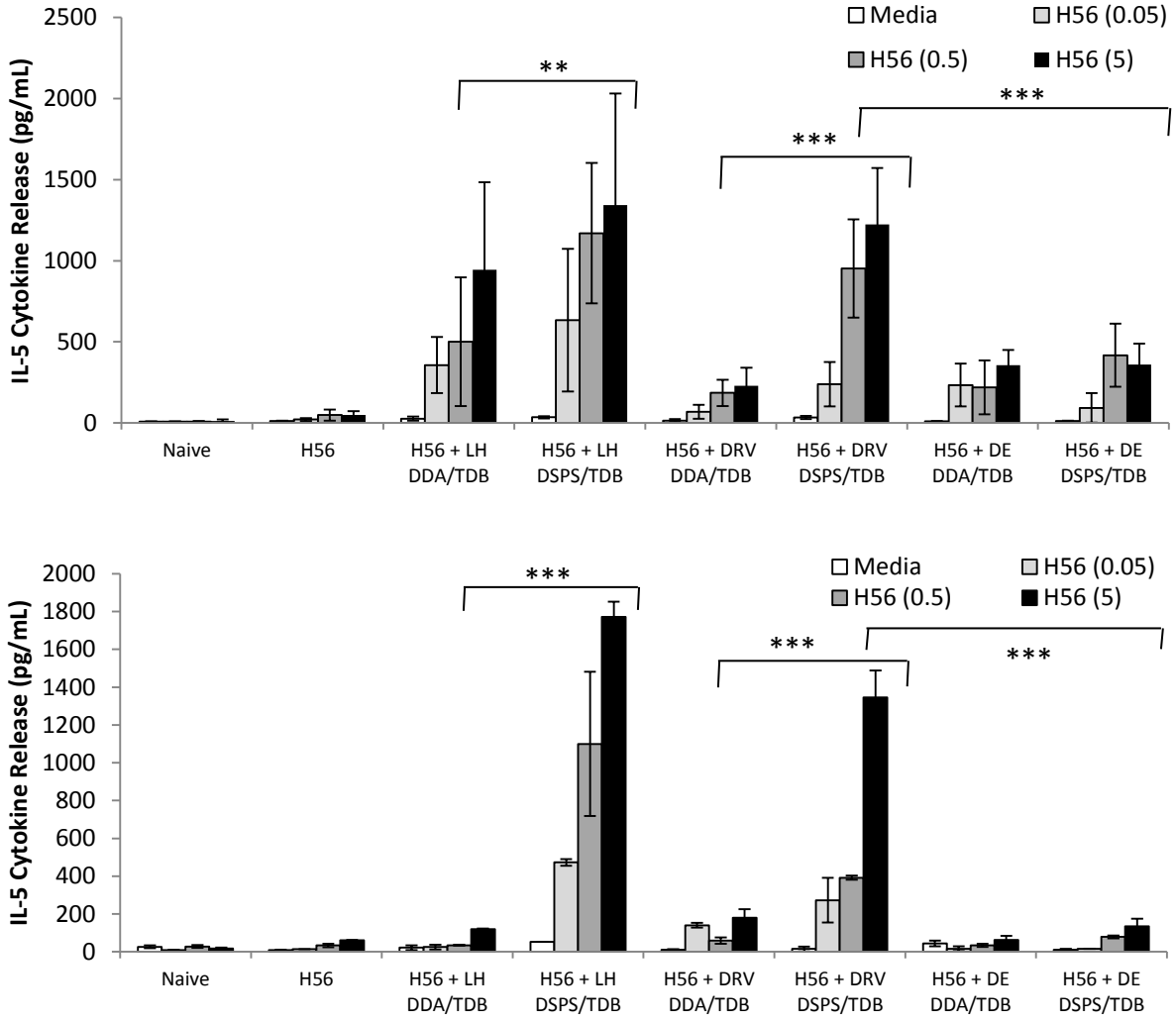


Figure 5-24. IL-5 cytokine analysis from H56 antigen restimulated splenocytes (A) and popliteal lymph nodes (B). Results are the mean of 6 mice per experimental group ± SD. Significance was measured between experimental groups by one-way ANOVA (*** $p<0.001$; ** $p<0.01$).

5.4. Conclusions

The results presented within this chapter showed that by varying the method of liposome preparation that antigen can become associated with the delivery system, either by adsorption to the liposome surface or encapsulation within the aqueous interior. Formulation of liposomal adjuvants by the DRV method led to entrapment of antigen within the delivery system, whereas double emulsion (DE)-prepared formulations have antigen associated at the liposome surface as well within the delivery system. The charged nature of the lipid head group conferred the surface charge of the delivery system with cationic DDA/TDB and anionic DSPS/TDB being cationic and anionic in charge respectively. In general DE-formulated liposomes displayed a reduced vesicle size in comparison to their DRV counterparts, with these differences being attributed to the variation in preparation method.

In terms of loading, DRV-prepared formulations showed enhanced loading and protection of antigen in comparison to their respective DE-formulations. Upon i.m. injection of these vaccines, cationic liposomes showed a higher retention profile at the injection site, whilst anionic liposomes drained significantly faster to the popliteal lymph node (PLN) irrespective of liposome preparation method. In term of the effect of antigen localisation on the observed biodistribution profile of liposomal vaccines, antigen entrapment offered no significant improvement on the retention of liposome (and associated antigen) at the injection site and the subsequent delivery to the draining lymph node. However, as described previously depot-forming cationic DDA/TDB liposome adjuvants showed a pattern of controlled release from the SOI to the PLN which was important in the generation of enhanced Th1 responses. During these studies, liposome retention at the SOI correlated with enhanced IFN-γ and IL-17 responses at both the spleen and PLN. However in terms of the effect of liposome preparation method on IL-5 production, anionic DSPS/TDB formulations (formulated by the LH- and DRV methods) gave rise to increased Th2 responses in comparison to their DE-counterparts

For all cytokines tested, mice injected with liposome-adjuvanted vaccines gave rise to significantly increased immune responses. Therefore whilst DRV formulations were shown to offer better antigen protection and higher antigen retention (compared to LH- and DE-prepared formulations) this did not translate into differences in the measured biodistribution or generated immune responses for cationic DDA/TDB formulations. However, H56 antigen entrapment within anionic DRV DSPS/TDB gave rise to higher levels of Th2 immunity as characterised by enhanced IL-5 production.

Chapter 6: The inclusion of TLR agonists within liposome adjuvants and the synergy between TLR and non-TLR agonists

6.1. Introduction

The flexible and versatile nature of liposomes allows for the potential of protein antigen and immunomodulatory components, including Toll-like receptor (TLR) and non-TLR agonists to be incorporated within the system. This chapter shall present data regarding the endosome-located TLR agonist, polyI:C and compare liposome systems comprising the mycobacterial- derived glycolipid non-TLR agonists, trehalose 6,6' – dibehenate and monomycoloyl glycerol.

6.1.1. Polyinosinic:polycytidylic acid (polyI:C)

PolyI:C is an immunostimulatory molecule consisting of a double stranded RNA (dsRNA), with one of the strands being a polymer of inosinic acid and the other strand being a polymer of cytidylic acid, which is known to interact with Toll-like Receptor 3 (TLR3). PolyI:C is located within the endosome in cells, most preferentially in the membrane of B-cells, macrophages and dendritic cells.

Recent studies have demonstrated the immunostimulatory ability of polyI:C following incorporation within cationic liposome vaccine adjuvants (Milicic et al., 2012). Addition of polyI:C significantly increased the adjuvant capabilities of DDA/TDB liposomes formed as MLV and antigen-entrapping DRV. However, DDA/TDB formed as SUV offered enhanced adjuvant capacity without the addition of TLR agonists to the system.

6.1.2. Trehalose 6,6' – Dibehenate (TDB)

TDB is a synthetic analogue of the mycobacterial 'cord factor', otherwise termed as trehalose dimycolate (TDM) and shows less toxicity *in vivo* (than TDM) whilst retaining its immunogenic properties. This is due to the reduced number of mycolate chains which results in a shorter chain length (Matsunaga and Moody, 2009). These properties allow for the successful inclusion of TDB into cationic liposome delivery systems, with an optimal ratio of 11 mol % (8:1 M/M) shown to display the highest titres of antibodies (Davidsen et al., 2005) in order to generate the optimal immune response. This inclusion of TDB into DDA-based liposomes leads to an increasingly stable (Christensen et al., 2007a, Christensen et al., 2009) and efficient adjuvant delivery system which has the advantage of greatly improving the Th1-mediated cell immune response and the antibody response (Davidsen et al., 2005). The enhanced stability of this cationic adjuvant formulation is

caused by the interaction of the glucose headgroups of TDB with the surrounding aqueous buffer which is used to hydrate the liposomes (Christensen et al., 2007a, Christensen et al., 2008). The incorporation of TDB into DDA-based liposomes confers an increased immune response against various subunit vaccine antigens due to the binding of TDB to a C-type Lectin Mincle Receptor (Ishikawa et al., 2009, Werninghaus et al., 2009, Schoenen et al., 2010) which can activate the TLR-independent FcR-Syk-Card9 pathway in APCs in order to cause a sufficient innate immune response, characterised by protective Th1- and Th17-immunity.

6.1.3. Monomycoloyl Glycerol

Studies (Andersen et al., 2009a, Andersen et al., 2009b) have also indicated monomycoloyl glycerol (MMG) as a prominent glycolipid from the mycobacterial cell wall which has an enhanced effect on the immune response. As described previously with trehalose 6,6' – dibehenate, MMG can also be incorporated into DDA-based liposomes.

This delivery system (DDA/MMG), in combination with the Ag85B-ESAT6 (H1) fusion protein was found to be highly efficient in the activation of APCs (by MMG) and the production of a Th1-biased immune response as characterised by high levels of interferon-gamma (IFN-γ) and IL2 as well as negligible levels of Th2 cytokines IL-4 and IL-5 (Rosenkrands et al., 2005). Also in more recent studies, synthetic analogues of MMG have been incorporated into DDA-based liposomes. These delivery systems in combination with vaccine antigen also lead to enhanced immune response as characterised by increased cytokine release including IFN-γ and IL-2, as well as Th17 responses as characterised by IL-17 production (Andersen et al., 2009a, Andersen et al., 2009b, Bhowruth et al., 2009, Nordly et al., 2011b).

6.1.4. Liposome formulations with and without inclusion of polyI:C TLR agonist

Within this chapter, both DDA/TDB and DDA/MMG liposome formulations (termed CAF01 and CAF04) can also be further enhanced following the addition of the TLR3 agonist, polyI:C. The addition of polyI:C to CAF01 leads to the formulation of CAF05 liposomes. Whereas the incorporation of polyI:C to CAF04 leads to the formulation of CAF09 liposomes (Figure 6.1).

Figure 6-1. Nomenclature of the cationic adjuvant formulations used within this chapter of the thesis. This figure was originally produced by Dennis Christensen from the Statens Serum Institute.

6.2. Aims and Objectives

The aim of the work reported within this chapter was to successfully incorporate Toll-like Receptor (TLR) and non-TLR immunostimulatory components within cationic DDA-based liposome formulations. In order to achieve this, the effect of the following will be investigated:

- the addition of immunostimulatory Toll-like Receptor (TLR) agonists on the physicochemical characteristics of cationic liposomal adjuvants,
- the long term stability of these systems in terms of shelf life storage under standard and accelerated conditions,
- varying the liposome preparation method for cationic liposomes associated with TLR3 agonist (with or without protein antigen) with antigen loading, agonist loading and physicochemical characteristics being measured,

- preparation of liposomes by high shear mixing (HSM) homogenisation with the aim of reducing vesicle size and retaining TLR agonist with the delivery system,
- stepwise incorporation of TLR agonist to DDA-based liposome formulations, in comparison to addition at the whole dose, in order to obtain desired physicochemical characteristics of liposomal adjuvants.

6.3. Results and Discussion

6.3.1. Incorporation of immunostimulatory compounds and the subsequent effect on vesicle characteristics

6.3.1.1. Multilamellar vesicles formulated by the lipid hydration method

Liposome vaccine formulations were prepared by LH method, with the addition of a range of increasing concentrations of the whole desired dose of polyI:C (between 0 and 50 μg) within the formulation to consider if the physicochemical characteristics of the liposome formulation was polyI:C concentration dependent. These liposomes were also formulated with or without the addition of the *in vivo* dose of H56 antigen (5 μg) and characterised for their size, polydispersity and zeta potential. During these studies, cationic DDA/TDB formulations with the inclusion of polyI:C are termed cationic adjuvant formulation 05 (or CAF05).

Upon increase in the concentration of polyI:C within these cationic formulations this resulted in a significant concentration dependent increase in vesicle size from ~600 nm to around 2.3 μm ($p < 0.001$) with a corresponding increase in polydispersity (Figure 6.2A). In general, antigen-loaded systems were larger in vesicle size than their empty counterparts; this could be due to loading of antigen at the surface of the delivery system. Antigen loading is mediated by electrostatic interactions between the cationic liposome and the anionic antigen at the surface of the delivery system (Henriksen-Lacey et al., 2010c).

Also upon increase in the polyI:C dose concentration within the formulation, a concentration dependent reduction in the zeta potential ($P < 0.001$) of the formulation was noted (Figure 6.2B). In combination with this decrease in zeta potential a reduction in antigen loading was seen (Figure 6.2B). These changes in vesicle characteristics were due to addition of anionic polyI:C, which will electrostatically bind (due to the opposing charge of these components) to the delivery system thus causing aggregation and neutralisation of the surface charge, and a reduced loading due to saturation of the ability to electrostatically bind polyI:C (Figure 6.2B).

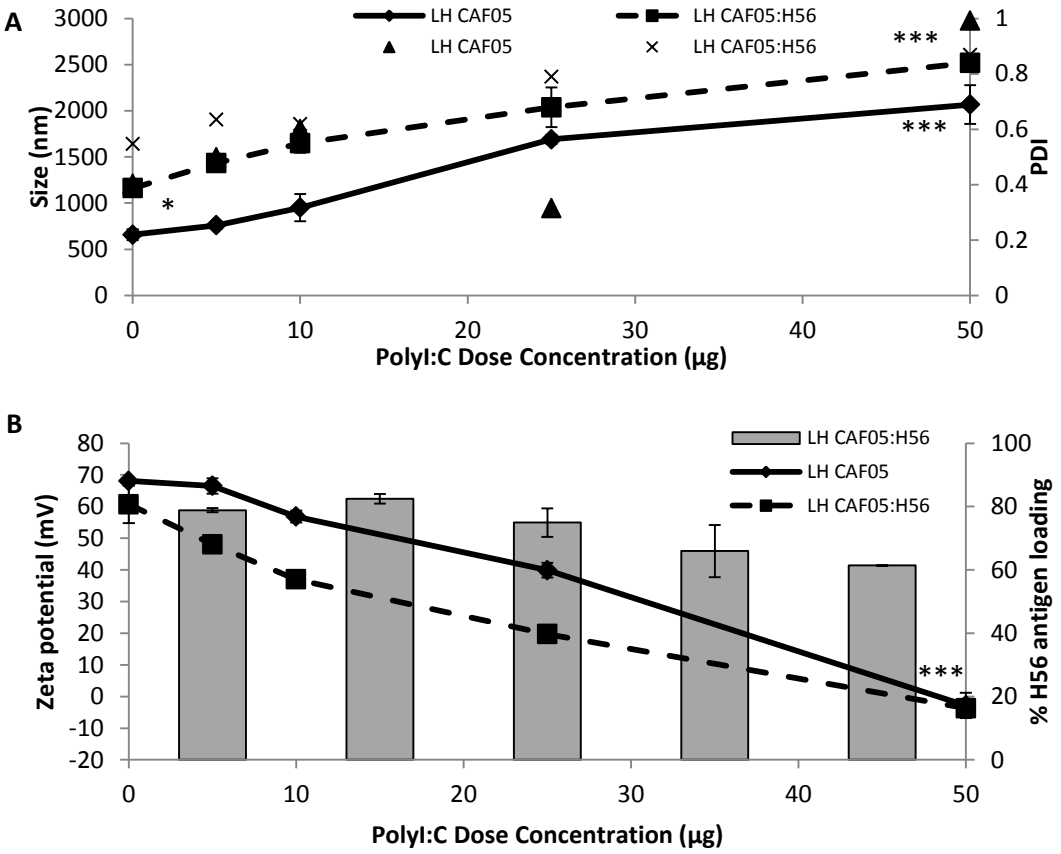


Figure 6.2. Vesicle size and polydispersity (A), zeta potential and antigen loading (B) of liposome vaccine formulations prepared by the lipid-film hydration method with addition of increasing concentrations of polyI:C (with or without H56 antigen). Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$; * $p<0.05$) as compared to the same formulation with no polyI:C (0 µg) included.

6.3.1.2. Alternative options for MLV; consideration of DRV and SUV systems

Liposome vaccine formulations were also prepared as DRV and SUV, with addition of increasing concentrations of the whole desired dose of polyI:C within the formulation. These liposomes were also formulated with or without the addition of the *in vivo* dose of H56 antigen and characterised for their size, polydispersity and zeta potential (Figure 6.3).

Upon increase in the concentration of polyI:C within these cationic formulations this resulted in a concentration dependent increase in vesicle size ($P < 0.05$; Figure 6.3A). This may be due to entrapment of polyI:C within the delivery system or association within the bilayer during the liposome preparation process. Also upon increase of the polyI:C concentration within the formulation this resulted in a dose-dependent reduction in the zeta potential of the formulation which occurred due to addition of the anionic TLR3 agonist polyI:C which was suggested to bind

electrostatically to the delivery system thus causing neutralisation of the surface charge (Figure 6.3B) however these changes were not as significant as for LH-formulations ($P < 0.05$; upon addition of 50 µg polyI:C per dose) which also suggested that agonist may become entrapped within the liposome itself. As described previously for MLVs (Section 6.3.1.1), a general trend existed that antigen-loaded DRVs were larger in size ($P < 0.05$) compared to their 'empty' counterparts suggesting antigen can become associated with these systems. Upon increasing the dose of polyI:C within the formulation, up to the *in vivo* dose of 50 µg, there was also a minimal reduction in initial antigen loading (Figure 6.3B). This minimal reduction in antigen loading was due to the reduction in zeta potential, due to increasing polyI:C concentration within the system, therefore the formulations had slightly less ability to electrostatically bind and entrap anionic H56 antigen (Figure 6.3B).

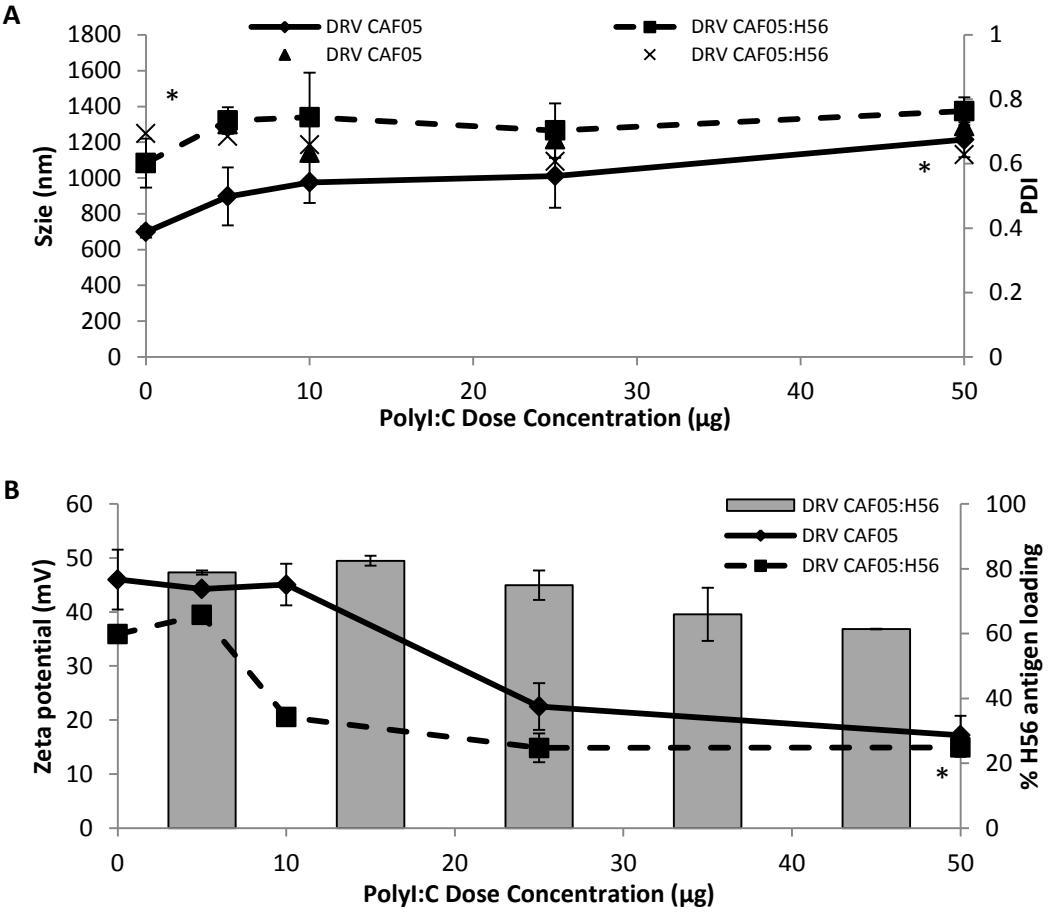


Figure 6-3. Vesicle size and polydispersity (A), zeta potential and antigen loading (B) of liposome vaccine formulations prepared by the dehydration-rehydration vesicle (DRV) method with addition of increasing concentrations of polyI:C (with or without H56 antigen) within the formulation. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (* $p<0.05$) as compared to the same formulation with no polyI:C (0 µg) included.

Liposome vaccine formulations were prepared as small unilamellar vesicles, with the addition of increasing concentrations of the whole desired dose of polyI:C within the formulation. These liposomes were also formulated with or without the addition of the *in vivo* dose of H56 antigen and characterised for their size, polydispersity and zeta potential (Figure 6.4).

Upon increase in the dose concentration of polyI:C, from 0 µg to 50 µg, within these cationic formulations this resulted in a significant dose-dependent increase in vesicle size and polydispersity (Figures 6.4A) from 150 nm to around 1.8 µm ($P < 0.001$). This was hypothesised to be due to electrostatic binding of the agonist at the surface of the delivery system. Also upon increase of the polyI:C concentration within the formulation, this resulted in a significant dose-dependent reduction in the zeta potential ($P < 0.001$) of the formulation which occurred due to addition of the anionic polyI:C electrostatically binding to the delivery system, thus causing neutralisation of the surface charge (Figure 6.4B) which became significantly different following addition of polyI:C at a concentration of 50 µg per dose. Upon increasing the dose of polyI:C within the formulation, up to the *in vivo* dose of 50 µg, there was also a reduction in initial antigen loading and enhanced antigen release kinetics from the formulation (results not shown).

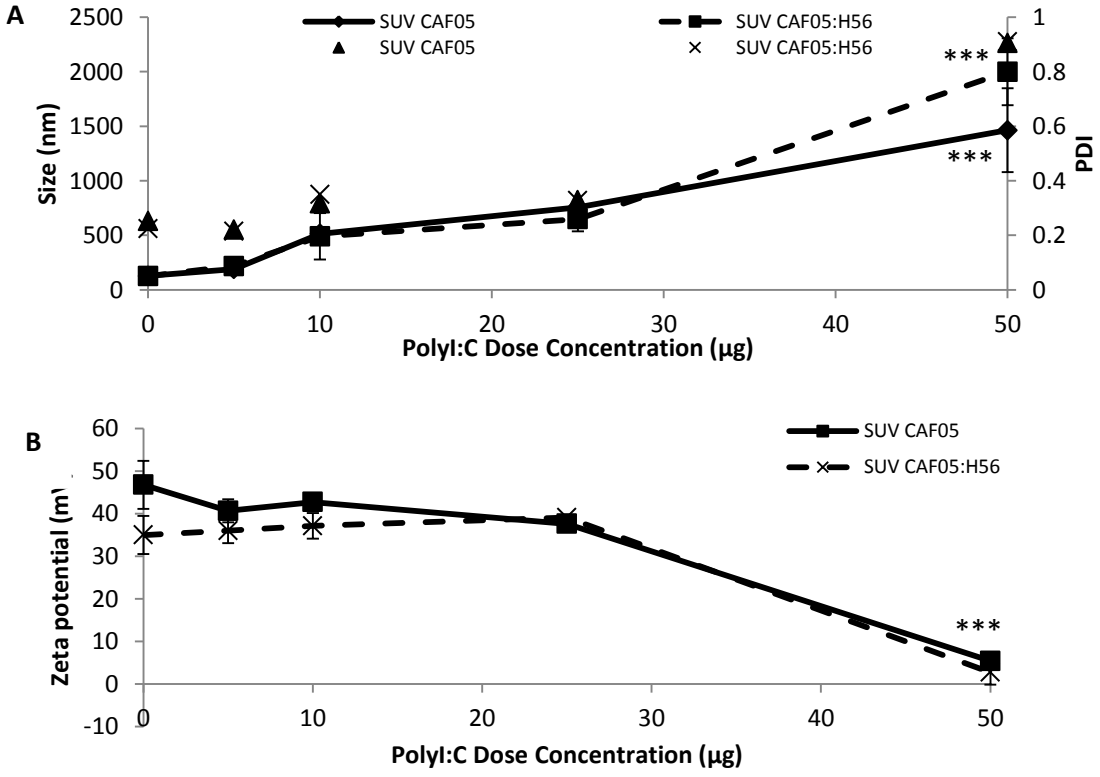


Figure 6-4. Vesicle size and polydispersity (A) and zeta potential (B) of liposome vaccine formulations prepared as small unilamellar vesicles (SUV) with addition of increasing concentrations of polyI:C (with or without H56 antigen) within the formulation. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$) as compared to the same formulation with no polyI:C (0 µg) included.

These studies have therefore reported the binding and association of the anionic dsRNA polyI:C TLR3 agonist with cationic DDA/TDB liposomes which caused a concentration dependent effect on vesicle characteristics when these systems were formulated as MLV and SUV. The association of higher concentrations of polyI:C within the system led to higher levels of polyI:C loading, which thus caused an increase in vesicle size and 'masking' of the cationic surface charge of the delivery system.

Similar interactions have also previously been reported for the delivery of DNA (Perrie and Gregoriadis, 2000, Moghaddam et al., 2011) as well as protein antigen to cationic liposome systems (Chapter 4). Upon addition of increasing concentrations of DNA or protein antigen to cationic liposomes this resulted in subsequent increases in vesicle size, thus causing vesicle aggregation and also reduction in zeta potential of the delivery system. These studies have also demonstrated that variation in preparation method in order to entrap polyI:C within the delivery system (by the DRV method) leads to lesser changes in vesicle characteristics, albeit with similar levels of loading of polyI:C.

6.3.1.3. The effect of liposome preparation method on polyI:C agonist loading

In order to further investigate the effect of TLR agonist upon vesicle characteristics it was required to be able to quantify whether indeed agonist was loading within the formulation, as has been shown that using varying methods of preparation and addition of agonist this has a significant effect upon vesicle characteristics (Sections 6.3.1.1 and 6.3.1.2). Hence, polyI:C was radiolabelled with ^{32}P before addition to liposome vaccine formulations. Subsequent centrifugation will allow quantification of agonist loading, as was determined by percentage recovery of ^{32}P-labelled polyI:C within the liposome pellet. Addition of the whole dose of polyI:C to cationic liposomal adjuvants (prepared as MLV, DRV and SUV) resulted in high levels of agonist loading in the region of 70-80 % (Figure 6.5). This polyI:C agonist loading led to resultant changes in MLV and SUV vesicle characteristics as has been described previously in Section 6.3.1, thus implicating charge-mediated electrostatic interaction between agonist and delivery system resulting in vesicle aggregation. However, for DRV-formulations, similarly high levels of polyI:C loading were noted, with lesser changes in vesicles characteristics measured (Figure 6.3) which suggested that TLR agonist may become entrapped within the delivery system as has been suggested in the previous literature for DNA delivery within DRV liposomes (Perrie and Gregoriadis, 2000).

The combination of nucleic acid-based TLR3 receptors and cationic liposomes constitutes an effective vaccine adjuvant approach for eliciting CD8+ T-cell responses. However, the relative

instability of these vesicles (with the addition of polyI:C at the *in vivo* dose) will limit their use in clinical applications (Nordly et al., 2011c) due to their rapid vesicle aggregation. Therefore even though polyI:C was efficiently loaded by these formulations, vesicle aggregation along with greater antigen release may limit the use of these vaccine adjuvants in further applications. Therefore the next set of studies will aim to formulate stable CD8-inducing adjuvants composed of high amounts of polyI:C associated with cationic DDA/TDB liposomes.

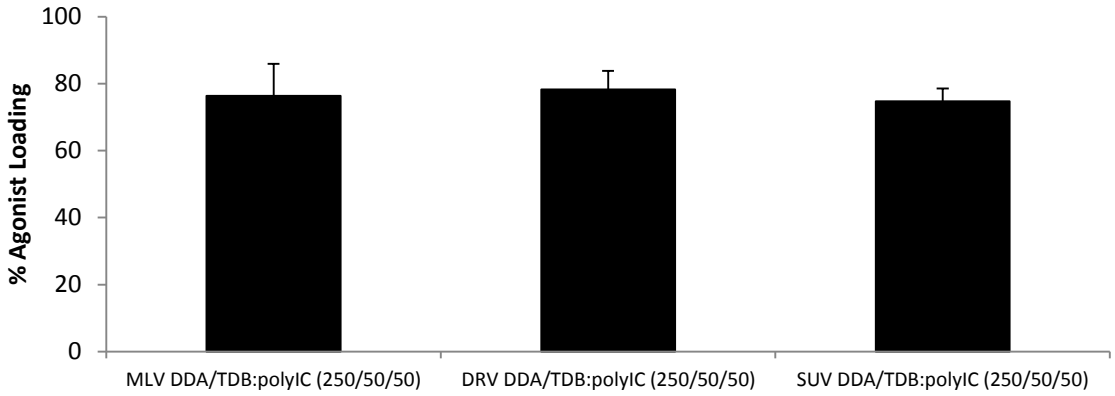


Figure 6-5. PolyI:C agonist loading to cationic liposome vaccine formulations, following whole dose addition. Results are the mean of triplicate experiments ± SD.

6.3.2. Optimisation of liposome adjuvants incorporating immunostimulatory compounds in order to control vesicle size

The next section of studies will aim to investigate the optimisation of liposomal adjuvants, including the TLR3 agonist polyI:C, in order to control and modulate the size of the delivery system.

6.3.2.1. Stepwise incorporation of TLR agonist within liposome formulations: effect of preparation method on vesicle characteristics and initial antigen loading

In order to control and modulate vesicle size, liposome vaccine formulations were initially prepared by the lipid-film hydration (LH) method, with the stepwise addition of polyI:C followed by vortexing every minute until the desired agonist concentration was reached. The aim of stepwise incorporation of polyI:C within the liposomal adjuvants was in order to prevent aggregation of the delivery system as described previously (Hansen et al., 2012). During this set of studies, DDA/TDB and DDA/MMG, without or with polyI:C (CAF01 and CAF05 & CAF04 and CAF09) were measured for their vesicle characteristics.

Monomycoloyl glycerol (MMG) is derived from the cell wall of mycobacteria. Through activation of antigen presenting cells (APCs), this non-TLR agonist has been shown to have an enhanced effect on the immune response (Andersen et al., 2009a). As described previously with TDB, MMG can also be incorporated into liposomes consisting of the cationic surfactant DDA. These liposomes were prepared at the same molar concentration as DDA/TDB (8:1 M/M) in order to serve as a direct comparison.

Upon stepwise addition of increasing concentrations of polyI:C within these cationic formulations, this resulted in a concentration dependent increase in vesicle size (Figure 6.6A) from around 400-500 nm to around 500-600 nm (following the addition of the *in vivo* dose of polyI:C), however these differences were not deemed significant as compared to whole dose addition of TLR agonist to MLV (Figure 6.2). Stepwise incorporation of polyI:C within the formulation also led to no significant changes in the zeta potential of the delivery system (Figure 6.6C) with the zeta potential being in the region of 60 mV and 40 mV (without and with antigen respectively) irrespective of the polyI:C concentration within the formulation. This was interesting as the same concentration of agonist was incorporated within the formulation with no such effects on vesicle characteristics. As expected and discussed previously, antigen loaded systems displayed a slight increase in vesicle size (from around 400 nm to around 500-550 nm) and reduction in zeta potential ($P < 0.05$) compared to their empty counterparts (Figure 6.6) which was due to electrostatic binding of anionic H56 antigen to the cationic surface of the delivery system as has been confirmed by antigen loading studies (Figure 6.6D). This has been described previously for the electrostatic interaction of anionic H1 TB antigen to the cationic surface of DDA/TDB liposomes (Henriksen-Lacey et al., 2010a).

These same liposome vaccine formulations were also prepared by the dehydration-rehydration vesicle method, with the addition of antigen and the stepwise addition of polyI:C to the SUV followed by vortexing every minute until the desired agonist concentration was reached. These SUV were then frozen at -70 ° C, before overnight freeze-drying at – 40 ° C. The freeze-dried lipid cake was then rehydrated with deionised water (above the transition temperature of the main lipid in the formulation) as described in Chapter 2 (Section 2.2.3).

Upon stepwise increase in the concentration of polyI:C within these cationic DRV formulations, this resulted in no significant effects on vesicle size, with vesicles measuring in the size range of 600 nm and 700 nm following addition of the *in vivo* dose of polyI:C (50 μg) within the formulation (Figure 6.7A) as compared to whole dose addition of TLR agonist (Figure 6.3A), which resulted in a dose-dependent increase in vesicle size. Stepwise incorporation of polyI:C within the formulation also resulted in no significant changes to the surface charge of the formulation (Figure 6.7C) as analysed

by the measured zeta potential which remained in the region of 60 mV for all formulations studied. These results suggested slow incorporation and entrapment of the TLR agonist within the formulation. This was interesting as the same concentration of agonist was incorporated within the formulation, as described previously (Section 6.3.1.2) with no such significant effects on vesicle characteristics. As expected and discussed previously, antigen loaded systems displayed an increase in vesicle size from around 450 nm to ~700 nm compared to their empty counterparts (Figure 6.7A). This was more significant at lower concentrations of polyI:C ($P < 0.05$) within the formulation (0, 5 and 10 µg polyI:C). Irrespective of the polyI:C concentration dose added within the formulation, anionic H56 antigen can be efficiently loaded with the delivery system, in the region of ~ 80 %, as was confirmed by antigen loading studies (Figure 6.7D).

These liposome vaccine formulations were also prepared as small unilamellar vesicles, with the stepwise addition of polyI:C followed by vortexing every minute until the desired agonist concentration was reached. Upon stepwise addition of increasing concentrations of polyI:C within these SUV cationic formulations this resulted in no significant changes in vesicle characteristics with vesicle size ranging in the region of 200 nm to 400 nm without and with antigen respectively (Figure 6.8), as compared to whole dose addition of TLR agonist to SUVs (Figure 6.4).

H56 antigen-loaded systems displayed a significant ($P < 0.05$) increase in vesicle size and reduction in zeta potential compared to their empty counterparts (Figure 6.8) which was due to electrostatic binding of anionic H56 antigen to the cationic surface of the delivery system as was confirmed by antigen loading studies (Figure 6.8D) and has been described in the previous literature for anionic H1 TB antigen (Henriksen-Lacey et al., 2010a). This was significant ($P < 0.01$; compared to LH- and DRV-prepared formulations) due to the fact that antigen interacted with a higher amount of the surface of these systems (due to their smaller initial vesicle size) so antigen loading led to increased vesicle size, however during this study this had no significant impact on the surface charge of the formulation.

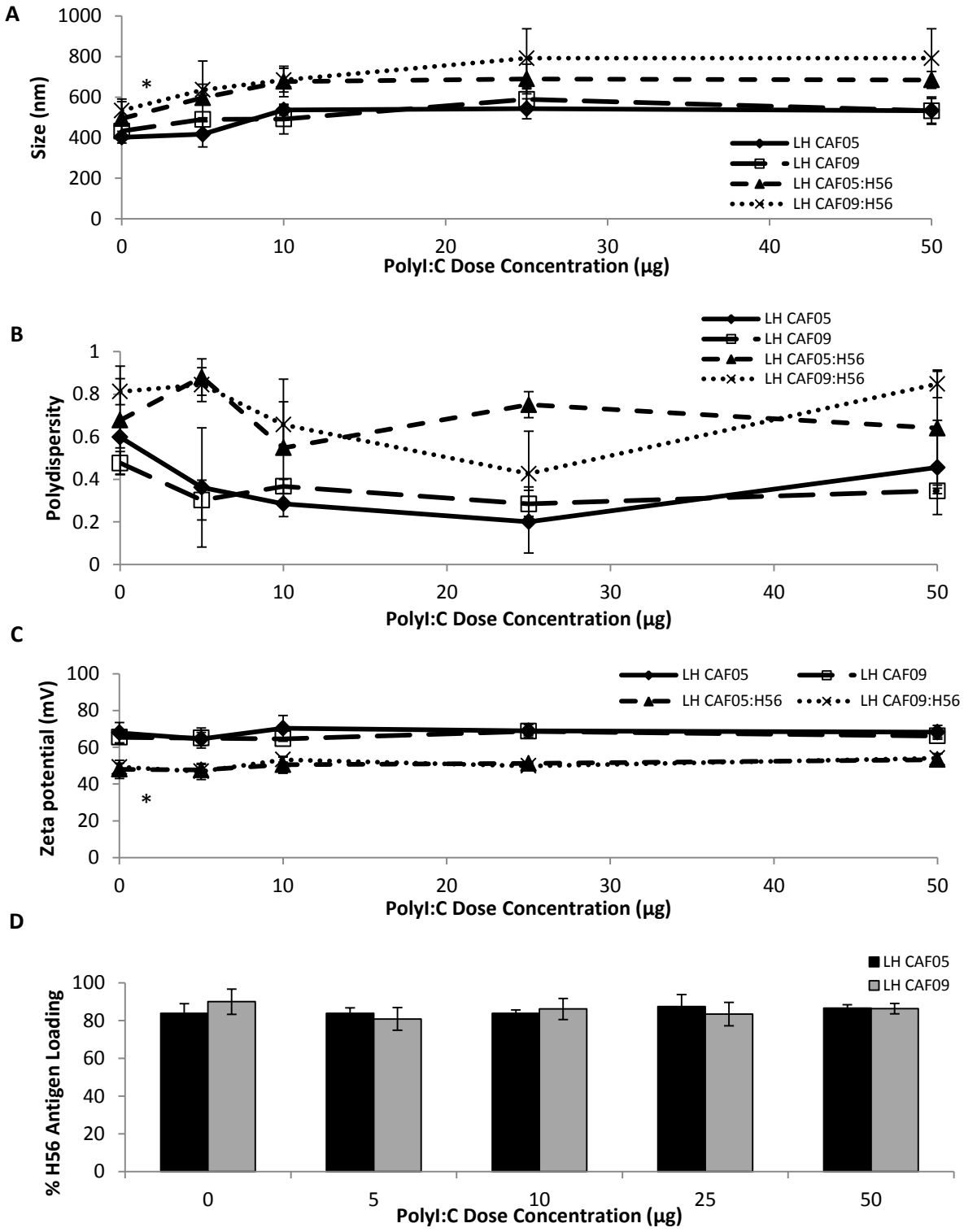


Figure 6-6. Vesicle size (A), polydispersity (B), zeta potential (C) and antigen loading (D) of liposome vaccine formulations prepared by the LH method with stepwise incorporation of polyI:C (with or without H56 antigen). Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (* $p<0.05$).

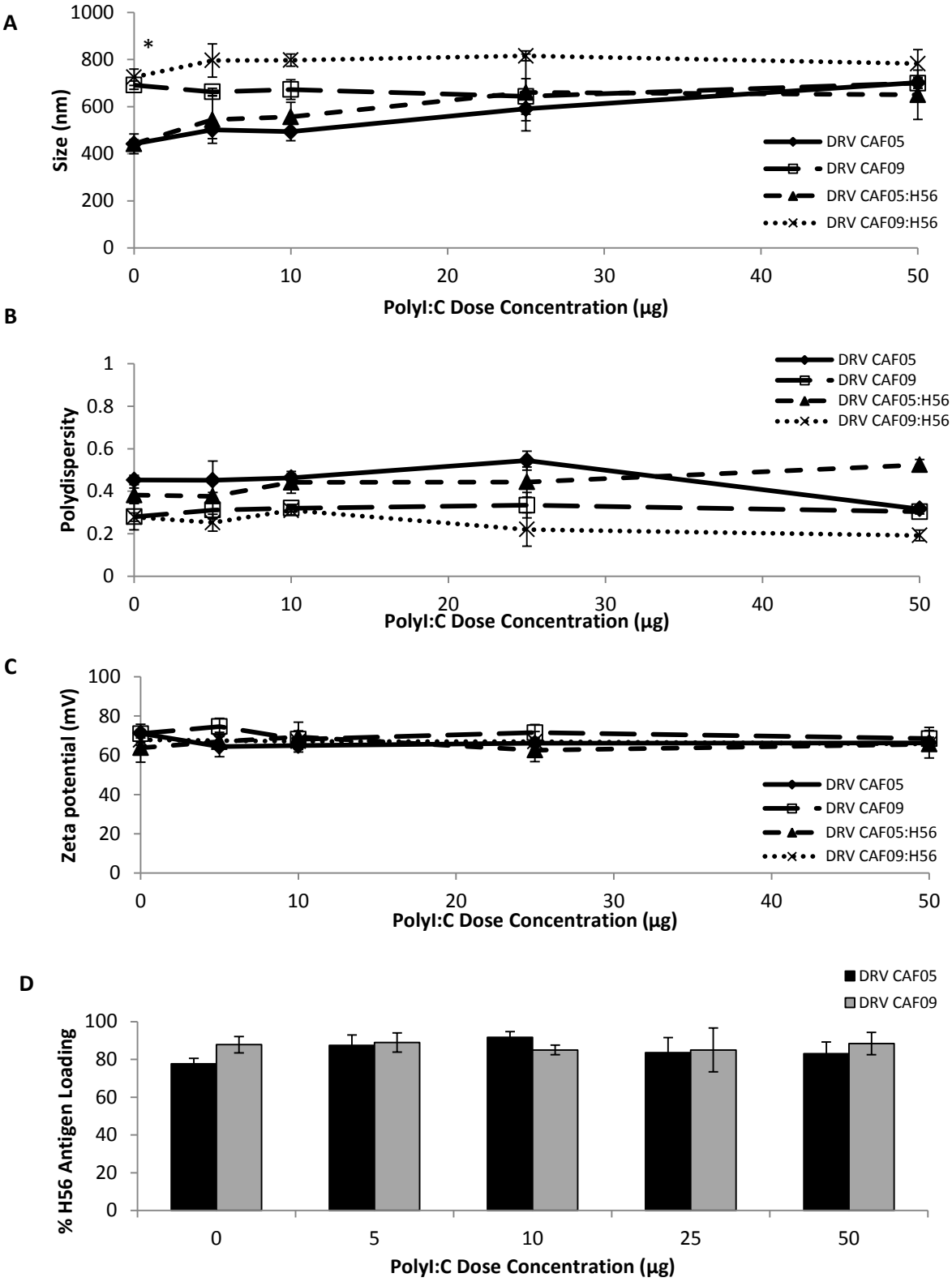


Figure 6-7. Vesicle size (A), polydispersity (B), zeta potential (C) and antigen loading (D) of liposome vaccine formulations prepared by the DRV method with stepwise incorporation of polyI:C (with or without H56 antigen). Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (* $p<0.05$).

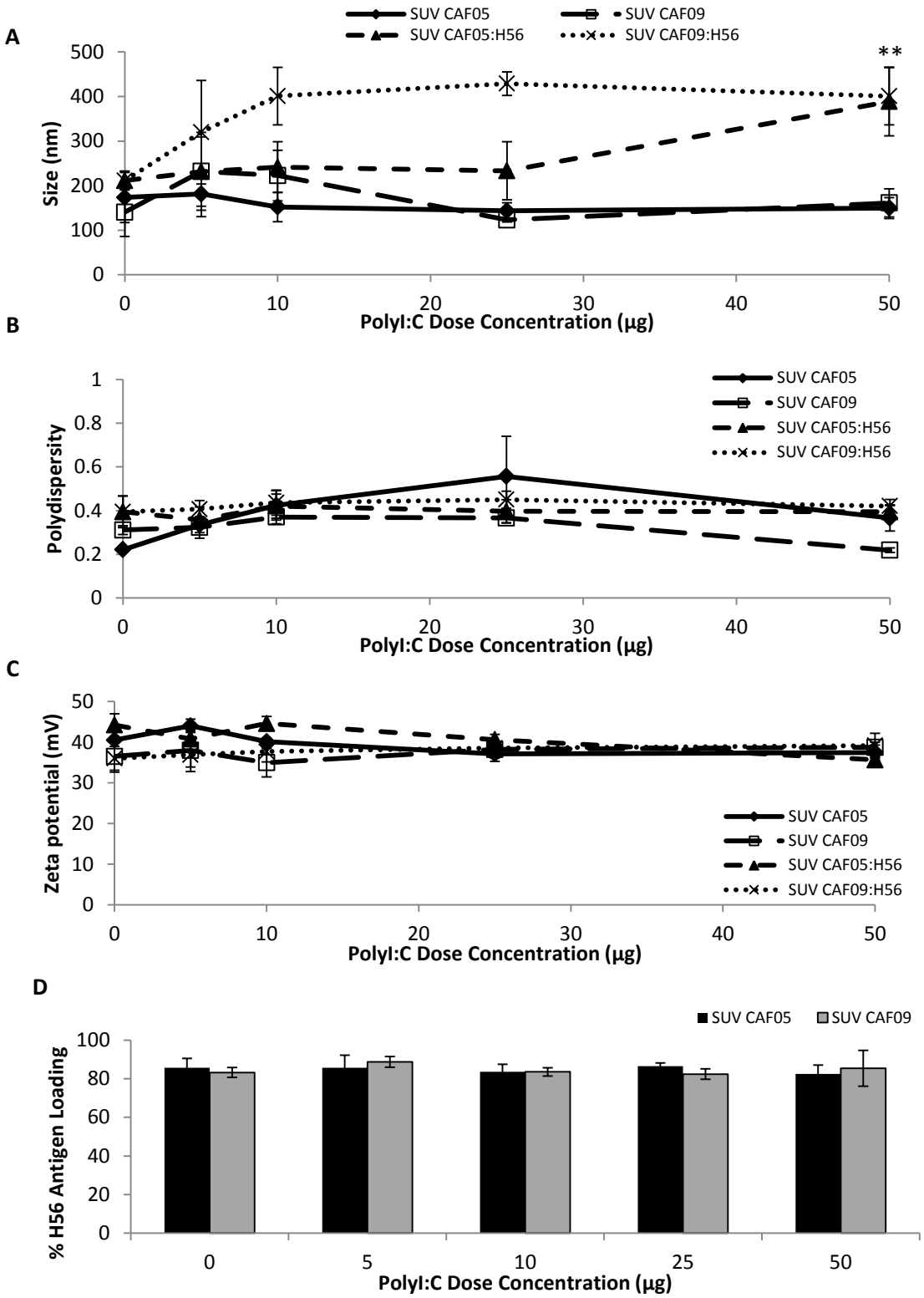


Figure 6-8. Vesicle size (A), polydispersity (B), zeta potential (C) and antigen loading (D)of liposome vaccine formulations prepared as SUV with stepwise incorporation of polyI:C (with or without H56 antigen). Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (** $p<0.01$).

6.3.2.2. The use of high shear mixing (HSM) homogenisation in order to control vesicle characteristics of polyI:C containing liposomal adjuvants

During initial studies at Statens Serum Institute, DDA/TDB (CAF01) liposomes were prepared by either the lipid-film hydration (LH) or high shear mixing (HSM) homogenisation methods, which gave rise to vesicles with sizes of 400 nm and 280 nm respectively (Figure 6.9A). Addition of the TLR3 agonist polyI:C led to the formulation of CAF05 (DDA/TDB/PolyI:C) liposomes. Upon stepwise addition of polyI:C (at the *in vivo* dose; 50 μg) to pre-formed CAF01, this resulted in a minimal increase in vesicle size to 440 nm and 310 nm (for LH method and HSM methods respectively). This small increase in vesicle size was partly ascribed to the step-wise addition of polyI:C (Hansen et al., 2012), rather than addition of the whole dose which resulted in significant changes in vesicle characteristics and neutralisation of the surface charge (as measured by zeta potential) especially at the *in vivo* dose of 50 μg (Section 6.3.1.1).

CAF01 and CAF05 prepared by the high shear mixing (HSM) method were of a reduced vesicle size compared to their LH-prepared formulation counterparts ($P < 0.05$). This was due to the introduction of energy and larger disruptive forces (compared to vortexing) to the liposome system which led to smaller vesicles being produced. Therefore these results demonstrated that the high shear mixing method was suitable for reducing the particle size of cationic liposome formulations, CAF01 and CAF05. This will be further tested with CAF04 and CAF09 formulations, which will be described in more detail below.

In terms of the vesicle surface charge of CAF01 (as was indicated by zeta potential), these formulations had measurements of 75 mV and 58 mV when formulated by the LH and HSM methods, respectively (Figure 6.9B). Upon addition of polyI:C to pre-formed CAF01 this led to formulation of CAF05 liposomes. This led to no significant reduction in the measured zeta potential of these liposomes (Figure 6.9B). This was in contrast to when the whole *in vivo* dose of polyI:C was added to the formulation post lipid-film hydration (LH) in section 6.3.1.1, which resulted in subsequent neutralisation of the surface charge. This showed that stepwise addition of polyI:C resulted in formulations, with or without HSM, which maintained their vesicle size as well as their cationic nature (as indicated by zeta potential).

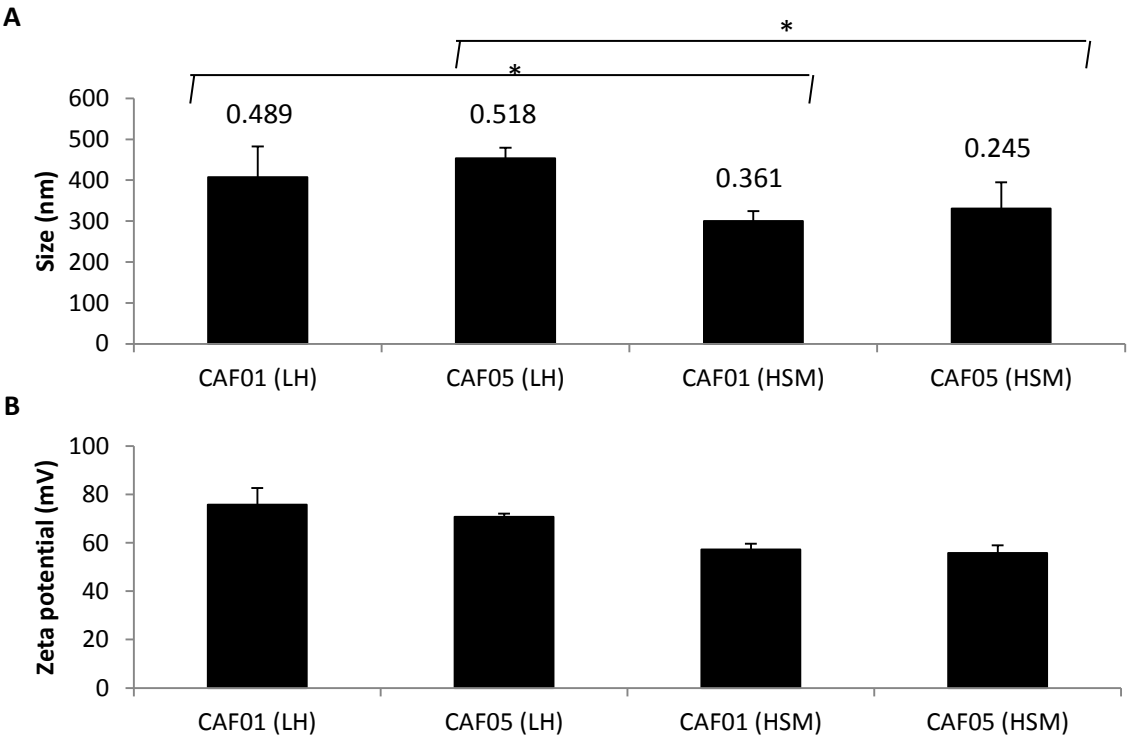


Figure 6-9. Vesicle size and polydispersity (A) and zeta potential (B) of CAF01 and CAF05 formulations prepared by either the lipid-film hydration (LH) or high shear mixing (HSM) method. PDI values are placed above their respective column bars. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (* $p<0.05$).

DDA/MMG (CAF04) liposomes were formulated by either the LH method or by high shear mixing (HSM). CAF04 gave rise to vesicles with a size of 550 nm (LH) and 320 nm (HSM) respectively. Upon addition of the TLR3 agonist polyI:C, this resulted in the production of CAF09 liposomes. These CAF09 liposome formulations displayed vesicle sizes of 560 nm and 370 nm for LH- and HSM methods respectively (Figure 6.10A). The addition of polyI:C within the formulation led to no significant changes to vesicle characteristics, including the vesicle size (Figure 6.10A). As described previously, formulations which were produced by HSM gave rise to vesicles with a significant reduction in size ($P < 0.01$), which was due to the introduction of larger disruptive forces to the liposome system. Therefore these results demonstrated that the HSM method was suitable for reducing the particle size of CAF04 and CAF09 formulations. The vesicle size distribution of CAF04 liposomes has been confirmed via cryo-TEM microscopy as displayed in Figure 6.11.

In terms of the zeta potential of DDA/MMG (CAF04), these formulations showed initial measurements of 64 mV and 61 mV, when formulated by the LH- and HSM methods respectively. For CAF09 liposomes, these had a zeta potential of 61 mV and 56 mV when prepared by either the LH-method or HSM respectively (Figure 6.10B). These results demonstrated that high shear mixing

led to only a slight decrease in the measured zeta potential of these formulations, however these differences were not significant. This showed that controlled, stepwise addition of polyI:C (with or without high shear mixing) resulted in formulations which maintain their vesicle size as well as their cationic nature (as indicated by zeta potential).

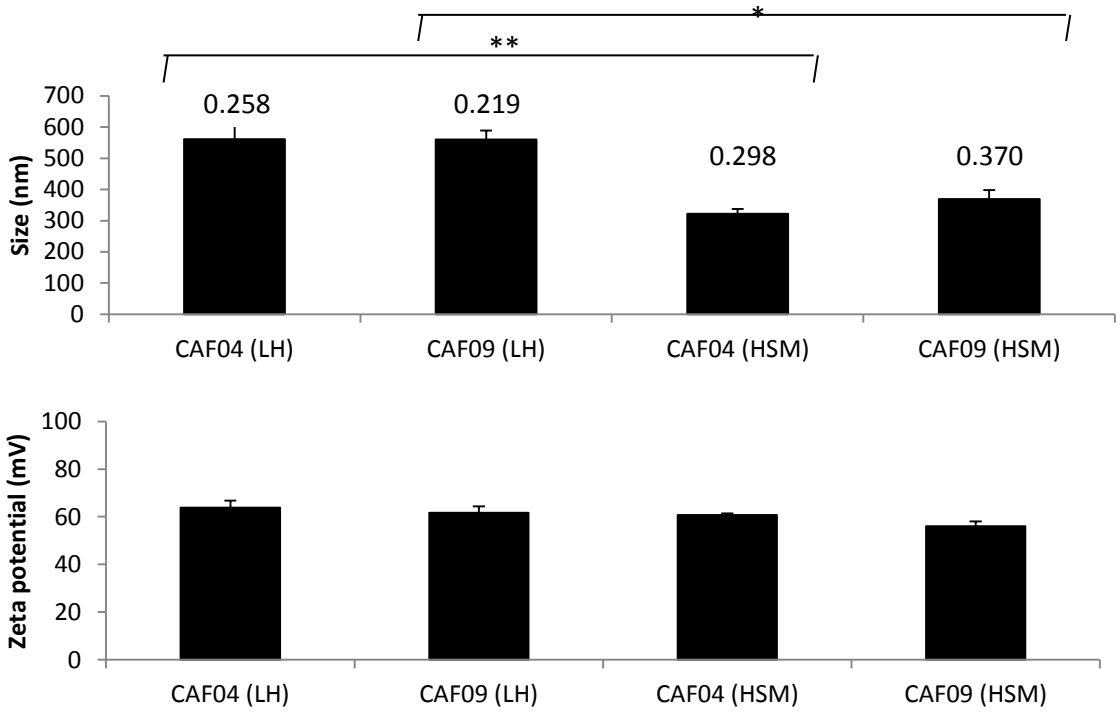


Figure 6-10. Vesicle size and polydispersity (A) and zeta potential (B) of CAF04 and CAF09 formulations prepared by either the lipid-film hydration (LH) or high shear mixing (HSM) method. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (** $p<0.01$; * $p<0.05$).

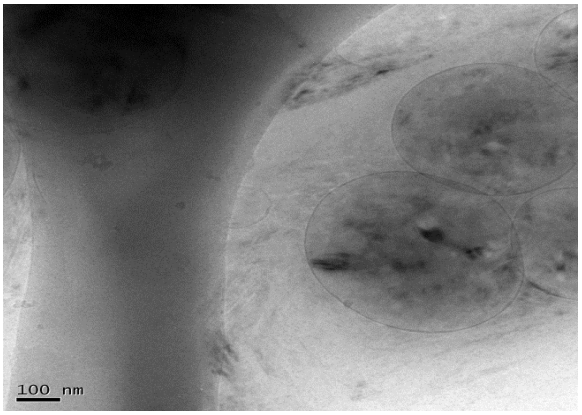


Figure 6-11. Cryo-TEM micrograph of DDA/MMG liposomes (or CAF04) formulated by the lipid-film hydration (LH) method. The long dark structures are the carbon grid onto which the sample was deposited before analysis.

These initial studies showed high promise with the inclusion of high amounts of polyI:C within the formulation which led to no significant effects on vesicle characteristics therefore will be further investigated in Section 6.3.2.3.

6.3.2.3. Liposome formulation by HSM: effect on vesicle characteristics and antigen loading

Through further optimisation of this method, upon stepwise addition of increasing concentrations of polyI:C (at varying dose concentrations between 0 and 50 μg) within these HSM-prepared cationic formulations this resulted in no significant measured differences in vesicle size and polydispersity (Figures 6.13 A and B) as compared to whole dose addition of TLR agonist which resulted in vesicle aggregation (as described previously in Section 6.3.1). Empty vesicles were measured in the region of 200 nm, as confirmed by cryo-TEM, (Figure 6.12) with vesicle size increasing to ~ 250 nm upon stepwise addition of the complete dose of polyI:C. Stepwise incorporation of polyI:C within the formulation also led to no significant changes in the zeta potential of the delivery system with measured values between 55-60 mV (Figure 6.13C). Upon high shear mixing (HSM), there was a reduction in vesicle size as compared to multilamellar vesicles formulated by the lipid-film hydration method. However, TLR agonist was still able to be retained and loaded by the cationic delivery system, as reflected by no such changes in vesicle characteristics. As expected and discussed previously, antigen loaded systems displayed slight increases ($P < 0.05$) in vesicle size (from around 200 nm to 300 nm) compared to their empty counterparts (Figure 6.13A) which was due to strong electrostatic binding of anionic H56 antigen to the cationic surface of the delivery system as was confirmed by antigen loading studies (Figure 6.13D).

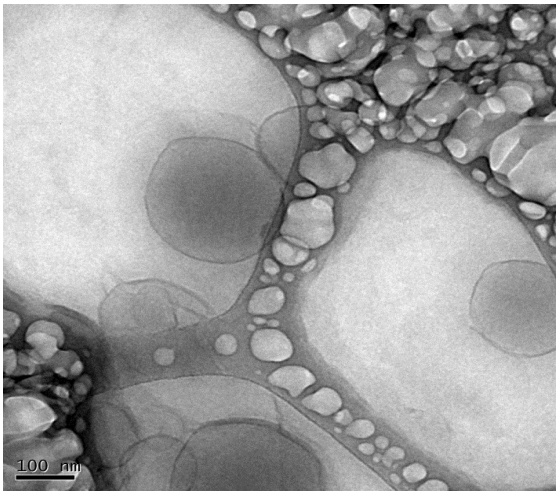


Figure 6-12. Cryo-TEM micrograph of DDA/TDB liposomes, formulated by the high shear mixing homogenisation (HSM) method. The long structures are the carbon grid the sample was loaded onto before analysis.

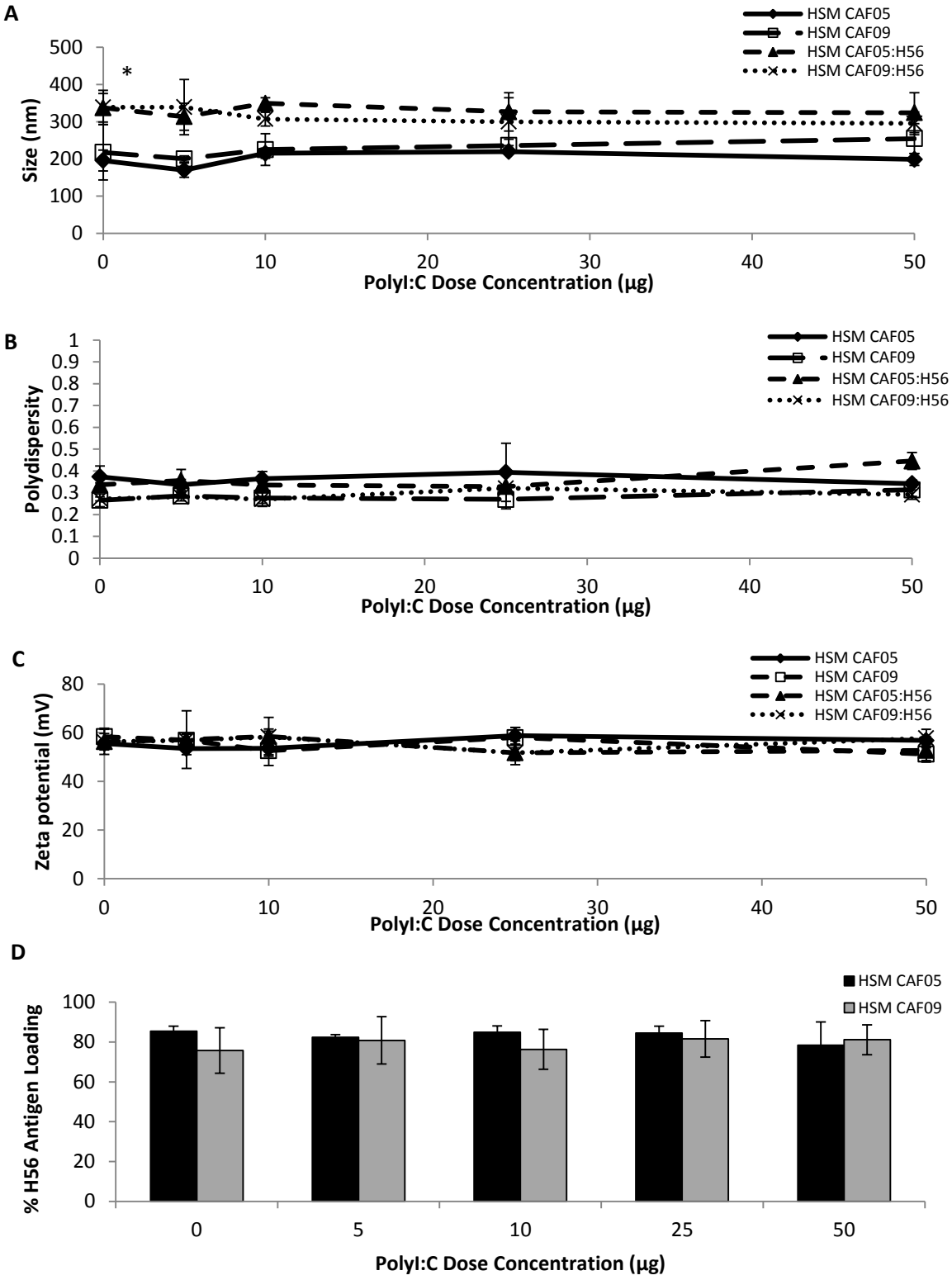


Figure 6-13. Vesicle size (A), polydispersity (B), zeta potential (C) and antigen loading (D) of liposome vaccine formulations prepared by the high shear mixing homogenisation method with stepwise incorporation of polyI:C (with or without H56 antigen). Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (* $p<0.05$).

6.3.2.4. Investigating the effect of stepwise incorporation of polyI:C on agonist loading within the formulation

Following the investigation into the stepwise incorporation of polyI:C on the measured vesicle characteristics (Sections 6.3.2.2 and 6.3.2.3) it was required to study whether the polyI:C agonist was in fact associated with the liposome formulation. These results were then to be compared to previous data in which whole dose addition of polyI:C was added within the formulation (Section 6.3.1.3). These results have suggested that stepwise incorporation of the *in vivo* dose of polyI:C (50 µg), followed by vortexing or high shear mixing, led to high levels of agonist loading in the region of 70-80 % (Figures 6.14A and B); however, this loading led to no observed changes in vesicle characteristics as was described previously (Section 6.3.2). The maintenance of reduced vesicle size, in combination with association of the TLR3 agonist polyI:C, has been suggested previously to lead to the enhanced immunogenicity of liposomal vaccines (Nordly et al., 2011c), which was due to the induction of CD8+ T-cell responses and the ability to cross-present antigen (Zaks et al., 2006, Milicic et al., 2012). The formulations summarised in Sections 6.3.2 thus showed high promise for the use as vaccine adjuvants. These formulations were able to efficiently load high levels of TLR3 agonist (Figure 6.14), as well as highly load vaccine antigen within the formulation causing no significant changes in vesicle characteristics. Therefore, these formulations have the ability to act as CD8-inducing adjuvants containing high amounts of the TLR agonist polyI:C.

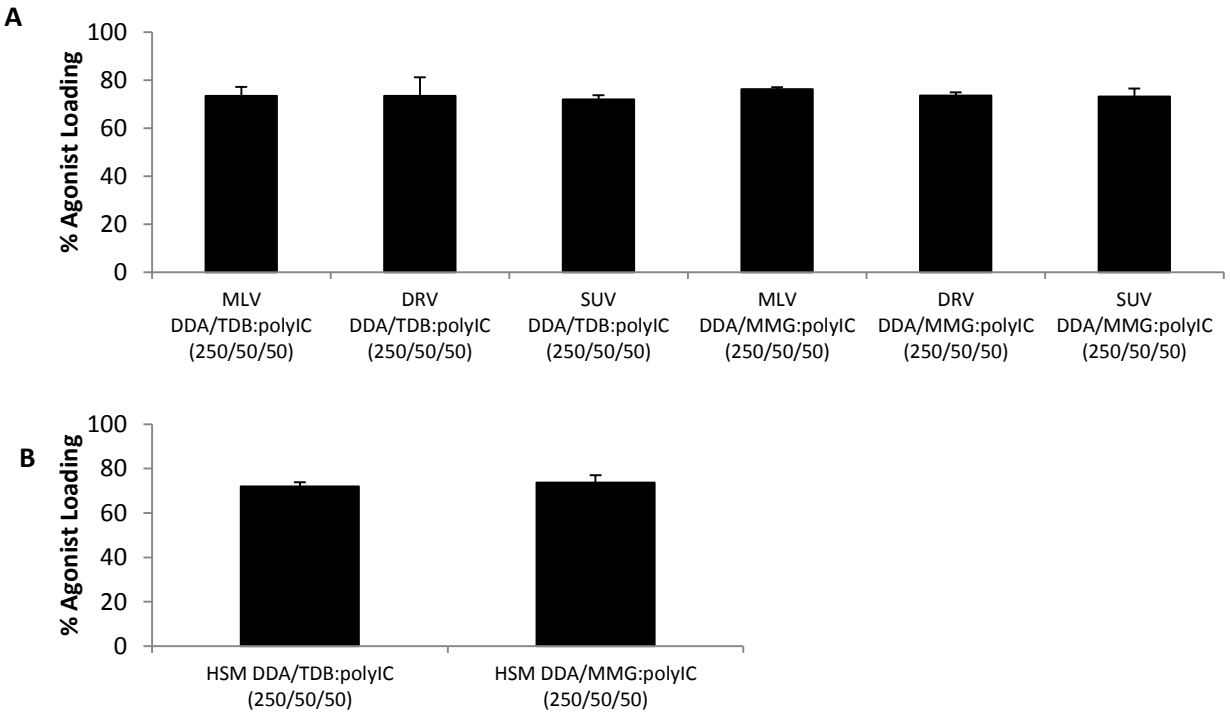


Figure 6-14. PolyI:C agonist loading to cationic liposome vaccine formulations, following (A) stepwise addition of agonist and (B) high shear mixing. Results are the mean of triplicate experiments ± SD.

6.3.3. Long term trials to investigate the stability of liposome systems

The long term stability of liposome formulations has been shown to be critically important in the successful development of these vaccines. During this investigation, liposomes were formulated by two separate methods and kept at 4 °C, 25 °C or 40 °C in order to show the effect of storage temperature on the vesicle characteristics of tested liposome adjuvants.

Cationic liposomal vaccine formulations were prepared by the lipid-film hydration method with the addition of the *in vivo* dose of H56 antigen, and with or without the *in vivo* dose of the TLR3 agonist polyI:C. These formulations were stored at 4 °C, 25 °C and 40 °C over a 6-month period and were assessed for their vesicle characteristics at a number of time points. These vesicles, without polyI:C, had an initial size, zeta potential and polydispersity of 650 nm, 65 mV and 0.41 respectively. Whereas inclusion of polyI:C within the formulation gave vesicle characteristics of 670 nm, 64 mV and 0.42 respectively. Therefore stepwise inclusion of polyI:C within the formulation led to no significant changes in initial vesicle characteristics (Figure 6.15).

Over the initial 28 days of this stability study, there were no significant changes measured in vesicle characteristics at all storage temperatures tested both for vesicle size and liposome surface charge (as measured by zeta potential). However storage at 40 ˚C had more impact on the vesicle size ($P < 0.01$) characteristics for both formulations tested, with and without the inclusion of polyI:C, with vesicles increasing in size to ~ 1.2 μm (Figure 6.15A). Therefore the storage of liposomes at higher temperatures, can lead to vesicle aggregation, which was not a desired property for liposomal vaccines as this may affect their properties and subsequent ability to generate immune responses.

Storage of liposomal formulations at 4 ˚C and 25 ˚C had no significant effect on vesicle characteristics up until the month 6 time point, with no significant differences in vesicle size and zeta potential measurements being measured. For zeta potential, up until the month 6 time point, the surface charge remained strongly cationic with storage temperature having no significant effect on the physicochemical parameter of liposomes. In terms of visual stability over the initial time points of this study, all samples remained homogenous which showed that vesicles had not significantly aggregated (Figure 6.16) during this study.

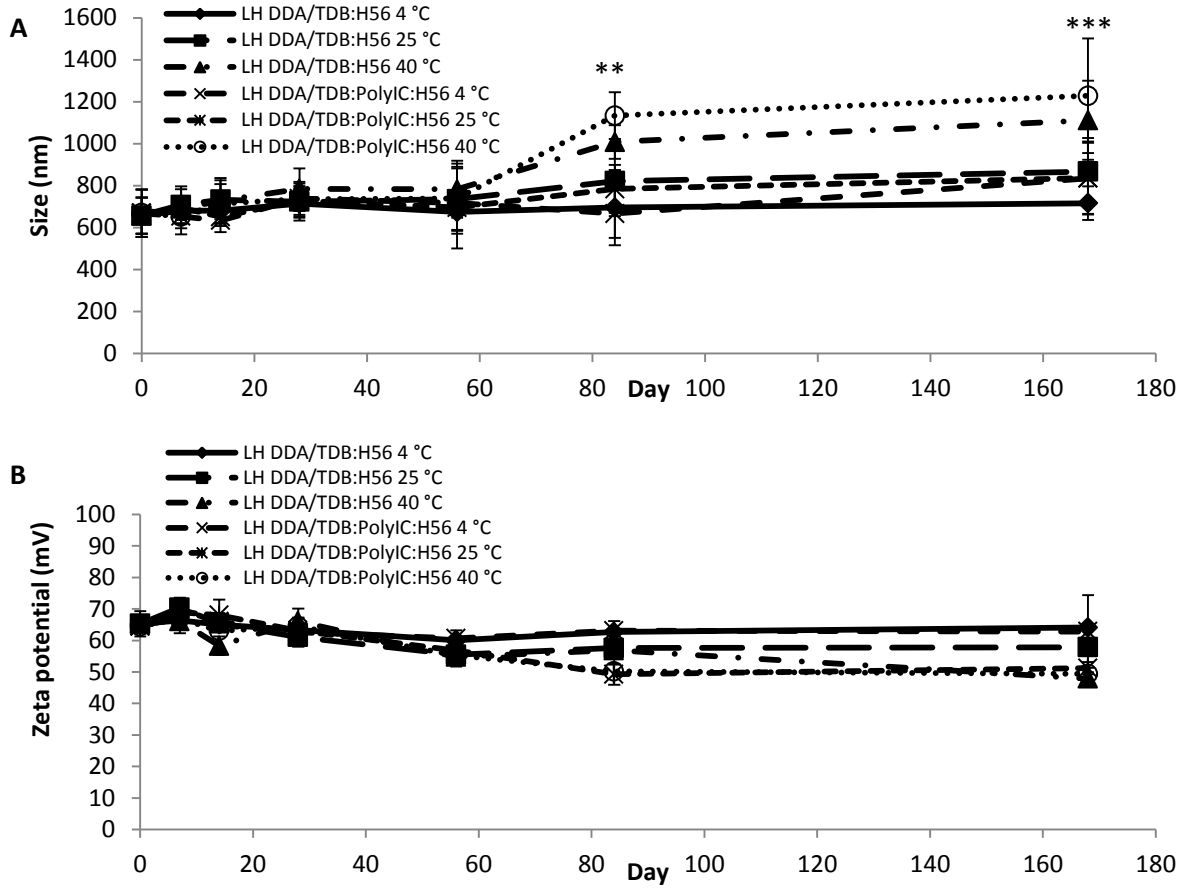


Figure 6-15. Vesicle size (A) and zeta potential (B) analysis of cationic liposomal vaccine formulations prepared by the lipid-film hydration method, with the addition of the in vivo dose of H56 antigen and also with and without stepwise addition of polyI:C at the *in vivo* dose. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$) as compared to initial characteristics at day 0.

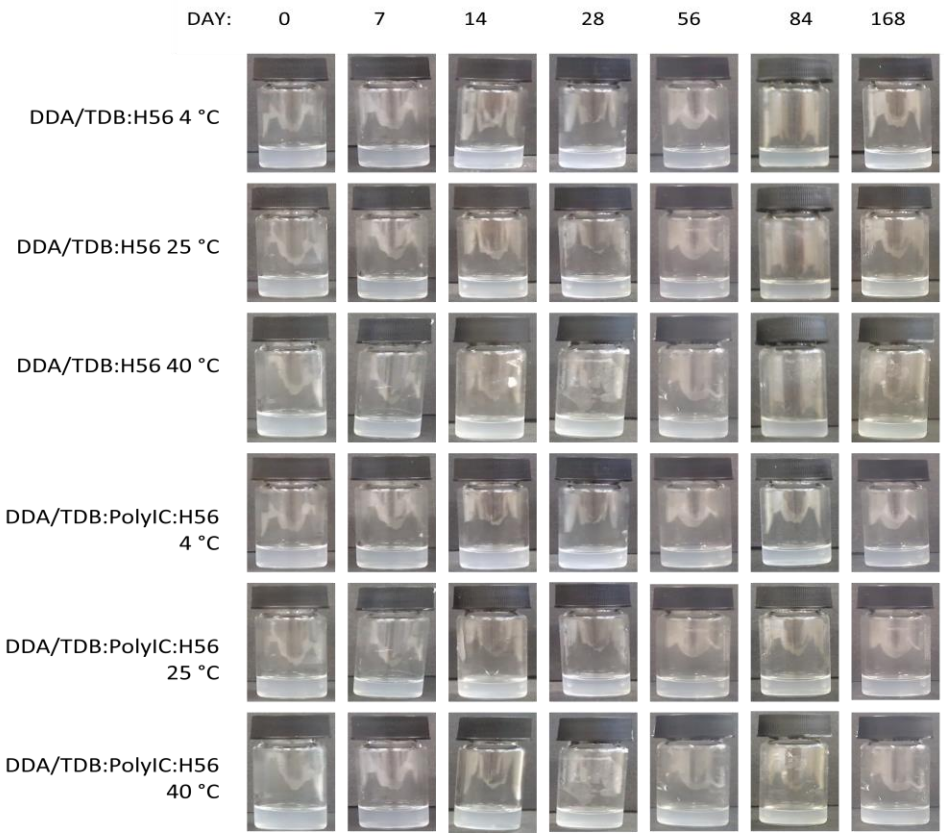


Figure 6-16. Visual stability of DDA/TDB liposome samples formulated by the lipid-film hydration (LH) method with the addition of the in vivo dose of H56 antigen, and also with and without the stepwise addition of the *in vivo* dose of poly(I:C). These liposomes were made in Tris buffer (10 mM; pH 7.4), and were stored at 4 ˚C, 25 ˚C and 40 ˚C.

Cationic DDA/TDB were also prepared by the high shear mixing (HSM) homogenisation method with the addition of the *in vivo* dose of H56 antigen, and with or without the in vivo dose of the TLR3 agonist poly(I:C). These formulations were stored at 4 °C, 25 °C and 40 °C over a 6-month period and were assessed for their vesicle characteristics at a number of time points.

At the initial time point, these formulations displayed an vesicle size of 324 ± 60 nm and 317 ± 78 nm (Figure 6.17A) for DDA/TDB:H56 and DDA/TDB/polyIC:H56 formulations respectively. In terms of the zeta potential, these formulations displayed initial surface charges of 54.6 ± 6.4 and 55.7 ± 3.5 mV, respectively (Figure 6.17B). These vesicle characteristics were in line with previously characterised HSM formulations prepared during this chapter (Section 6.3.2.3). Upon storage of these formulations at 4 °C, 25 °C and 40 °C, this led to measured differences in the characteristics of these formulations (Figure 6.17).

Up until the month 2 time point, liposome formulations displayed similar size (400-500 nm) and surface charge characteristics (50 to 60 mV) irrespective of the storage temperature. However, by

month 3, liposome formulations stored at 40 °C tended to aggregate with vesicle size significantly increasing ($P < 0.001$) to between 1 and 1.2 µm (without and with polyI:C respectively), with zeta potential showing no further significant changes (Figure 6.17). These stability trials are currently ongoing up until the month 6 time point.

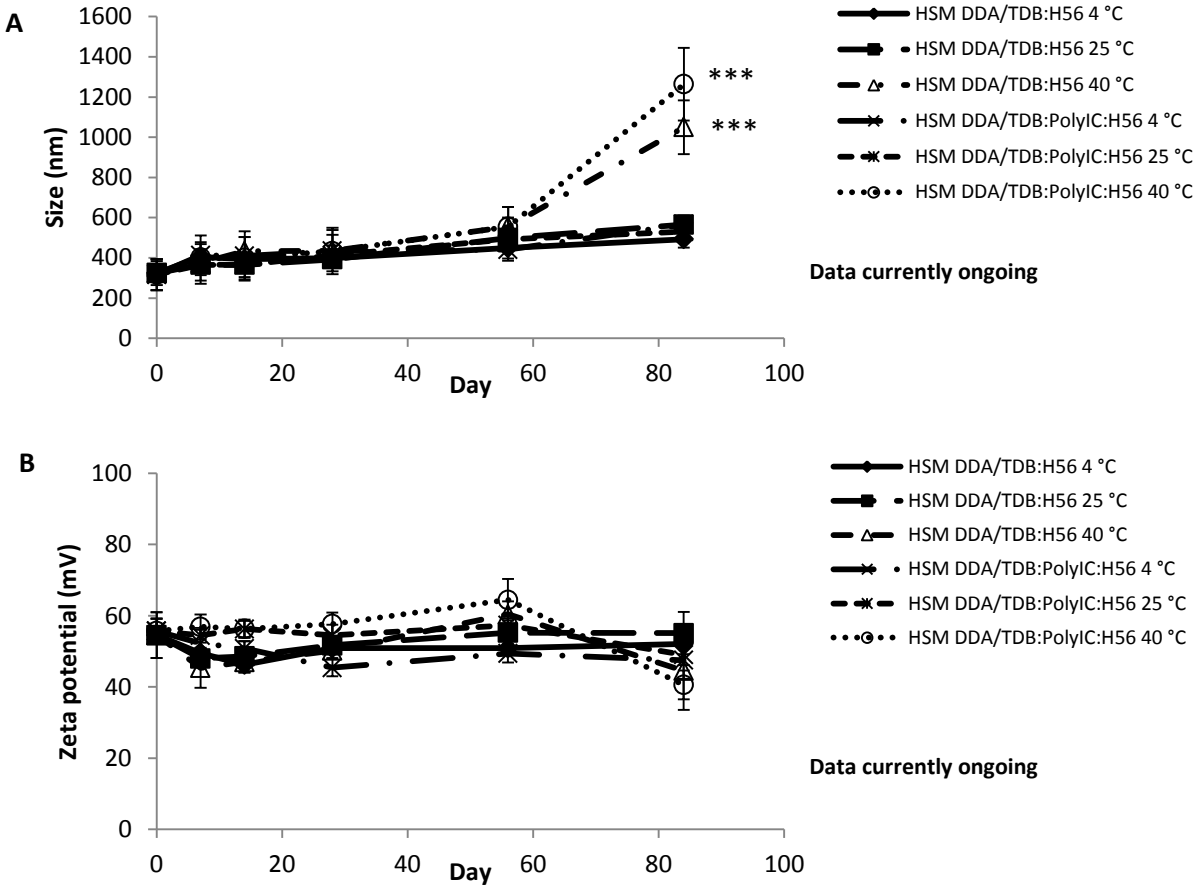


Figure 6-17. Vesicle size (A) and zeta potential (B) analysis of cationic liposomal vaccine formulations prepared by the high shear mixing (HSM) homogenisation method, with the addition of the *in vivo* dose of H56 antigen and also with and without stepwise addition of polyI:C at the *in vivo* dose. Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$) as compared to initial characteristics at day 0.

6.3.3.1. Investigating the effect of long term stability on antigen integrity within liposome vaccine formulations

In order to investigate the effect of long term stability on the integrity of antigen within the liposome formulation it was required to run SDS-PAGE (Figure 6.18). These results showed that antigen integrity was mainly dependent on the formulation storage temperature as the same concentration of antigen was present within each formulation. H56 antigen (within these liposome vaccine formulations) remained stable up until the 6 month, 3 month or 2 month time points

following formulation storage at 4 °C, 25 °C or 40 °C, respectively, as shown by SDS-PAGE (Figure 6.18) irrespective of whether polyI:C was present within the formulation or not. Inclusion of the polyI:C had no further effect on antigen integrity within the formulation (Figure 6.18). The high stability of these vaccines, with or without the inclusion of high amounts of polyI:C suggested that these formulations may have increased potential for use as vaccine adjuvants. The presence of polyI:C within the formulation results in the ability to 'cross-present' antigen through interaction with MHC I complexes therefore leading to the activation of CD8+ T-cells (Chikh and Schutze-Redelmeier, 2002, O'Hagan and Valiante, 2003, Zaks et al., 2006) which leads to enhanced T-cell responses.

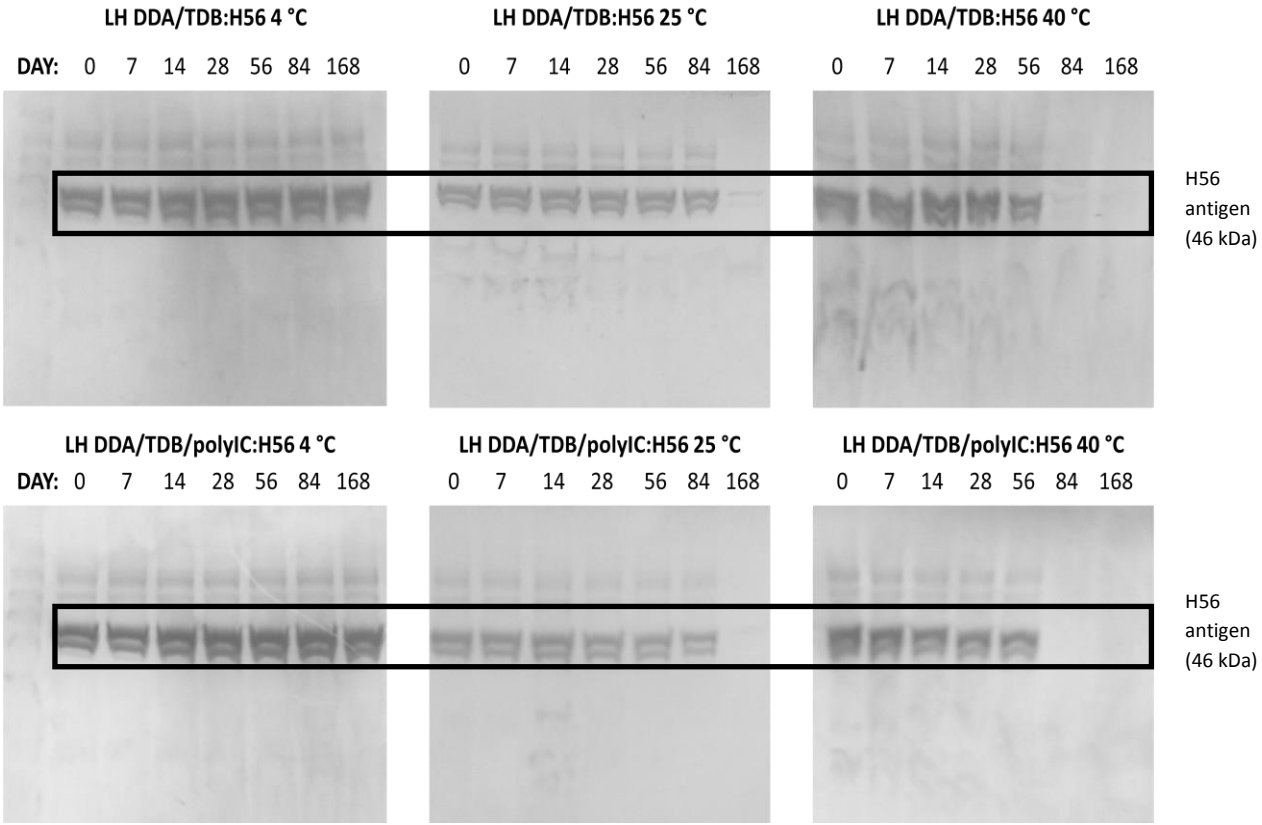


Figure 6-18. SDS-PAGE to show the effect of liposome preparation method and storage temperature upon H56 antigen integrity for LH-prepared liposome vaccine formulations.

6.3.4. Generation of an immune response from cationic liposomes with complexed-TLR agonists

The immunostimulatory ability of cationic liposomes complexed with TLR3 agonists has previously been investigated in detail therefore this was not further explored during this chapter. The association of these agonists within cationic liposomes was able to promote the entry of antigens

into the MHC class I pathway in order to be presented to $CD8^+$ T-cells (O'Hagan and Valiante, 2003). Further it has been shown that cationic liposomes can facilitate the interaction of TLR3 and TLR9 agonists with their endosomal receptors and therefore promote immune activation (Gursel et al., 2001, Suzuki et al., 2004, Zaks et al., 2006, Nordly et al., 2011c). Further studies within our laboratory have demonstrated the ability of cationic DDA/TDB liposomes (formulated as MLV, DRV and SUV) in combination with polyI:C and/or CpG DNA with the aim of eliciting CD8 responses (Milicic et al., 2012) as has been discussed by Christensen and colleagues (Christensen et al., 2011). These complexed TLR agonists were able to enhance immune responses with MLV and DRV cationic DDA/TDB liposomes, however SUV liposomes were able to induce stronger immune responses without the need for addition of these TLR agonists (Milicic et al., 2012).

6.4. Conclusions

The flexible and versatile nature of liposomes allowed the incorporation of immunostimulatory lipids and TLR-agonists with or within the delivery system, which has the potential to further enhance the generated immune response.

Controlling the size of liposomal adjuvants can be achieved using high shear mixing (HSM) in which the immunostimulatory TLR3 agonist, polyI:C, can be incorporated with cationic liposome delivery systems (DDA/TDB and DDA/MMG) with no further significant changes in vesicle characteristics being measured. Immunostimulatory TLR agonists can also be added stepwise (at higher doses) to vesicles prepared by the lipid-hydration method, without vesicle characteristics being significantly altered. This was important as reduction in vesicle size in combination with TLR agonist association within the formulation is important in vaccine design in order to further stimulate CD8+ T-cell responses (Nordly et al., 2011c).

This was in contrast to whole dose addition of these TLR agonists to pre-formed vesicles (MLV and SUV), which at the higher doses (*in vivo* dose; 50 µg polyI:C) resulted in vesicle aggregation as was noted through increases in vesicle size and neutralisation of the surface charge. Through variation in liposome preparation method, the localisation of TLR agonist in regards to the delivery system can be modified, similar to that of antigen (as discussed in Chapter 5). However through preparation of liposomes by the DRV method, this was suggested to lead to the entrapment of TLR agonist with the delivery system as shown by lesser effects on vesicle characteristics.

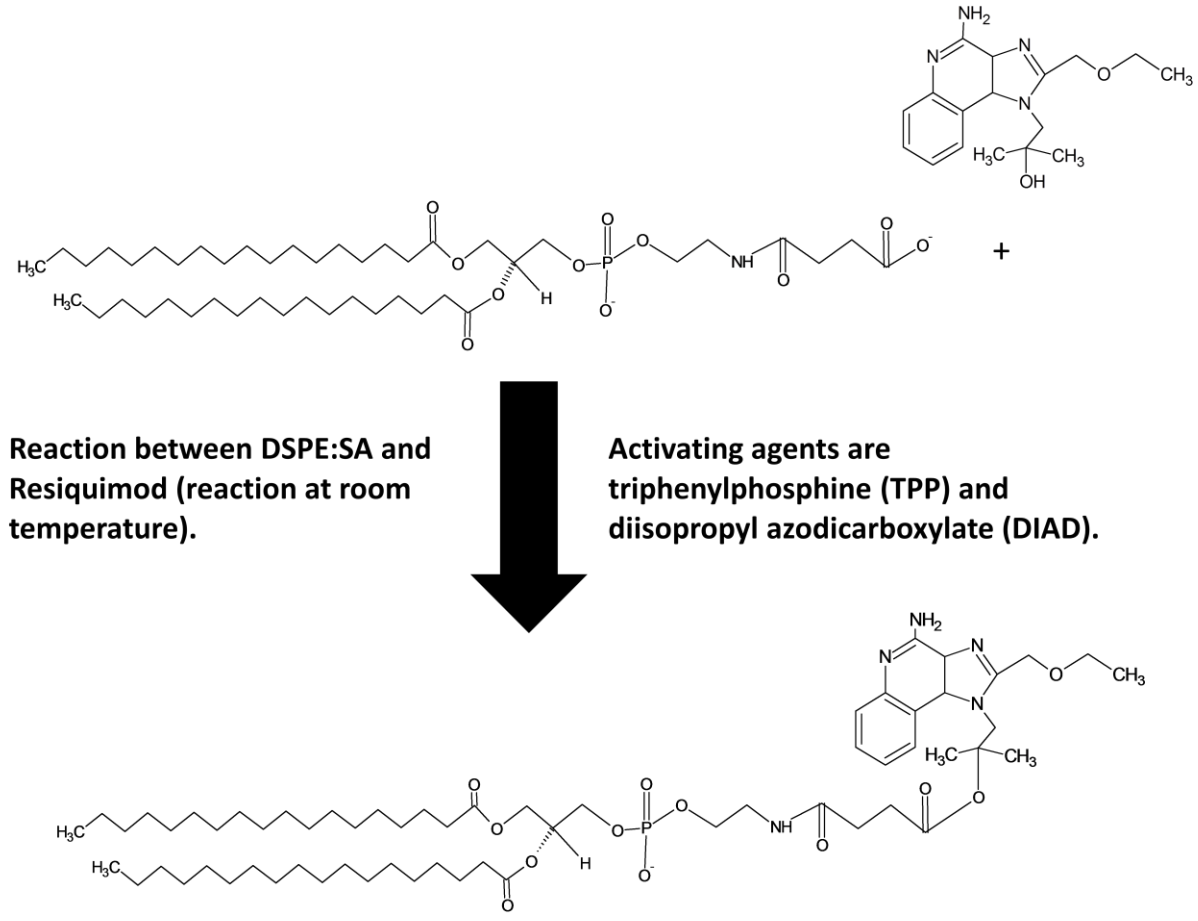
Antigen loading studies, using the anionic H56 subunit vaccine antigen, have been carried out which have demonstrated high loading due to the general trend of retention of the cationic surface charge of the liposome delivery system (especially after stepwise TLR agonist addition and HSM). A slight reduction in antigen loading was observed following whole dose addition of TLR agonist within the liposome formulation, which was attributed to measured changes in the physicochemical characteristics due to slight vesicle aggregation through interaction with agonist at the liposome surface.

A number of formulations were entered into long term stability trials over a 6 month time period in which cationic DDA/TDB liposomes were formulated, through either the lipid hydration (LH) or high shear mixing (HSM) method, in combination with the *in vivo* dose of H56 TB vaccine antigen and with or without polyI:C. These studies have demonstrated that storage of liposome-based vaccines at 4 °C and 25 °C resulted in lesser changes compared to storage in accelerated conditions (40 °C), for LH-formulated vaccines. Initial studies for HSM liposomal vaccine formulations have shown no significant changes in vesicle characteristics, irrespective of the storage temperature, up to the month 2 time point of the study. However following month 3 of storage, vesicles stored at 40 °C tended to aggregate as shown by significant increase in vesicle size.

To conclude, through modification in liposome preparation method, as well as the incorporation of TLR agonists, it was suggested that electrostatics were the main source of interaction between anionic systems combined with cationic liposomes. However, electrostatic interactions had the potential to lead to issues with regards to vesicle aggregation. This has been solved through the stepwise incorporation of TLR agonist within liposomal adjuvants, with or without high shear mixing homogenisation in order to control and modulate vesicle size and surface charge characteristics.

However as an alternative, another method in which to incorporate TLR agonists and control vesicle size could be to conjugate lipid to TLR agonist and incorporate within the membrane bilayer of cationic liposomes, as will be discussed in further detail within Chapter 7.

Chapter 7: The inclusion of immunomodulatory resiquimod within liposome vaccine adjuvants



Some of the results presented in this chapter have been published in the paper:

Wilkinson A., Lattmann E., Perrie Y. "The development of a novel cationic liposome vaccine adjuvant using DDA/TDB as a platform with bilayer conjugation of a lipid=TLR7 agonist conjugate within the formulation" (Manuscript in preparation).

7.1. Introduction

Imidazoquinoline molecules are able to activate B-cells to proliferate and differentiate into antibody-secreting cells therefore leading to enhanced antibody production (Bishop et al., 2000, Tomai et al., 2000). This family of molecules are able to activate both Toll-like receptor (TLR) 7 and 8 agonists leading the skewing of immunity towards a Th1 immune response (Jurk et al., 2002). These molecules present in the imidazoquinolines are termed synthetic immune response modifiers (IRM) due to their ability to selectively activate these specific TLRs. These TLRs are located within the endosomal compartment of the cell and are able to recognise guanosine- and uridine-rich single stranded (ss)RNA (Diebold et al., 2004, Heil et al., 2004, Gorden et al., 2006) as well as IRM such as imidazoquinolines. Therefore activation of these TLRs occurs at the endosome, which has been shown to be due to activation of these TLRs (by imiquimod and resiquimod) leading to endosomal acidification and downstream events leading to the activation of the specific immune response (Tomai et al., 2007). This has been further demonstrated by agents that block acidification of endosomes and hence block the subsequent activation of the immune response (Hart et al., 2005).

Although resiquimod and other analogues (which activate through TLR7 and/or TLR8) have been demonstrated as effective vaccine adjuvants in a number of model systems, several other studies have given mixed success in their ability to act as vaccine adjuvants (Wille-Reece et al., 2005). The short half life, in combination with small size (Mw = 314.38) and high aqueous solubility, may lead to the rapid distribution throughout the body rather than staying at the injection site which is not ideal for the local activation of dendritic cells at the injection site. Therefore, these TLR7/8 agonists may need to be co-localised with other stimuli (e.g. antigen and adjuvants) at the injection site in order to offer enhanced immunity (Ahonen et al., 2004). Therefore, for optimal vaccine adjuvant formulation it may be required to retain the TLR agonist at the site of injection in order to induce local adjuvant effects without inducing systemic cytokines, which may give side effects. This could be achieved by formulating the agonist within a delivery system such as liposomes.

During this investigation, DSPE was selected as a lipid which could be conjugated to the TLR7 agonist resiquimod; this was due to this lipid containing a primary amine group ($-NH_3^+$) which could initially react with succinic anhydride to form a carboxylate, which could subsequently form an ester upon reaction with resiquimod.

7.2. Aims and Objectives

The aim of the work reported in this final chapter addressed the inclusion of the TLR7 agonist resiquimod within the cationic DDA/TDB liposome formulation. In order to achieve this it was required to;

- chemically synthesise a conjugate between lipid and TLR agonist, which will be confirmed by chemical analysis,
- formulate cationic DDA/TDB liposomes with the inclusion of the lipid-TLR agonist conjugate, with the physicochemical characteristics and stability of these systems being measured,
- determine TLR agonist and antigen loading and retention within the formulation,
- carry out *in vivo* biodistribution studies to investigate the presence of liposome and TLR agonist at the injection site, draining lymph nodes and other organs,
- investigate these formulations as vaccine adjuvants for the induction of cell mediated immune (CMI) and humoural responses after immunisation with the tuberculosis subunit vaccine antigen Ag85B-ESAT6-Rv2660c (H56).

7.3. Results and Discussion

7.3.1. Chemical Synthesis of Lipid-TLR conjugate

It was proposed to design and synthesise a novel lipid-TLR agonist conjugate consisting of the lipid 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and the TLR7 agonist resiquimod. Both lipid and TLR agonist are displayed in Figures 7.1 A and B.

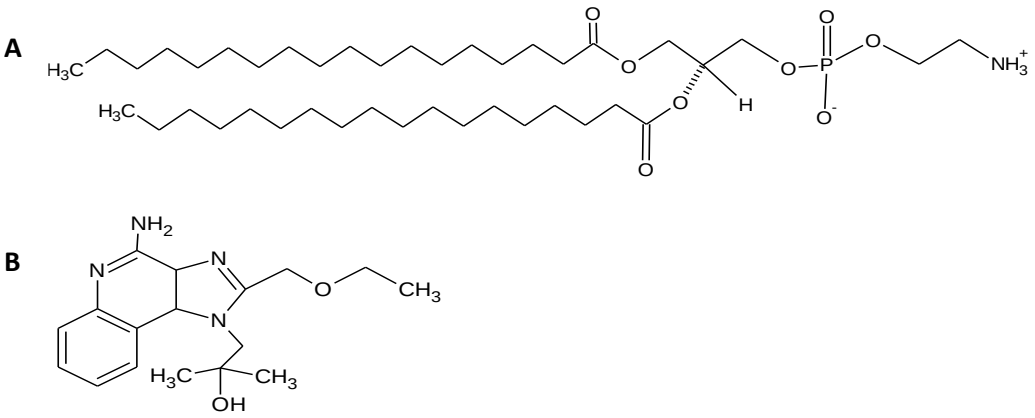


Figure 7-1. Chemical structures of (A) DSPE lipid and (B) Resiquimod.

This study will require two important chemical reactions (Figure 7.2); firstly DSPE and succinic anhydride will be reacted together (under heat and magnetic stirring) in order to form the DSPE lipid with amide bond and terminal carboxylate. Upon formation of this structure, subsequently the TLR agonist resiquimod was added (which contains an alcohol group (-OH)) in the presence of the diisopropyl azodicarboxylate (DIAD) activating agent and triphenylphosphine (TPP). This will lead to the formation of an ester (due to the Mitsunobu reaction) made up of DSPE conjugated to the resiquimod TLR7 agonist.

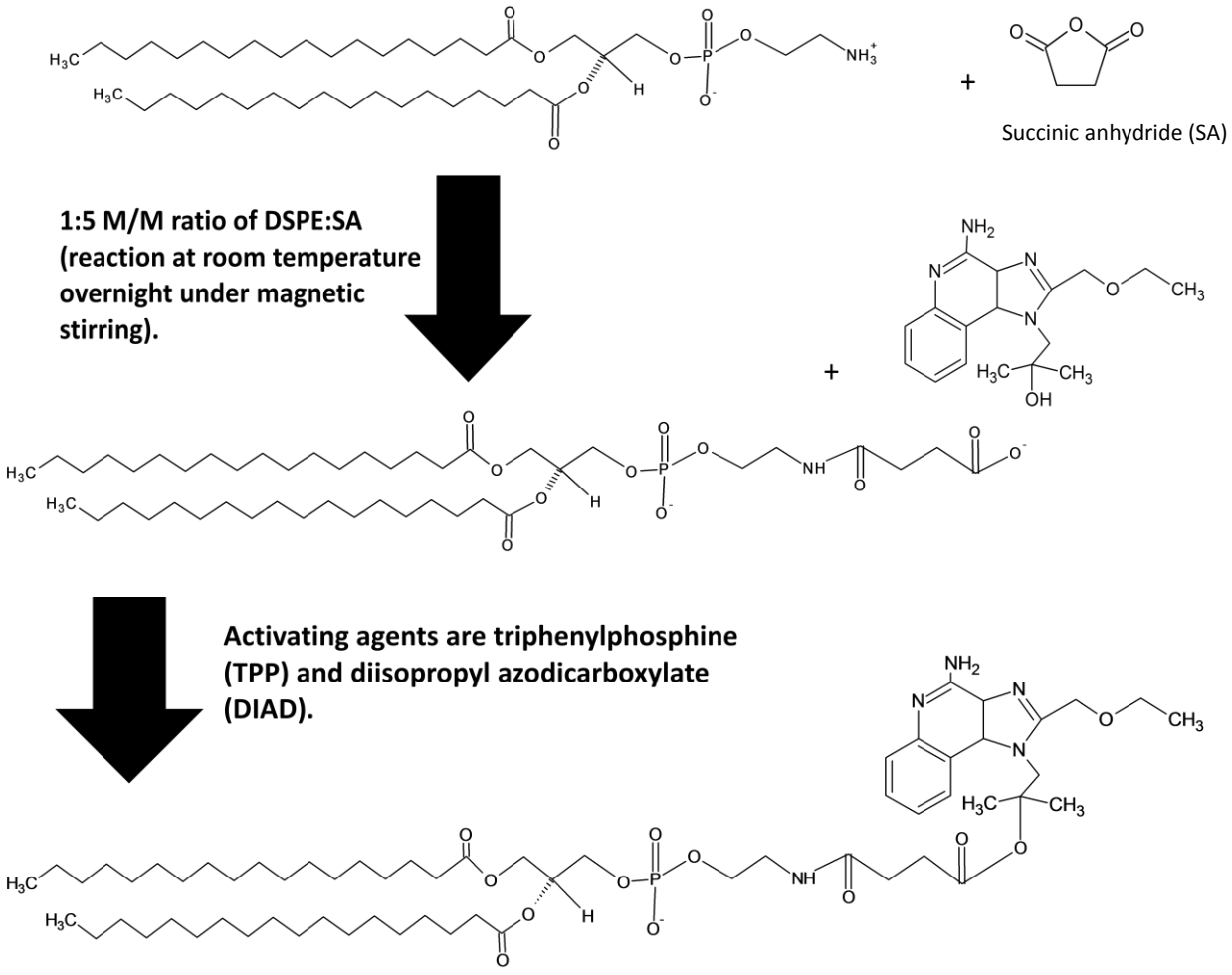


Figure 7-2. Overview of chemical synthesis reactions.

7.3.1.1. Initial Optimisation of Step 1 Reaction

To synthesise the lipid-TLR conjugate, DSPE and succinic anhydride (SA) were initially reacted together at various molar ratios (either at 1:2, 1:5 or 1:10 M/M ratio) at either room temperature or 40 °C. These variables were tested to show which concentration of succinic anhydride was required

to synthesise the correct product, as it was previously suggested that this may have an effect on the reaction (Hermanson, 2008). Variation in reaction temperature was also tested in order to investigate whether this variable had an effect on the kinetics of this reaction. In general, increasing the temperature of a substance increases the average speed (or energy) of the particles and consequently the number of particles colliding with sufficient energy to react. So, at higher temperatures there are more successful collisions (between reaction materials) and therefore a faster reaction.

The aim of this reaction was to produce a reaction product, with the DSPE lipid containing an amide bond and terminal carboxylate (Figure 7.2). This reaction product should form due to a ring-opening process (Klotz, 1967), using succinic anhydride (SA) in order to react with the amine group of the DSPE lipid.

Both at 1:2 and 1:10 M/M ratios of DSPE to SA this reaction did not prove efficient with incorrect product formed, as per analysis by TLC, NMR and IR (results not shown). However, the kinetics of the reaction can be altered upon altering the temperature in which the reaction takes place or by altering the molar ratio between starting materials. Therefore, upon 1:5 M/M ratio reaction between DSPE and SA (at 40 °C) this led to the formation of two products as shown by TLC, showing the reaction product was not pure; however, these products can be separated by column chromatography. During this investigation, the effect of the choice of solvent as the mobile phase was investigated. Upon optimisation, ethyl acetate was determined to be the chosen mobile phase for all subsequent analyses by TLC due to this solvent providing good separation between reaction products (data and results not shown).

7.3.1.2. Optimised Step 1 Reaction

In an optimised reaction, DSPE lipid and succinic anhydride were reacted together at a 1:5 M/M ratio at room temperature under magnetic stirring. As the initial reaction product (following reaction at 40 °C) was not completely pure and required an additional separation stage (which was not ideal), it was chosen to carry out the reaction now at room temperature with the aim of synthesising the pure correct reaction product. This reaction was allowed to take place for several hours with aliquots (50 µL) of the reaction mixture taken at various time points for analysis by thin layer chromatography (TLC). Products from this reaction (and the initial starting materials) were visualised under UV light (Figure 7.3). These results showed that a reaction product was formed with an R_f

value of ~ 0.75. This reaction product, following 24 hours, was shown to be different from the starting materials by simultaneously spotting these materials onto the TLC plate. The starting materials were shown not to leave a spot on the TLC plate, which was suggested due to the highly lipophilic and hydrophilic nature of DSPE lipid and SA respectively. This was shown in all analyses by TLC during this investigation. However the reaction product left a spot on the plate thereby showing the DSPE lipid has been modified during this reaction (Figure 7.3). Based on this, the reaction mixture was dried under vacuum and dessication in order to form a powder residue. This powder, as well as the starting materials (DSPE and SA), were subsequently analysed by infrared spectroscopy (IR) and nuclear magnetic resonance (NMR) spectroscopy, with the resulting spectra being displayed in Figures 7.4 and 7.5 respectively.

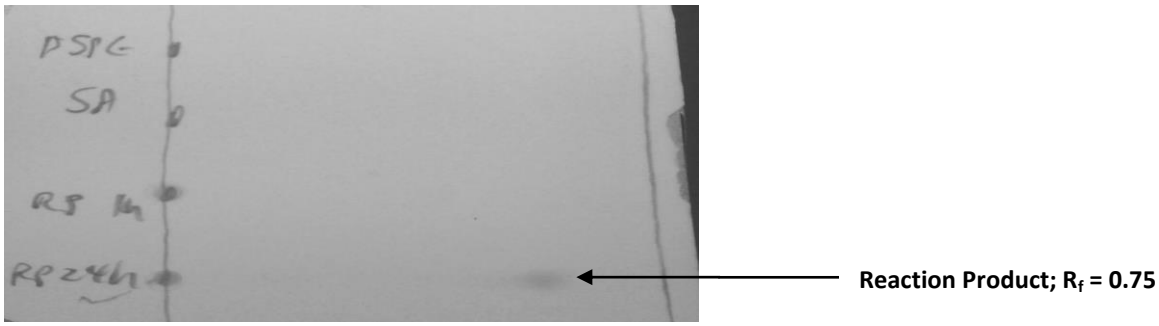
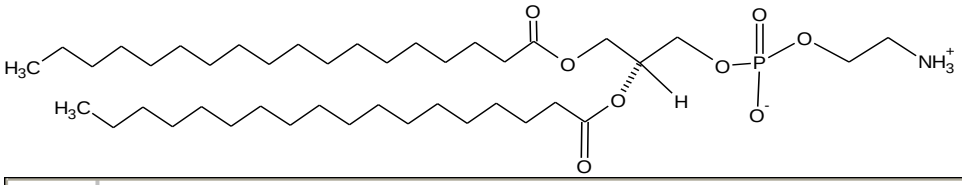
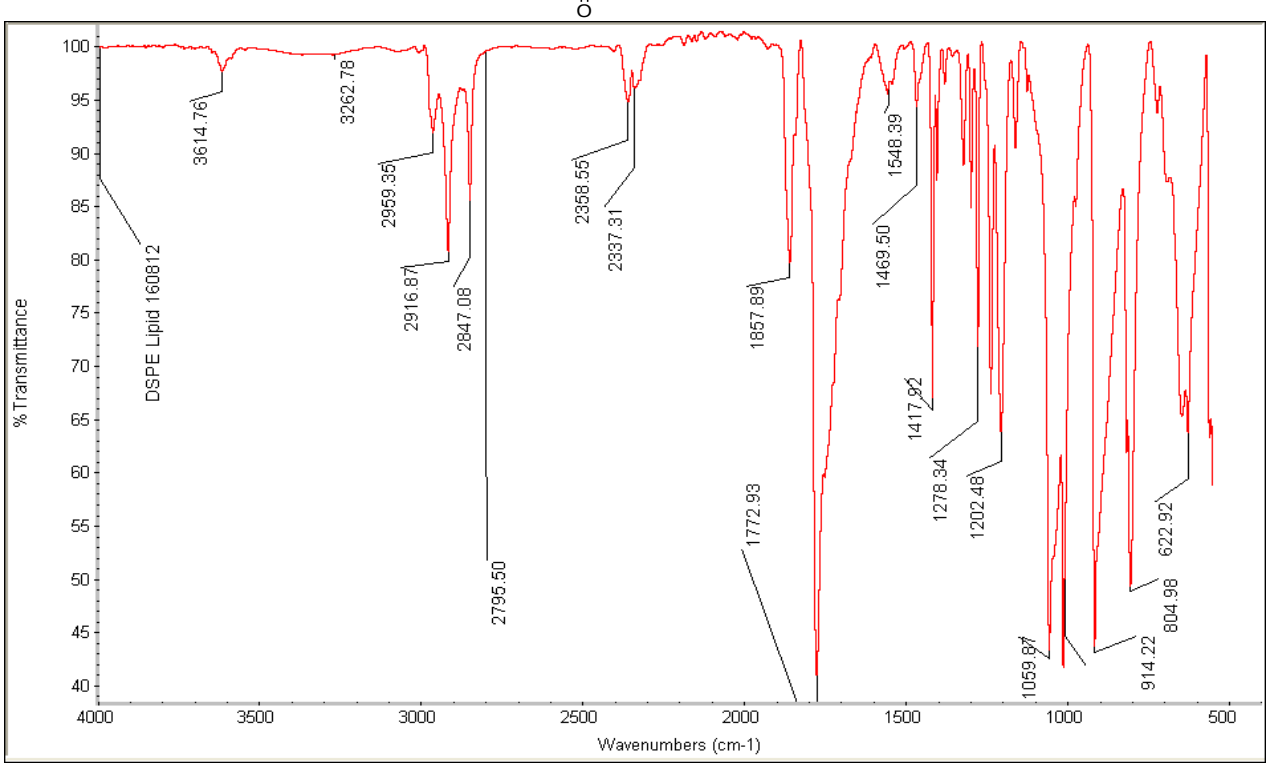


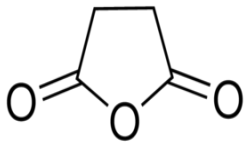
Figure 7-3. TLC plate analysis of starting materials and various aliquots of reaction product (RP) from the succinylation reaction between DSPE and SA (1:5 M/M ratio at room temperature under magnetic stirring).

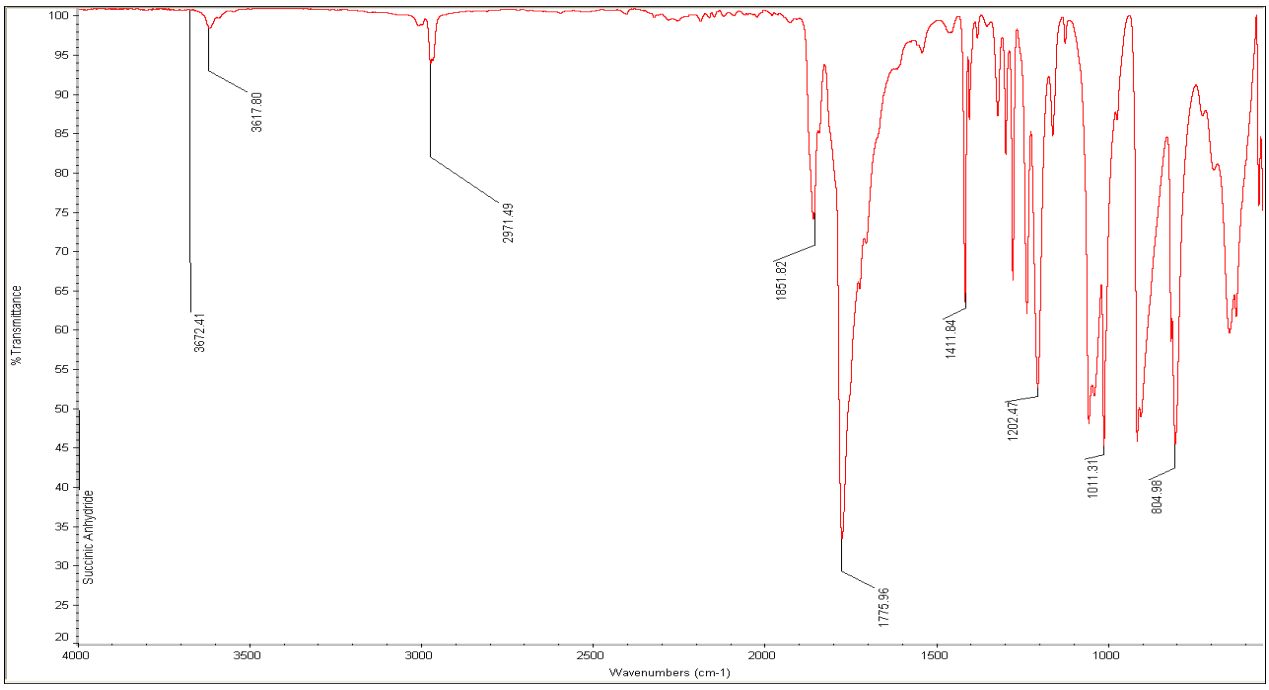
A




DSPE Lipid 160812
% Transmittance
Wavenumbers (cm-1)
3614.76
3262.78
2959.35
2916.87
2847.08
2795.50
2358.55
2337.31
1857.89
1772.93
1548.39
1469.50
1417.92
1278.34
1202.48
1059.97
914.22
804.98
622.92

B




Succinic Anhydride
% Transmittance
Wavenumbers (cm-1)
3672.41
3617.80
2971.49
1851.82
1776.96
1411.84
1202.47
1011.31
804.98

C

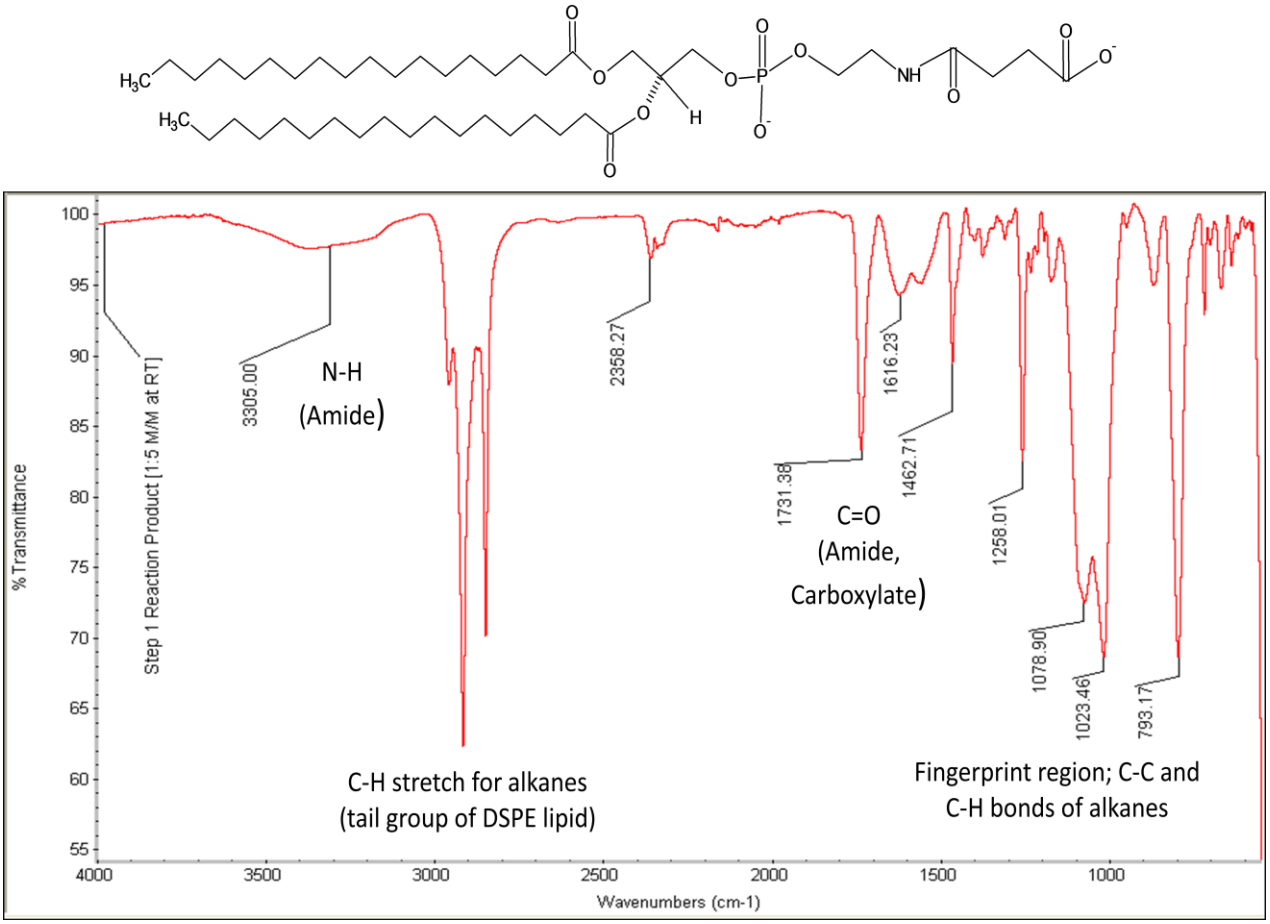


Figure 7-4. Infra-Red (IR) Spectra of (A) DSPE lipid, (B) succinic anhydride and (C) the reaction product formed during the succinylation reaction between DSPE and SA (at a 1:5 M/M ratio at room temperature under magnetic stirring).

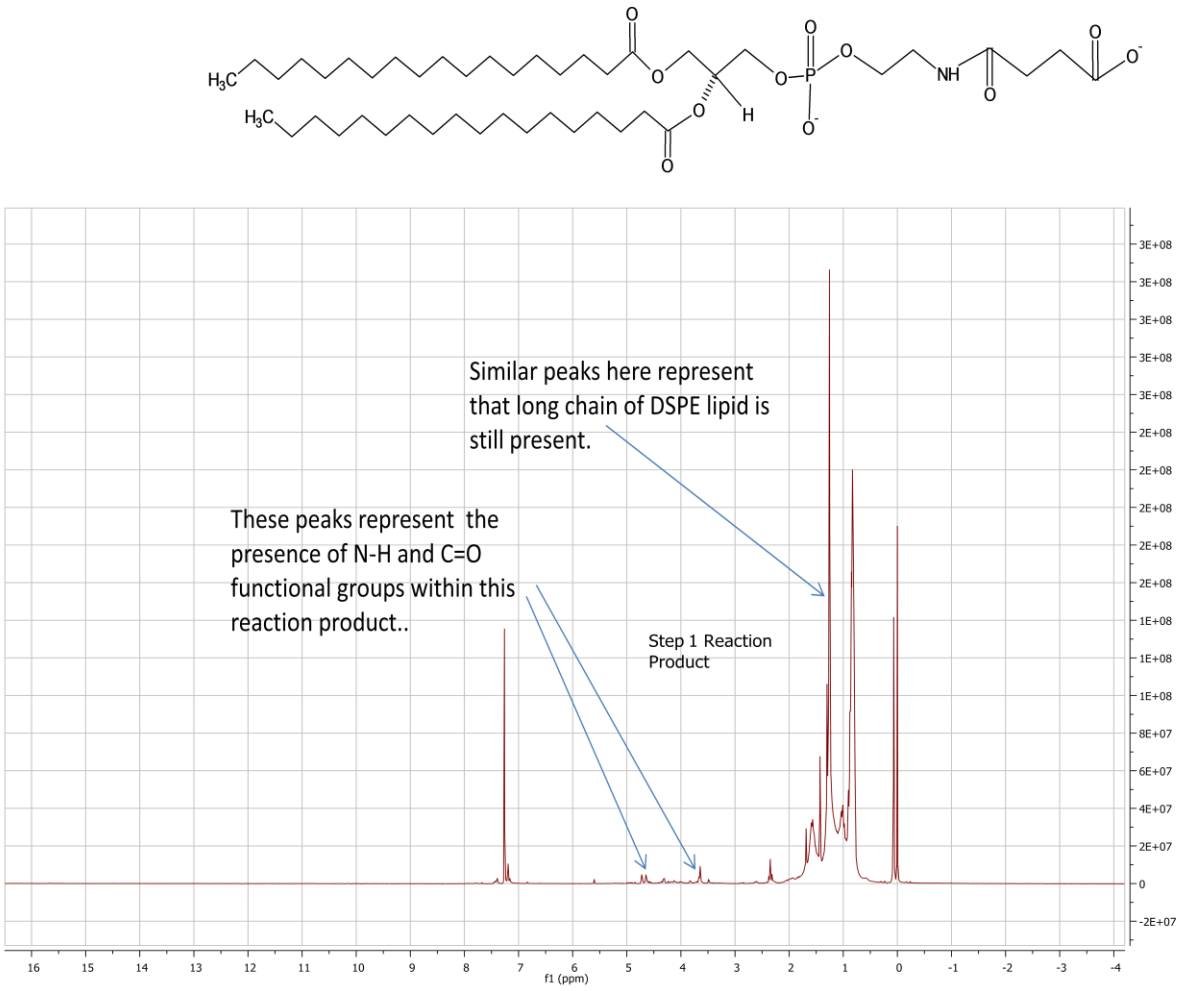


Figure 7-5. NMR Spectra of the Reaction Product formed during the succinylation reaction between DSPE and SA (at a 1:5 M/M ratio at room temperature under magnetic stirring).

For the IR spectra of the step 1 reaction product there was a triplet peak at around 3000 cm^{-1} with these peaks also occurring in the spectra for the DSPE lipid (Figure 7.4A) thus showing the retention of the lipid tail group (C-H stretch for alkanes) within this reaction product. There was also the presence of a new peak present at 3300 cm^{-1} which represented the presence of an amide (N-H) bond within the reaction product. There was also a peak at around 1700 cm^{-1} which represents the C=0 bonds present within the structure of the newly formed amide and carboxylate (Figure 7.4C). This showed that the lipid head group was modified upon successful completion of this reaction.

Furthermore, for the NMR spectra of this Step 1 reaction product there was the presence of multiple peaks between 0.9 and 1.7 ppm. These peaks represented the C-H bonds in the tail group of the DSPE lipid therefore this suggested that the DSPE lipid tail was retained within this reaction product. There were also small peaks at 3.5 and 4.8 ppm which represented the presence of additional functional groups within the reaction product, such as amide bond (N-H) and carboxylate (C=O),

which were not present in the DSPE lipid itself (Figure 7.5). These peaks were small due to the large major presence of the DSPE lipid within the reaction product. The peaks at 7.2 ppm and 0 ppm represented chloroform (solvent) and tetramethylsaline (TMS) respectively (Figure 7.5).

7.3.1.2. Step 2 Reaction

Upon formation of the correct structure in step 1 of the reaction, the TLR agonist resiquimod (which contains an alcohol group (-OH)) was added to the reaction mixture in the presence of the diisopropyl azodicarboxylate (DIAD) activating agent and triphenylphosphine (TPP) with the aim to form a conjugate structure between DSPE and resiquimod TLR agonist. Therefore we hypothesised that under the optimal conditions (1:1 M/M ratio at room temperature under magnetic stirring) this would lead to the formation of an 'ester' bond, due to the Mitsunobu reaction (But and Toy, 2007, Swamy et al., 2009, Otera and Nishikido, 2010), conjugating the DSPE lipid to the resiquimod TLR7 agonist (Figures 7.1 and 7.2). As described previously (Section 7.3.1.1) in an initial reaction the DSPE:SA conjugate (formed in the step 1 reaction) was reacted with TLR7 agonist resiquimod in the presence of the activating agent diisopropyl azodicarboxylate (DIAD) and triphenylphosphine (TPP) in order to form the correct reaction product. In an initial reaction, DIAD and TPP were reacted together at room temperature at a 1:1 M/M ratio in order to form the betaine 1 (Morrison-Bruns-Huisgen) intermediate, which was zwitterionic and required for the subsequent activation step in the reaction (Otera and Nishikido, 2010). The formation of this intermediate was determined by TLC analysis (Figure 7.6A). Following the production of this intermediate, subsequently the step 1 reaction product (DSPE:SA conjugate containing amide bond and terminal carboxylate) followed by resiquimod were added to the reaction mixture (at a 1:1 M/M ratio) at room temperature under magnetic stirring.

Subsequently, upon the addition of the DSPE lipid bearing a terminal carboxylate (step 1 reaction product) this led to the formation of a separate reaction product (Figure 7.6B) which had an R_f value of 0.4. Subsequently upon addition of the TLR7 agonist resiquimod to the reaction mixture, this resulted in a further shift in the R_f value of the reaction product, from 0.4 to 0.15 (Figure 7.6C), with a small by-product present at R_f(0.4). This suggested that a new product was formed, however there was a by-product from this reaction which was suggested to be triphenyloxide or TPhP=O (Otera and Nishikido, 2010).

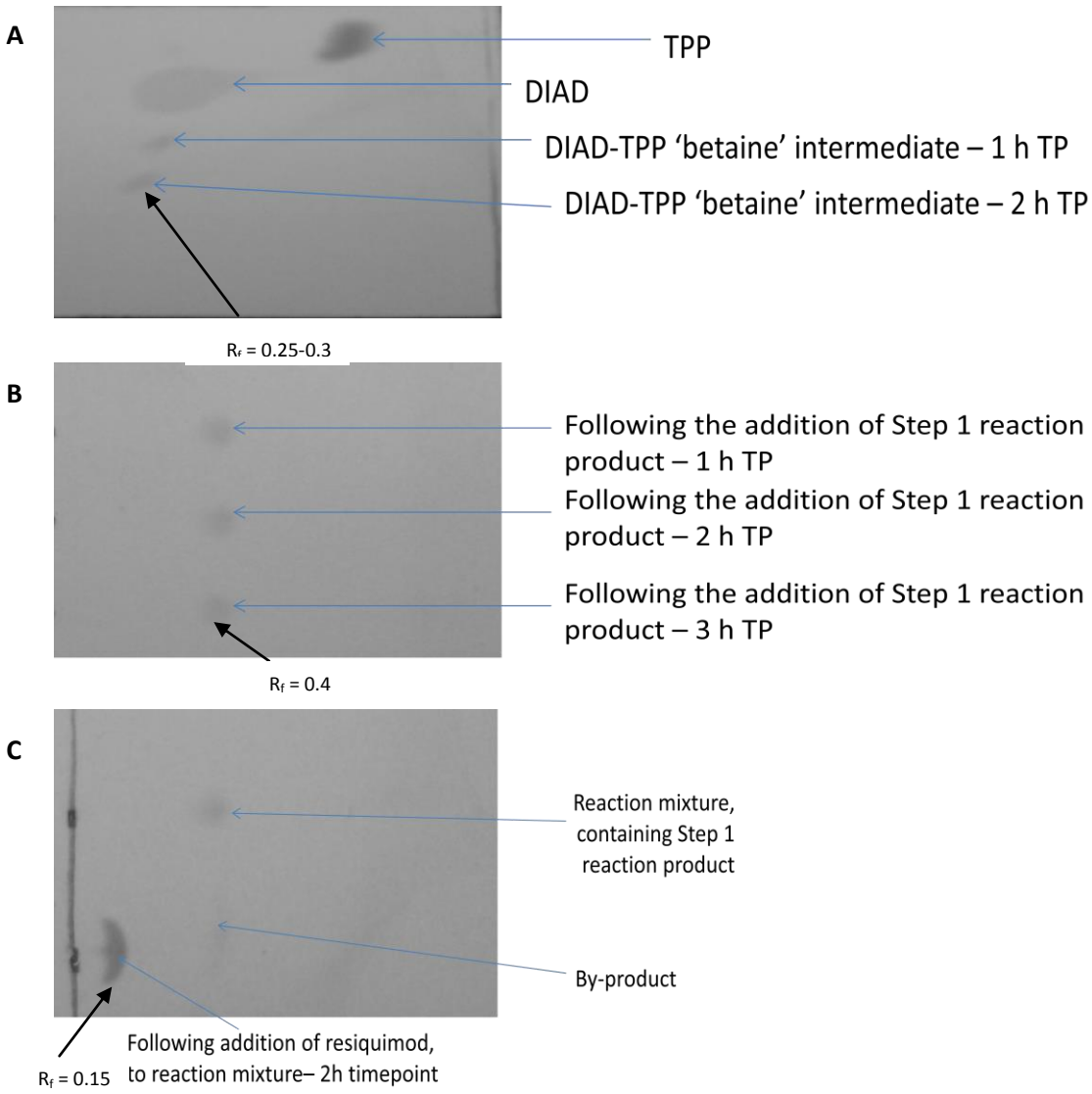


Figure 7-6. TLC plate analysis of various aliquots (taken at 1 hour time points) taken from the Mitsunobu esterification reaction between step 1 reaction product and resiquimod, in the presence of diisopropyl azodicarboxylate (DIAD) and triphenylphosphine (TPP) as activating agents.

Following this analysis, the reaction mixture was initially subjected to silica-sand column chromatography in order to extract, isolate and separate the correct reaction product which was present at R_f(0.15). This reaction product was dried under vacuum and dessication in order to form a powder residue. This product was subsequently analysed by Infrared spectroscopy (IR) and nuclear magnetic resonance (NMR) spectroscopy with the resulting spectra being displayed in Figures 7.7 and 7.8 respectively. Analysis of the IR spectra from this newly formed product suggested the retention of the major functional groups of the step 1 reaction product (DSPE:SA conjugate) in the final product including the amide (N-H) bonds at 3300 cm-1 and 1700 cm-1 (C=O) as well as the C-H stretch triplet peak at 2800-3000 cm-1 (Figure 7.7). However, there were additional peaks in this

spectra suggesting the presence of an ester group, which was required as the 'linker' in this product as represented by peaks at 1100 and 1700 cm-1 (C=O and C-O peaks respectively). There were also additional peaks at 3600-3700 cm-1 and ~ 1500 cm-1 which represented C-H and C-C bonds present in the aromatics of resiquimod, respectively (Figure 7.7). This showed that resiquimod was able to be conjugated to DSPE lipid, which can then be subsequently used in liposome formulation.

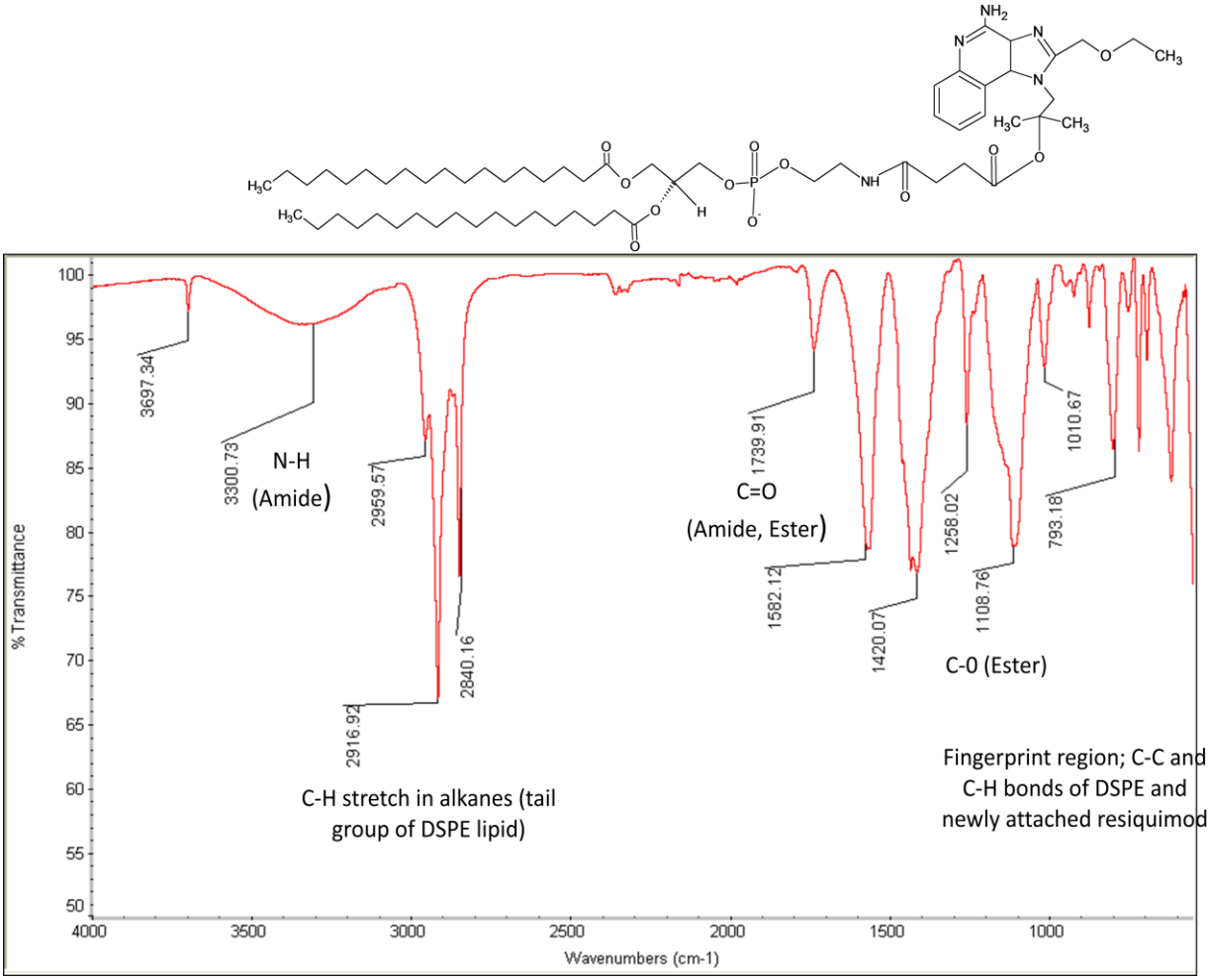


Figure 7-7. IR Spectra of the Reaction Product formed during the Mitsunobu esterification reaction between DSPE:SA conjugate (Step 1 reaction product) and Resiquimod (at a 1:1 M/M ratio at room temperature under magnetic stirring).

The NMR spectrum of this Step 2 reaction product displayed the retention of peaks between 0.9 and 1.7 ppm, which indicated the retention of the DSPE lipid component. There were also more pronounced peaks at 3.5 and 4.8-5.0 ppm (Figure 7.8), which represented newly attached bonds in the attached resiquimod molecule. The additional peaks between 7.5 and 8.0 ppm suggested the presence of an aromatic molecule or residues, which was shown due to the presence of resiquimod

in the conjugate structure. The peaks at 7.2 ppm and 0 ppm represented chloroform and tetramethylsilane (TMS) respectively (Figure 7.8).

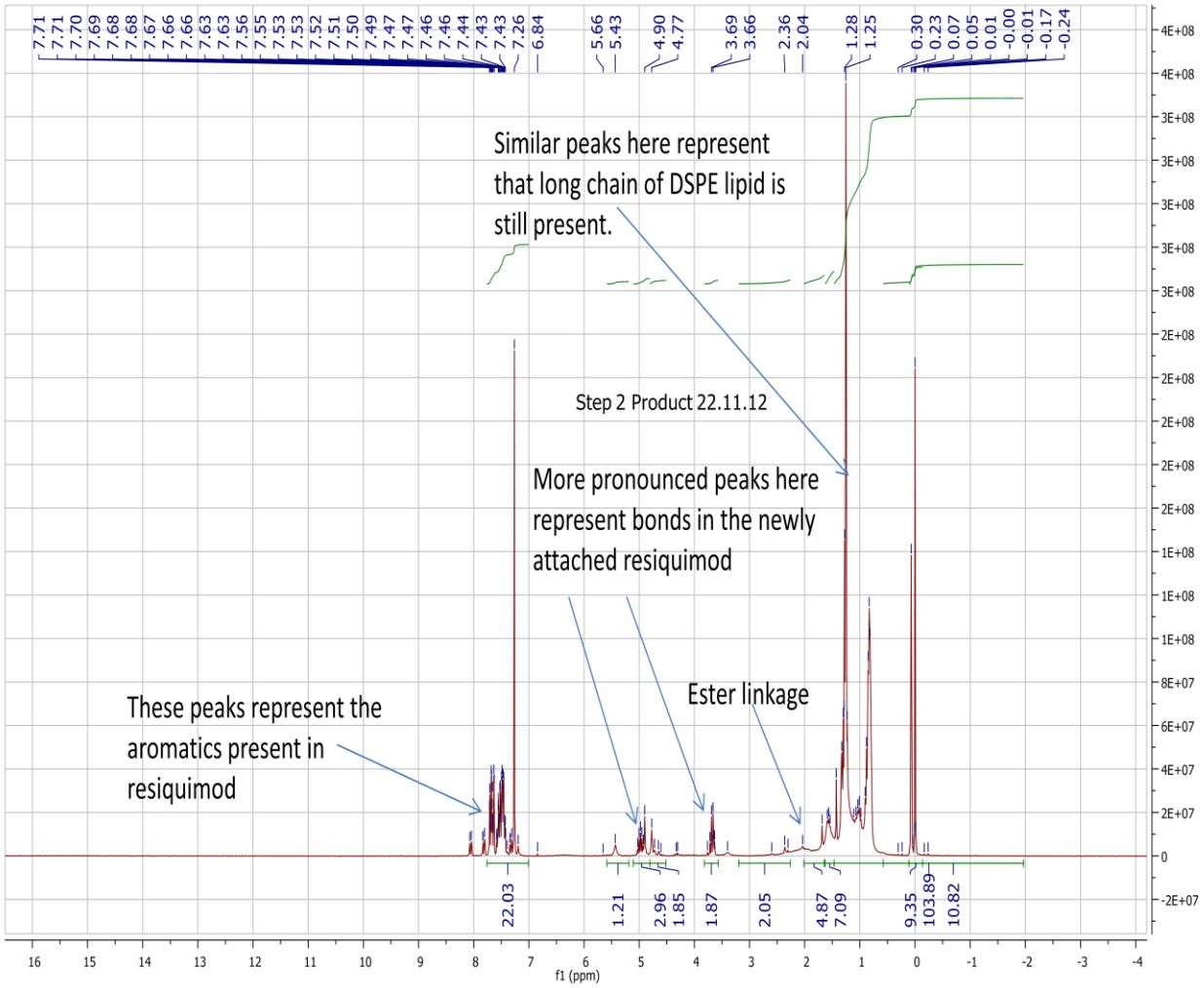


Figure 7-8. NMR Spectra of the Reaction Product formed during the Mitsunobu esterification reaction between DSPE:SA conjugate and Resiquimod (at a 1:1 M/M ratio at room temperature under magnetic stirring).

7.3.2. Incorporation of immunostimulatory resiquimod within multilamellar vesicles

During these studies, DDA/TDB MLVs were formulated with DSPE-conjugated resiquimod (DSPE=Resiquimod; Figure 7.9A) and also with inclusion of DSPE (within the bilayer) and addition of resiquimod post-LH (Figure 7.9B).

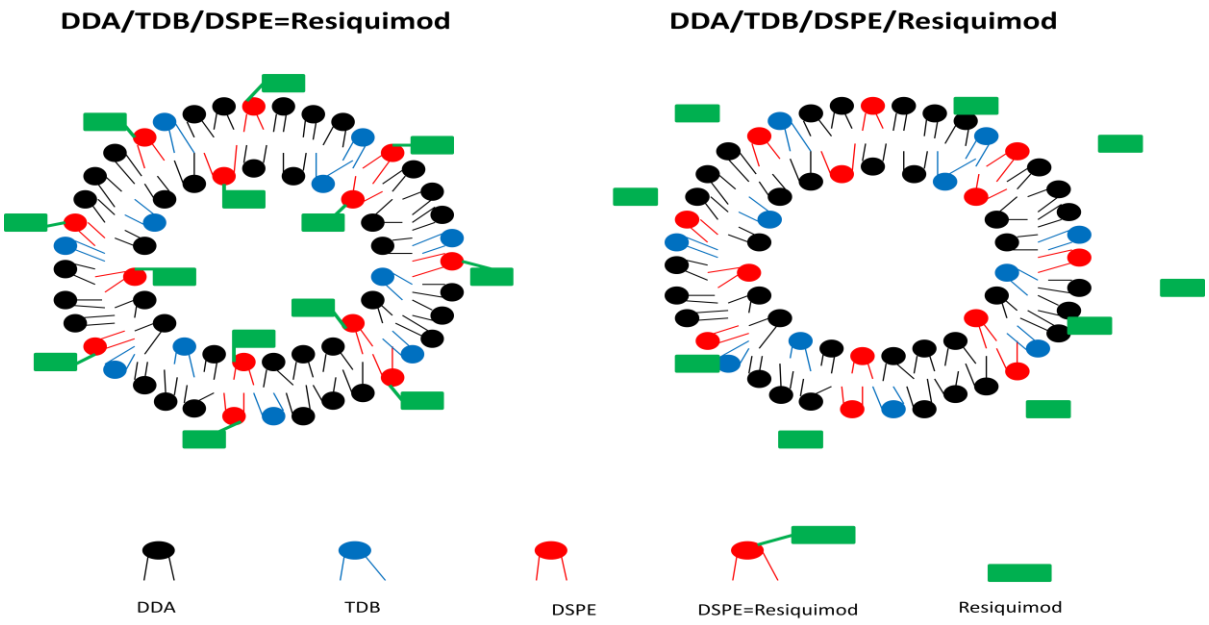
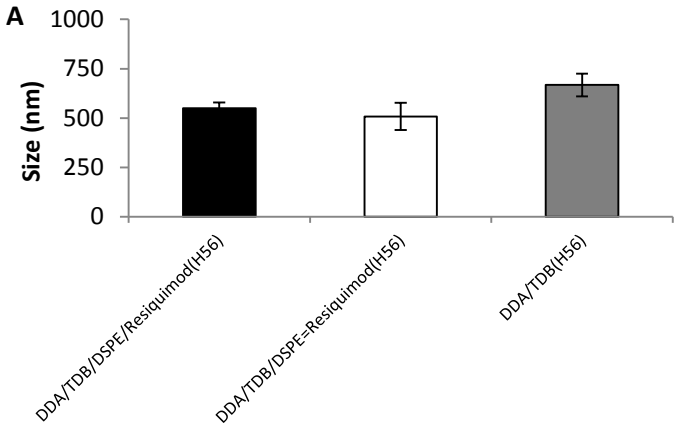


Figure 7-9. Multilamellar vesicles; with bilayer incorporation of DSPE=Resiquimod conjugate to DDA/TDB (A) or incorporation of DSPE and post-LH addition of resiquimod to DDA/TDB (B).

7.3.2.1. Characterisation and stability of resiquimod/MLV formulations

All liposome-adjuvanted formulations displayed vesicle sizes in the region of 500-600 nm. There were also no significant changes in the polydispersity (PDI) of these tested formulations (Figures 7.10 A and B). This showed that association of DSPE within the bilayer of the formulation, with either conjugated resiquimod or the addition of resiquimod post lipid film-hydration, had no significant impact on the vesicle characteristics of these liposome systems.

Zeta potential analysis of all-tested liposome formulations gave rise to surface charges in the region of 60 to 65 mV, which were strongly cationic, as would be expected by the positively-charged head group of DDA present within the formulation (Figure 7.10C). Therefore association of DSPE and resiquimod within cationic liposome vaccine formulations resulted in no significant changes in the zeta potential of these formulations. This was suggested to be due to the fact that resiquimod, due to its small size and high aqueous solubility (Tomai et al., 2007) was unable to remain with the formulation (when added post lipid film-hydration). However, conjugating resiquimod to the bilayer of vesicles led to efficient incorporation of this TLR agonist without further significant effects on vesicle characteristics, which has been hypothesised to be optimal for the adjuvant action of these formulations (Tomai et al., 2007).



B

Formulation	PDI
DDA/TDB/DSPE/Resiquimod(H56)	0.464
DDA/TDB/DSPE=Resiquimod(H56)	0.298
DDA/TDB(H56)	0.440

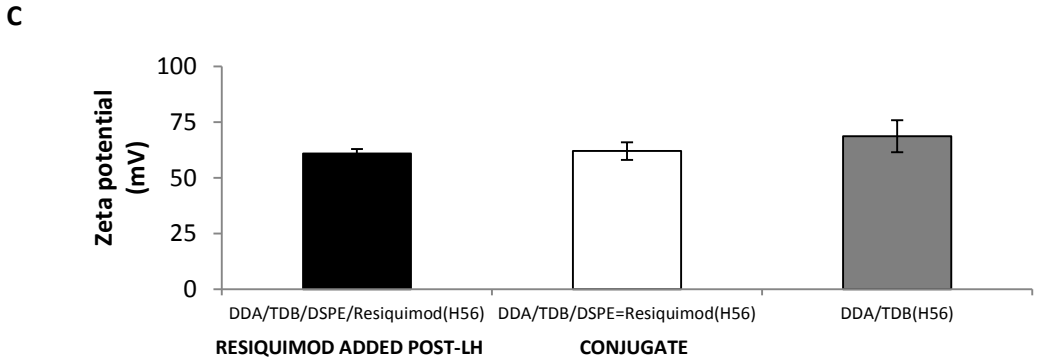


Figure 7-10. Characterisation of liposome vaccine formulations (A: Size; B: Polydispersity; C: Zeta potential) with addition of the *in vivo* dose of H56 antigen (5 µg per dose). Results are the mean of triplicate experiments ± SD.

The stability of liposomes is a crucial factor in the development and formulation of novel liposome vaccine delivery systems. Cationic DDA/TDB liposomes were incorporated with DSPE lipid within the bilayer and resiquimod post-LH (DDA/TDB/DSPE/Resiquimod), or were incorporated with the addition of the DSPE=Resiquimod (lipid=TLR) conjugate within the bilayer (DDA/TDB/DSPE=Resiquimod). Both of these liposome formulations contained the same molar ratio of all lipids and TLR agonist. These liposomes were also combined with and without the addition of the *in vivo* concentration of H56 antigen (5 µg). The stability of vesicles was tested over a 28 day period following storage at 4 °C and 25 °C respectively.

Cationic DDA/TDB/DSPE liposomes (mixed post lipid film-hydration with resiquimod) displayed an initial vesicle size of either 500 or 550 nm (without and with H56 antigen respectively; Figure 7.11A). In terms of storage conditions, liposome systems (both with and without antigen) stored at 25 °C displayed a slight increase in vesicle size (over time) compared to when stored in 4 °C standard conditions however these changes were not significant over a 28-day study period. Polydispersity analysis (Figure 7.11B) showed that cationic DDA/TDB/DSPE/Resiquimod, both with and without antigen, gave rise to vesicles which had a fairly narrow size distribution and a uniform and

homogenous population. In terms of zeta potential these liposome vaccine formulations displayed initial zeta potential values of 65 mV and 60 mV, both without and with antigen respectively (Figure 7.11C). Over the time period of this study both empty and antigen-loaded liposome systems showed no significant changes in zeta potential. This showed that the vesicle characteristics of these liposomes remained stable over this study period, both following storage at 4 °C and 25 °C. These stability studies have shown that cationic DDA/TDB/DSPE/Resiquimod liposomes (both with and without H56 antigen) remained homogenous and exhibited minimal phase separation even after a 28-day storage period (at both temperatures of 4 ˚C and 25 ˚C; Figures 7.12 A and B). The homogenous nature of these liposome samples when stored at 4 ˚C at 25 ˚C conditions was reflected by no hugely significant changes in the vesicle size, polydispersity and zeta potential over the time period (Figure 7.11) therefore these formulations are recommended for storage at both of these temperature conditions in an aqueous format.

Cationic DDA/TDB/DSPE=Resiquimod liposome formulations (incorporating the TLR agonist conjugate within the bilayer) were measured for their vesicle size, polydispersity and zeta potential. This liposome formulation displayed an initial vesicle size of either 420 or 500 nm (without and with H56 antigen respectively; Figure 7.13A). Empty and antigen-loaded liposome systems displayed similar vesicle sizes following storage at 4 °C and 25 °C. By day 28 both empty and antigen-loaded systems displayed vesicle sizes in the region of 600 and 730 nm respectively (Figure 7.13A). Polydispersity analysis (Figure 7.13 B) showed that cationic DDA/TDB/DSPE=Resiquimod, both with and without antigen displayed vesicles which have a fairly narrow size distribution. In terms of zeta potential these liposome vaccine formulation displayed initial zeta potential values of around 65 mV and 61 mV, both without and with antigen respectively (Figure 7.13C). Over the time period of this study both empty and antigen–loaded liposome systems showed no significant changes in zeta potential. This showed that these systems remained stable over this study period, following storage at 4 °C and 25 °C respectively. Also antigen and agonist incorporation within the liposome system resulted in no significant changes in measured vesicle characteristics for either of these liposome systems (Figures 7.11 and 7.13). These stability studies have shown that cationic DDA/TDB/DSPE=Resiquimod liposomes (both with and without H56 antigen) remained homogenous and also exhibited minimal phase separation even after a 28-day storage period (at both temperatures of 4 ˚C and 25 ˚C; Figures 7.14 A and B). The homogenous nature of these liposome samples when stored in both standard (4 ˚C) and accelerated (25 ˚C) conditions was reflected by no hugely significant changes in the vesicle size and zeta potential respectively over the time period of this study (Figures 7.13), therefore suggesting these liposome systems are able to stored in an aqueous format, over a 28-day storage period with no significant effects on vesicle characteristics.

Therefore resiquimod, whether bilayer-incorporated or surface-attached within DDA/TDB liposomes, had no further subsequent effect on the vesicle characteristics and stability of these formulations.

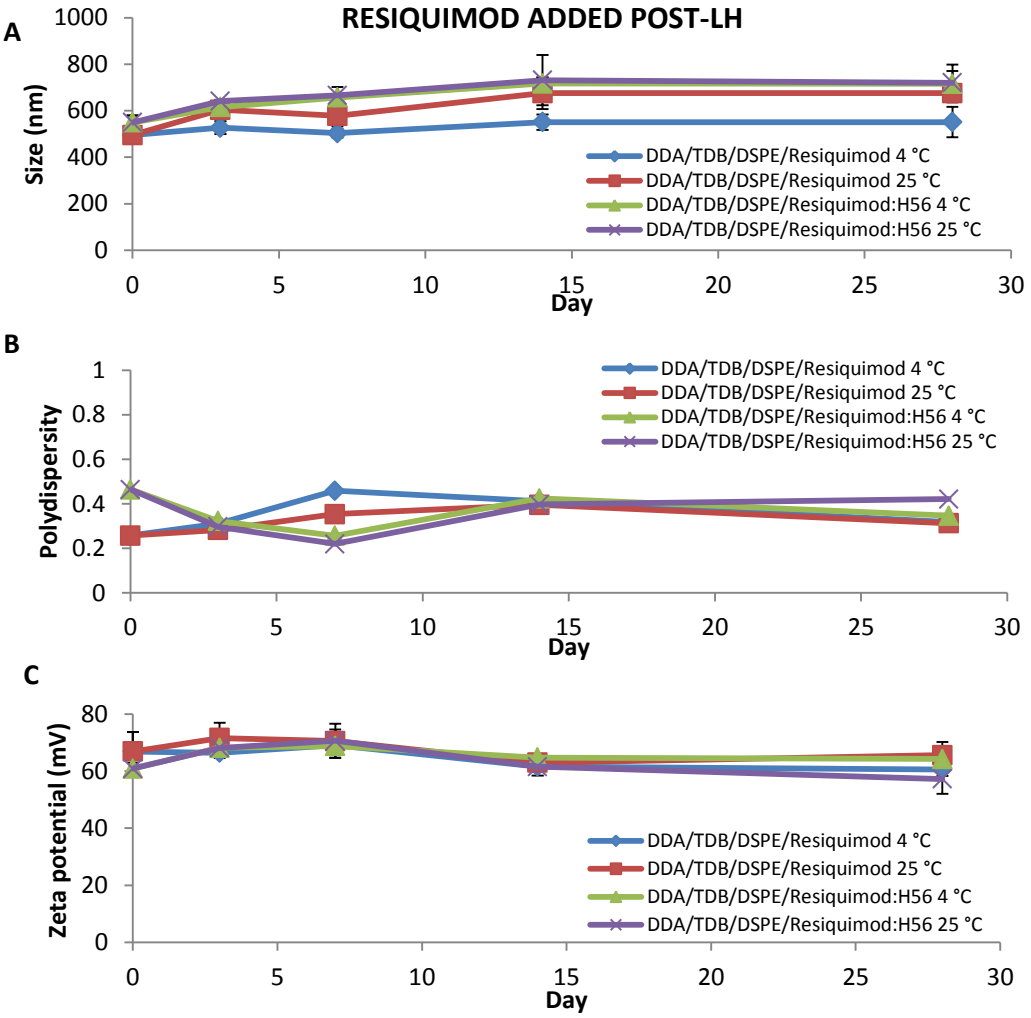


Figure 7-11. Vesicle size (A) analysis, polydispersity (B), zeta potential (C) and stability (over a 28-day period) of DDA/TDB incorporating DSPE lipid and resiquimod (mixed post-LH) with and without H56 antigen following storage at 4 °C and 25 °C. Results are the mean of triplicate experiments ± SD.

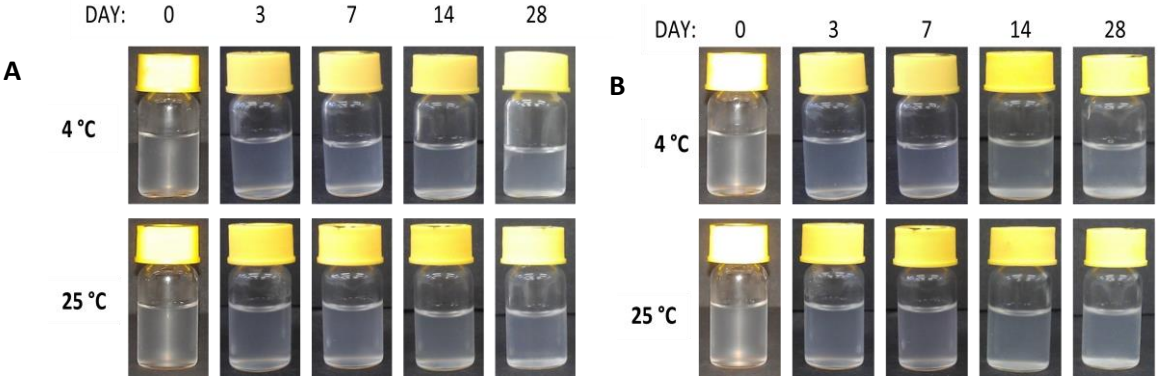


Figure 7-12. DDA/TDB/DSPE/Resiquimod (A) and DDA/TDB/DSPE/Resiquimod:H56 (B) liposome samples stored in glass vials were prepared by the lipid-film hydration (LH) method and were stored for 28 days at 4 ˚C and 25 ˚C.

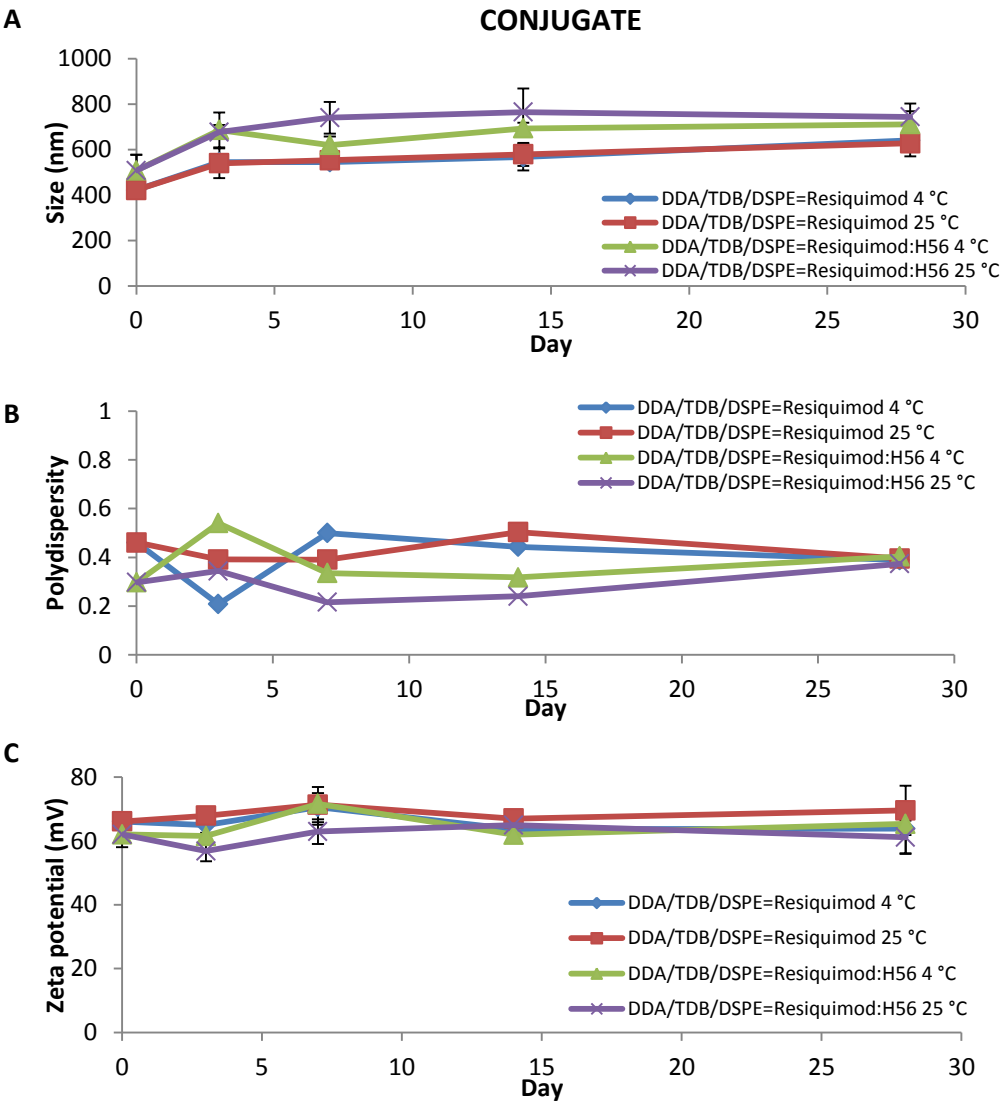


Figure 7-13. Vesicle size (A), polydispersity (B) and zeta potential (C) analysis and stability (over a 28-day period) of DDA/TDB incorporating DSPE=Resiquimod TLR conjugate, with and without H56 antigen following storage at 4 °C and 25 °C. Results are the mean of triplicate experiments ± SD.

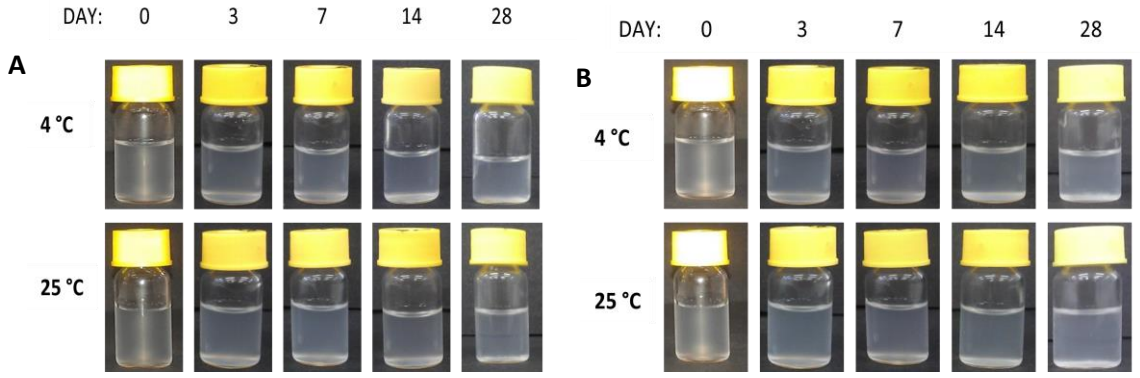


Figure 7-14. DDA/TDB/DSPE=Resiquimod (A) and DDA/TDB/DSPE=Resiquimod: H56 (B) liposome samples stored in glass vials were prepared by the lipid-film hydration (LH) method and were stored for 28 days at 4 ˚C and 25 ˚C.

7.3.3. Quantification of antigen loading and retention

These liposome vaccine formulations were further tested for their ability to associate and retain antigen within the delivery system. These parameters were quantified by determining the amount (percentage) of radiolabelled antigen (H56) with the delivery system. These results showed that both conjugate and control formulations, DDA/TDB/DSPE=Resiquimod and DDA/TDB/DSPE/Resiquimod respectively, were able to associate high levels of the anionic H56 TB antigen with antigen loading levels of 80-90 % being determined (Figure 7.15).

Both of these formulations showed high levels of antigen loading (Figures 7.15) and low levels of antigen release over this study period therefore this showed that these systems were stable (Figures 7.16 A and B) and were able to efficiently retain the vaccine antigen associated to the surface of the liposome delivery system. There was an initial burst release of antigen ($P < 0.001$) from the formulation over the initial 3 to 5 hours of the study, however this release stabilised throughout the rest of the 96-hour study period (Figures 7.16 A and B). Therefore the presence of resiquimod within the formulation (either bilayer-conjugated or surface-adsorbed) had no subsequent effect on the ability to load and retain vaccine antigen.

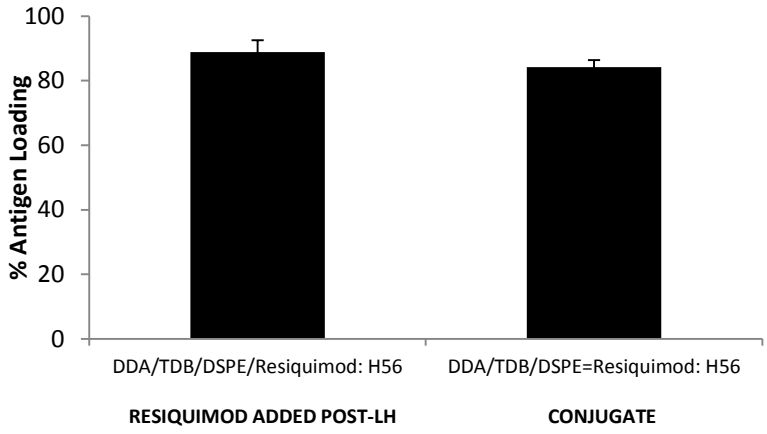


Figure 7-15. Initial loading of H56 Antigen by cationic liposome-TLR formulations. Results are the mean of triplicate experiments ± SD.

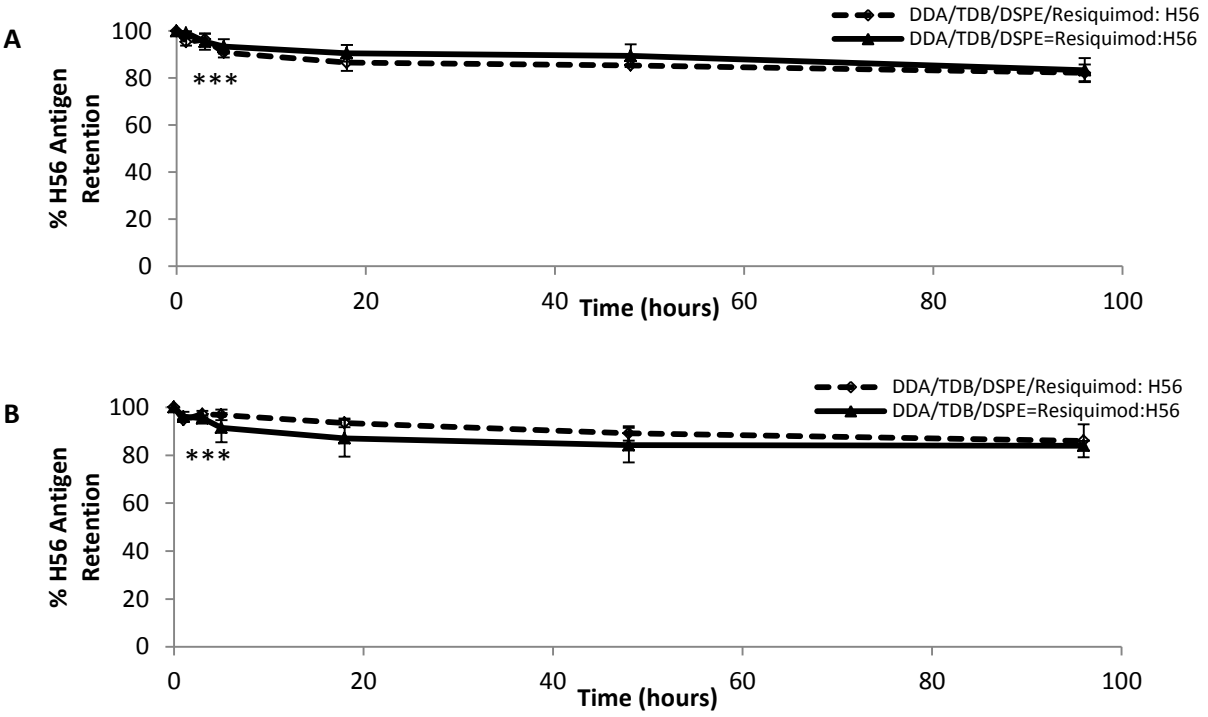


Figure 7-16. Antigen Release Kinetics from Cationic Liposome-TLR formulations in (A) simulated in vivo conditions (FCS/Tris; 50:50 v/v; 37 °C) and (B) control conditions (Tris 10 mM pH 7.4; 37 °C). Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$) as compared to initial antigen retention at 0 h.

7.3.4. Quantification of resiquimod loading and retention

7.3.4.1. Proof of radiolabelling

During these studies, the radiolabelling of resiquimod was proven by agonist loading and dialysis studies as well as biodistribution studies (Section 7.3.6). Following radiolabelling of this TLR agonist with ^{125}I, this process was shown not to be completely efficient by dialysis (Figure 7.17). Radiolabelled resiquimod or DSPE-conjugated resiquimod, following association with cationic DDA/TDB/DSPE or DDA/TDB respectively, were placed in separate dialysis tubing in Falcon tubes containing Tris buffer (10 mM, pH 7.4). Gamma counts were subsequently measured in the dialysis tubing (total counts) and release media following dialysis, in which by the 24-hour time point only 30 % of the total counts (Figure 7.17A) were found in the release media (which were released in the first hour of dialysis; $P < 0.001$). Therefore free ^{125}I was released through the dialysis tubing with retention of liposomes with radiolabelled resiquimod or DSPE-conjugated resiquimod within the tubing (Figure 7.17A). Removal of free ^{125}I following the radiolabelling of resiquimod or DSPE-conjugated resiquimod was by the use of sodium thiosulphate, which had the ability to convert free iodine to iodide (see equation 7.1). Subsequently, free iodide and tetrathionate were removed using

dialysis in order to obtain an efficiently radiolabelled TLR agonist free from contaminants (Figure 7.17B).

$$I_2 + 2\ S_2O_3^{2-} \rightarrow 2\ I^- + S_4O_6^{2-} \qquad \textbf{Equation 7.1}$$

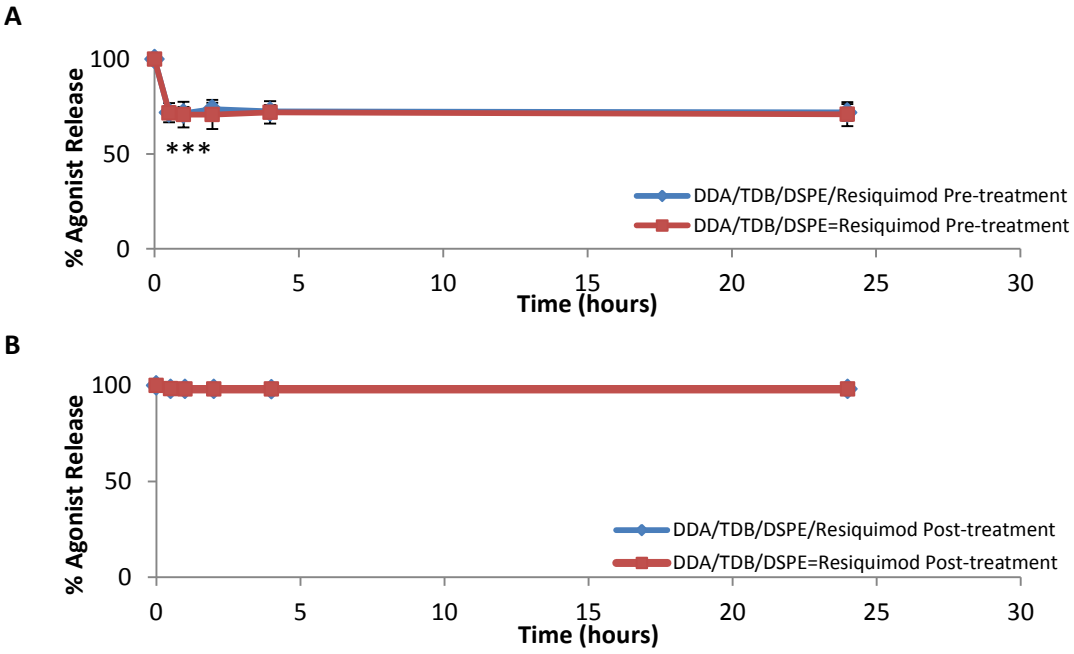


Figure 7-17. Radiolabelling of resiquimod-containing liposome formulations and subsequent removal of free radiolabel following extended dialysis, (A) or treatment with sodium thiosulphate and extended dialysis (B). Results are the mean of triplicate experiments ± SD. Significance between experimental groups was measured by one-way ANOVA (*** $p<0.001$).

Following radiolabelling and dialysis (in order to remove free radiolabel) of resiquimod-containing liposome vaccine formulations, the loading of resiquimod within these formulations was determined. Incorporation of the DSPE=Resiquimod conjugate within the cationic DDA/TDB formulation resulted in high agonist retention (87 %) within the liposome pellet, therefore this showed that this agonist had been successfully incorporated within the liposome delivery system (Figure 7.18). In contrast the addition of radiolabelled resiquimod, post lipid film-hydration to the DDA/TDB/DSPE liposome delivery system resulted in significantly ($P < 0.001$) lower levels of agonist loading in the region of 18-20 % (Figure 7.18). This was due to the fact that resiquimod became less associated with the delivery system due to the lack of charge-related electrostatic interaction (Zaks et al., 2006, Tomai et al., 2007) and, therefore, this agonist required either entrapment by the delivery system or incorporation within the liposome bilayer through chemical conjugation with the DSPE lipid as has been displayed through these studies (Section 7.3.1).

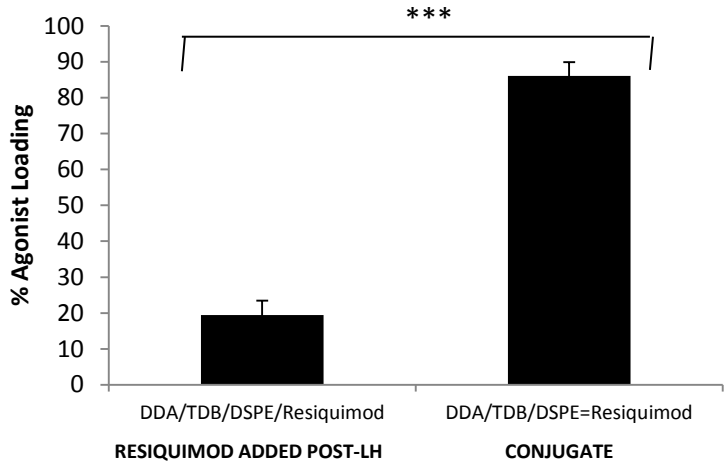


Figure 7-18. Resiquimod loading ability of cationic liposome vaccine formulations, either added post-LH and incorporated within DDA/TDB/DSPE delivery system or conjugated to DSPE and incorporated within DDA/TDB delivery system. Results are the mean of triplicate experiments ± SD. Significance between experimental groups was measured by one-way ANOVA (*** $p<0.001$).

7.3.4.2. Resiquimod release profile from multilamellar vesicles in simulated *in vivo* conditions

Following radiolabelling, the loading and release kinetics of the TLR agonist resiquimod (either when conjugated to DSPE and incorporated into DDA/TDB, or added post lipid film-hydration to the DDA/TDB/DSPE delivery system) were also determined (Figure 7.19). Unlabelled H56 antigen was also added at the *in vivo* (0.1 mg/mL) dose to these formulations as this antigen was added during *in vivo* studies. For both formulations, there was an initial significant release of agonist from the delivery system ($P < 0.001$) within the first hour of the study. However following this initial burst release, both formulations displayed high levels of agonist retention (Figure 7.19 B). In terms of agonist retention, both formulations shared similar release profiles, whether resiquimod was conjugated within the bilayer or added post lipid film-hydration to the delivery system (Figure 7.19 B). However, the DDA/TDB formulation (with bilayer-conjugated resiquimod) was able to initially load a higher amount of resiquimod (Figure 7.18), therefore this formulation was hypothesised to be advantageous in terms of its ability to exert downstream immune responses (Tomai et al., 2007) which will be investigated in Section 7.3.7.

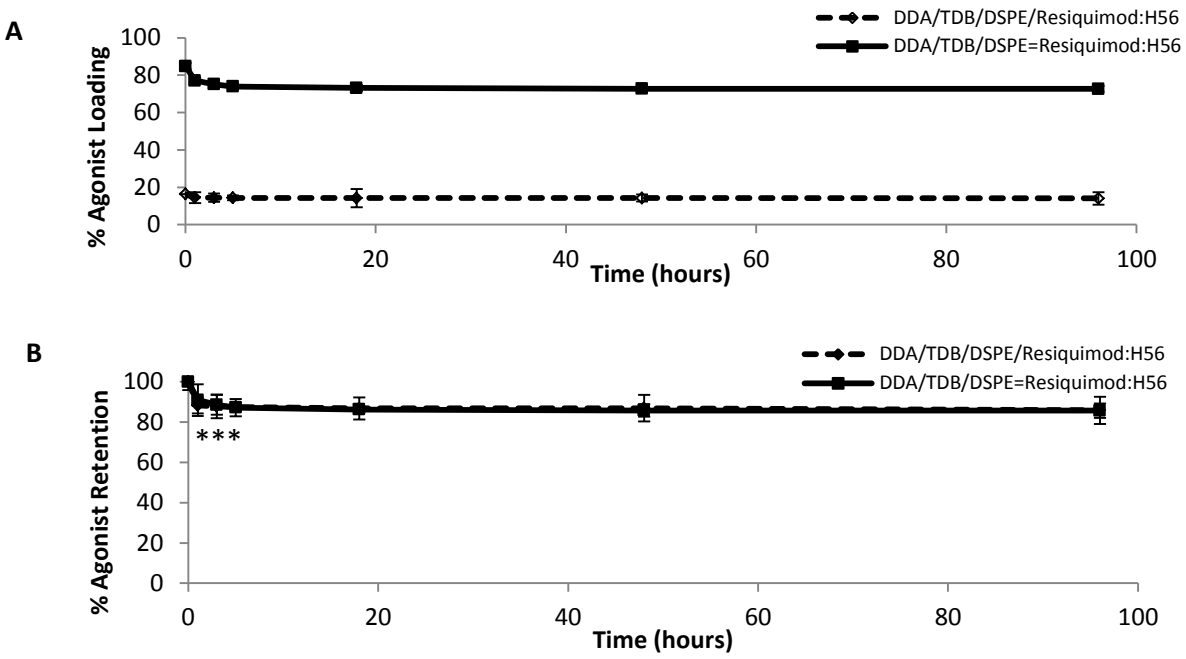


Figure 7-19. Resiquimod TLR Agonist Loading (A) and Release Kinetics (B) from cationic Liposome-TLR formulations in simulated *in vivo* conditions (FCS/Tris; 50:50 v/v; 37 °C). Results are the mean of triplicate experiments ± SD. Significance was measured by one-way ANOVA (*** $p<0.001$) as compared to initial agonist retention at 0 h.

7.3.5. Stability of radioactive markers within resiquimod-containing liposome formulations in order to investigate membrane stability

As discussed previously in Chapters 4 and 5, the membrane stability of liposomes can be determined upon the introduction of trace amounts of ^{3}H-Cholesterol within the bilayer membrane. Liposomes (DDA/TDB/DSPE/Resiquimod and DDA/TDB/DSPE=Resiquimod) were prepared by the lipid film-hydration method, with the incorporation of trace amounts of ^{3}H-Cholesterol (0.17 nM). Dialysis was used to study the retention of ^{3}H-Cholesterol within the liposome membrane. At various time points (1h, 3h, 5h, 18h, 48h and 96h) over a 96 hour study period, 1 mL aliquots of dialysis media were removed and the amount of ^{3}H present were measured. These results showed that incorporation of DSPE and either bilayer-conjugated or surface-attached resiquimod had no effect on the membrane stability of these liposome formulations or release of ^{3}H-Cholesterol from the membrane with these formulations shown to display a maximum ^{3}H-Cholesterol release of ~ 4.5 % from the liposome bilayer (Figure 7.20) over the 96 hour period of the study. These formulations were then taken forward into *in vivo* biodistribution studies.

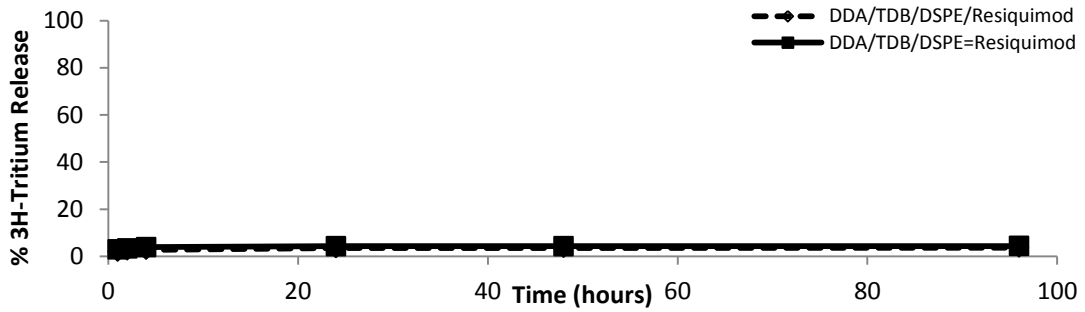


Figure 7-20. Membrane stability of DDA/TDB/DSPE/Resiquimod and DDA/TDB/DSPE=Resiquimod liposomes (prepared by the lipid film hydration method) was studied by the addition of a trace amount of ^{3}H-Cholesterol within the liposome formulation. Results express the % of the original ^{3}H-Cholesterol dose added to liposomes that is detected in the dialysis buffer over a 96 hr time period. The samples were stored at 37 °C in 50 % FCS in Tris buffer (10 mM; pH 7.4). Results denote the mean of triplicate experiments ± SD.

7.3.6. Tracking the *in vivo* biodistribution profile of liposome and TLR agonist

The biodistribution of radiolabelled liposomes and agonist was studied at day one, four and eight post-injection. The day 8 time point was studied (instead of day 14 p.i), as generally very low doses of liposome and antigen were detected at day 14 p.i in previous studies. The percentage liposome and agonist dose retention was examined at these time points in a number of organs including the vaccination site (left quadricep), draining lymph nodes, spleen, muscle, liver, lung, kidney, heart, blood, small intestine and brain.

Previous studies have suggested that the ability to form a depot is important for the function of many adjuvants (Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2011a, Kaur et al., 2011, Christensen et al., 2012). Incorporation of DSPE (either just lipid or lipid=TLR conjugate, however was present at the same molar ratio) was shown to have no significant effect on the liposome dose retention at all time points, with all formulations studied showing a similar liposome 'depot' effect. At day 1 p.i, DDA/TDB:H56 displayed high liposome dose retention of ~ 85 % (which was similar to previous studies (Henriksen-Lacey et al., 2010a, Henriksen-Lacey et al., 2010c)) with still around 30 % of the dose remaining at day 8 p.i. For both novel vaccine formulations incorporating resiquimod, a significant liposome 'depot' was observed with around 70-75 % remaining at day 1 p.i. whereas still 40 % and 25 % of the dose remained at days 4 and 8 p.i, respectively (Figure 7.21A). These results suggested that liposome was retained at a high percentage at day 1 p.i before controlled drainage of liposomal adjuvants by day 8 p.i ($P < 0.001$), as was discussed previously in Chapters 4 and 5 as well as in the previous literature (Henriksen-Lacey et al., 2010a). In terms of resiquimod retention at the SOI, when conjugated to DSPE and incorporated within the DDA/TDB

vaccine delivery system, resiquimod was well retained at the site of injection with around 70 % of the dose remaining at day 1 p.i, 40 % at day 4 p.i and 24 % at day 8 p.i. However, resiquimod agonist adsorbed to the delivery system gave rise to only minimal retention with 6 %, 1 % and 0.5 % of the dose remaining at the injection site at days 1, 4 and 8 p.i respectively (Figure 7.21B). Also resiquimod TLR agonist injected alone was shown to rapidly drain from the injection site. These results showed that resiquimod was unable to adsorb to the delivery system due to the lack of electrostatic interactions. However, when resiquimod was conjugated to lipid and incorporated within the DDA/TDB delivery system this led to the formation of significant 'depot' of agonist ($P < 0.001$) at this site, compared to surface-associated resiquimod (Figure 7.21B). For this formulation, the retention of both delivery system and TLR agonist (as well as antigen, as discussed previously in Chapters 4 and 5) was important in the mechanism of action of liposome vaccines termed the 'depot effect' (Storni et al., 2005, Henriksen-Lacey et al., 2010a).

In terms of liposome drainage to the PLN (Figure 7.21C), all formulations tested showed similar drainage profiles. At day 1 p.i, there was ~ 0.01 % of the dose drained to the PLN (for all formulations tested). However by day 8 p.i, there was a significant increase ($P < 0.001$) of these liposome vaccines draining to the PLN. These results therefore suggested that these cationic liposome vaccines formed a superior depot at the injection site which resulted in a more delayed controlled release of the adjuvant to the draining lymph nodes which was important for the mechanism of action of these vaccine formulations. Also incorporation of the DSPE lipid (either alone, or conjugated to resiquimod) within the DDA/TDB formulation was shown to have no significant effect on the drainage of the liposome delivery system to the PLN (Figure 7.21C). In terms of agonist drainage to the PLN, the levels of resiquimod were very low (< 0.05 %). At day 1 p.i, resiquimod alone was present in higher levels however the levels of this agonist were significantly decreased by day 8 p.i ($P < 0.01$), thereby suggesting rapid drainage of agonist from the injection site (Figure 7.21D). Conjugation of resiquimod to DSPE and incorporation within the bilayer of the delivery system resulted in enhanced drainage to the popliteal lymph node (PLN) by day 8 p.i, compared to when just adsorbed to the delivery system ($P < 0.05$). Therefore the controlled release of delivery system in combination with resiquimod retention by the delivery system resulted in a significant increase in drainage from the injection site to the PLN by day 8 p.i. Pontamine blue was used a marker for the staining of infiltrating monocytes at the left quadricep (site of injection) as described in previous research studies (Tilney, 1971, Henriksen-Lacey et al., 2010a) and in Chapters 4 and 5. The injection of DDA and DDA/TDB liposomes had been shown in earlier studies (Korsholm et al., 2007) to result in the significant recruitment of circulating monocytes and neutrophils at the SOI, which linked with the observed pontamine blue staining in these studies for DDA/TDB:H56. Therefore, this

demonstrated an enhanced monocyte infiltration to this site (Figure 7.21E; also see chapters 4 and 5). This activation of the innate immune system occured due to the presence of the immunostimulatory component TDB within the liposome system which was able to activate a non-TLR pathway (Werninghaus et al., 2009) as opposed to a TLR-based response which acts through the Myd88 pathway. Both other cationic formulations tested in this study (DDA/TDB/DSPE/Resiquimod:H56 & DDA/TDB/DSPE=Resiquimod:H56) also activated localised pontamine blue staining at the injection site (Figure 7.21E). Pontamine blue staining at the injection site correlated with the formation of a 'liposome depot' at the injection site (Figure 7.21A) which suggested that this staining was due to the TDB-induced infiltration of monocytes to the SOI, therefore this showed that this formulation may be able to produce a population of circulating monocytes and neutrophils at this site. These monocytes were then able to transport liposome delivery system (and associated antigen) from the injection site to other areas in the body mainly including the draining lymphatics (as described previously in Chapter 4). However the negative control formulation used in this study (resiquimod alone) was unable to induce pontamine blue staining, which was due to the lack of a delivery system in this formulation hence no liposome depot (Figure 7.21E). Therefore these studies suggested that the high cationic charge of the delivery system was the key driver in showing monocyte influx, as was demonstrated by high localised pontamine blue staining at the SOI for all liposome formulations studied. The inclusion of resiquimod within these cationic liposomal adjuvants had no further effect on the intensity of pontamine blue staining at this site.

As a control, the distally located popliteal lymph node (termed the blank lymph node) was processed in order to determine the % liposome and agonist dose drainage (Appendix Figures 1 and 2). These results showed that only minimal trace levels (< 0.005 %) of the delivered liposome and resiquimod TLR agonist were found at this site with no general trend observed over the time period of this study between formulations. Therefore this showed that the vaccine was draining to the LN on the same leg as injection. The drainage of liposome and TLR agonist to a number of other organs was also investigated during this study. Muscle was excised from the uninjected leg, as well as dissection of other key organs such as the heart, brain, lung, liver, kidney, small intestine and blood. For these organs tested, < 1 % of both the liposome and/or agonist dose was detected. In terms of liposome drainage (Appendix, Figure 3), there was no general trend noticed between formulations in terms of their drainage to other organs at all experimental time points (days 1, 4 and 8 p.i). Whereas in terms of drainage of the TLR agonist resiquimod (Appendix, Figure 4), a slightly higher percentage dose was present at the day 1 time point p.i. This indicated more rapid drainage of the TLR agonist to these organs studied at earlier time points.

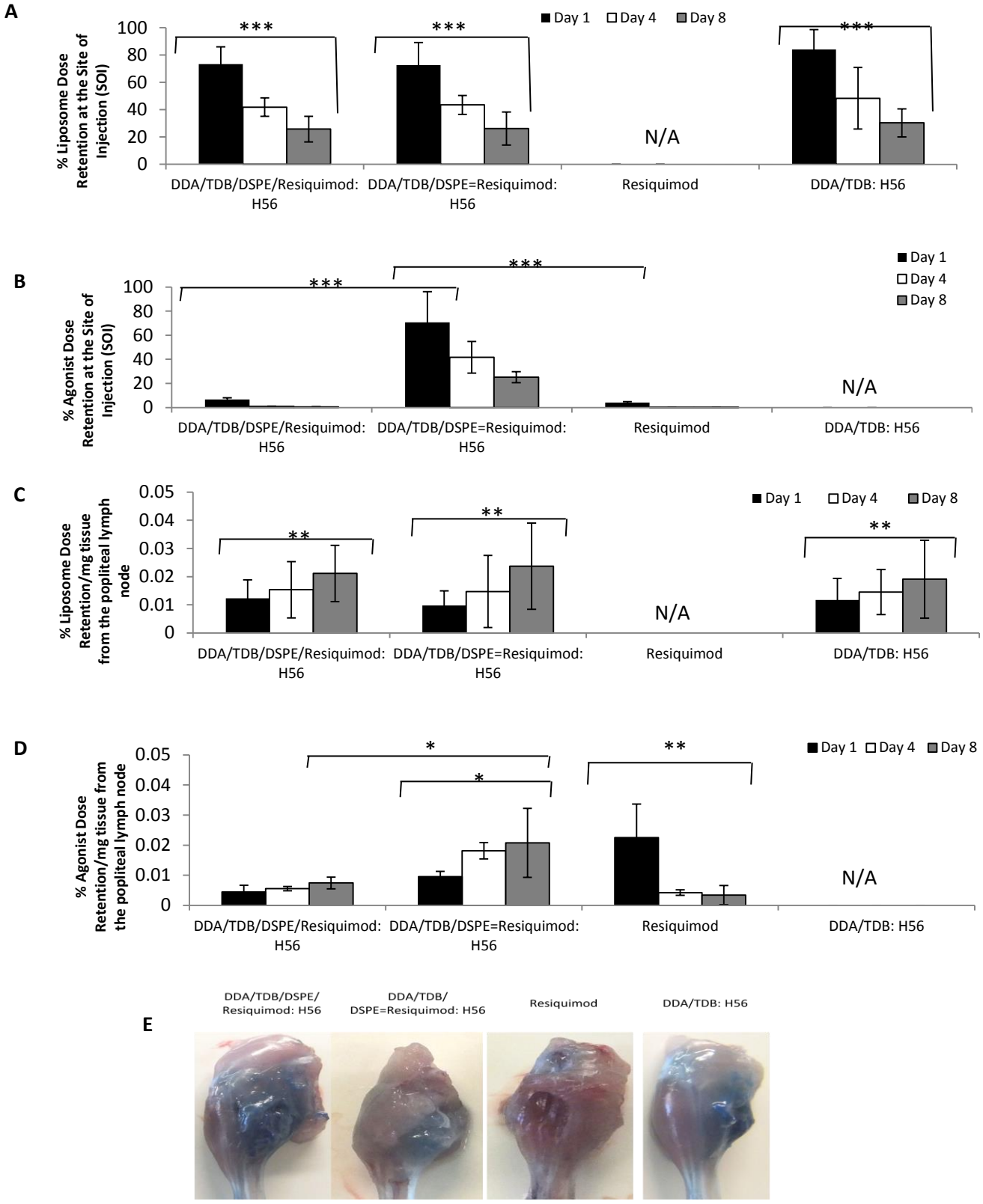


Figure 7-21. Liposome (A, C) and agonist dose retention (B, D) at the SOI and PLN respectively following i.m. injection of either DDA/TDB/DSPE/Resiquimod, DDA/TDB/DSPE=Resiquimod and DDA/TDB (all adsorbing H56 antigen) or resiquimod alone (negative control). (E) shows monocyte influx as indicated by pontamine day staining at day 1 p.i following i.m. injection of these liposome vaccine formulations. The proportion of ^{3}H or ^{125}I radionucleotides at the SOI and PLN as a percentage of the initial dose were calculated. Results represent the mean ± SD of four mice. Significance between experimental groups and time points was measured by one-way ANOVA (*** p<0.001; ** p<0.01; * p<0.05).

The biodistribution profile of these vaccine formulations was important due to their subsequent ability to initiate immune responses. Therefore, the ability of liposomes to successfully retain associated TLR agonist was crucial in order to deliver these components to the same monocyte recruiting cells (Korsholm et al., 2007, Kamath et al., 2009, Kamath et al., 2012). Previous literature has stated that it was apparent that in order for imidazoquinolines (such as resiquimod) to be both safe and effective, that the immune activation at the application site without systemic activation was important (Tomai and Vasilakos, 2011). Therefore the results during these studies showed effective retention of liposome (with bilayer-conjugated resiquimod) at the SOI, followed by controlled drainage of both liposome and resiquimod to the PLN. However, if not conjugated, the liposomes were retained at the SOI but the resiquimod was cleared rapidly, which was similar to resiquimod administered without liposomes.

A number of other studies in the previous literature have suggested that conjugation of TLR agonists to lipid has a subsequent effect on the maintenance of a 'reservoir' of TLR agonist at the injection site (Chan et al., 2009, Andrews et al., 2011, Smirnov et al., 2011). Therefore, the retention and controlled drainage of liposome and TLR agonist from the SOI to the PLN was hypothesised to be advantageous for the induction of immune responses.

7.3.7. Immunisation studies

The aim of these studies was to identify a formulation with the ability to induce strong cell-mediated immune (CMI) responses, characterised by cytokine production at the injection site, spleen and popliteal lymph node (PLN). Cationic DDA/TDB liposomes were prepared as MLVs combined with the H56 TB vaccine, with or without the incorporation of resiquimod; either bilayer incorporated (DDA/TDB/DSPE=Resiquimod:H56) or surface attached (DDA/TDB/DSPE/Resiquimod:H56).

7.3.7.1. The effect of resiquimod on antibody response in the blood sera

The ability of H56 vaccine antigen either delivered alone or in combination with DDA/TDB liposomes without or with resiquimod (bilayer-incorporated or surface attached) to induce IgG (total), IgG1 and IgG2b antibody isotypes was investigated using ELISAs. Mice immunised with resiquimod alone were also used as a negative control during this set of studies. Blood was collected on days 13 and 46 and mice were injected with vaccines on days 0, 14 and 28. In general, there were a number of noted

differences in antibody responses in which liposome-adjuvanted vaccines induced significantly enhanced antibody titres at day 46 ($P < 0.001$) following triple homologous vaccination. At day 13 (following one immunisation), low and minimal responses were noted for all formulations apart from resiquimod which gave no response at all (Figure 7.22). For IgG and IgG1 antibody responses (at day 46) all cationic formulations gave rise to similarly high titres (Figures 7.22 A and B), whereas in contrast DDA/TDB/DSPE=Resiquimod:H56 liposomes induced significantly higher ($p < 0.01$) IgG2b responses (Figure 7.22C). Therefore, this showed that conjugating resiquimod to the bilayer of the vesicles resulted in higher anti-H56 specific IgG2b antibody titres. This indicated that this formulation (efficiently incorporating resiquimod) gave rise to increased antibody responses as has been suggested in the previous literature (Weeratna et al., 2005, Tomai et al., 2007) for formulations that maintained agonist locally at the site of administration.

7.3.7.2. Cytokine Analysis at the Site of Injection

During this investigation there was a need to monitor and study 'leg immune responses' at the site of injection (SOI). This was in order to monitor the immune response in regards to cytokines associated with the inflammasome complex such as interleukin1-beta (IL-1β) as well as interleukin-18 (IL-18) and interleukin-33 (IL-33) as discussed previously in Chapter 4. Immunisation of mice with liposome-adjuvanted H56 vaccines gave rise to significantly increased cytokine production at the SOI, in comparison to immunisation with resiquimod or H56 antigen alone ($P < 0.001$). Interestingly, cationic formulations with bilayer-conjugated resiquimod gave rise to further significantly increased production (Figure 7.23) for all cytokines tested (IL-1β ($p < 0.001$), IL-18 ($p < 0.05$) and IL-33 ($P < 0.01$)) in comparison to the other cationic formulations (DDA/TDB:H56 and DDA/TDB/DSPE/Resiquimod:H56 respectively). These results therefore suggested that bilayer incorporation of DSPE=Resiquimod within the formulation boosted local innate immune responses at the SOI and enhanced the Th1 bias of H56-adsorbed cationic liposomes. This could be due to the fact that resiquimod has the potential to bind the endosome-located TLR7 receptor thus initiating further cellular immune responses that boost the immune response. These results further concluded that proinflammatory cytokine production at the SOI was a result of activation of the inflammasome, causing downstream activation of cellular signalling pathways hence this boosted the immune response (Ogura et al., 2006, Sharp et al., 2009, Tomai and Vasilakos, 2011).

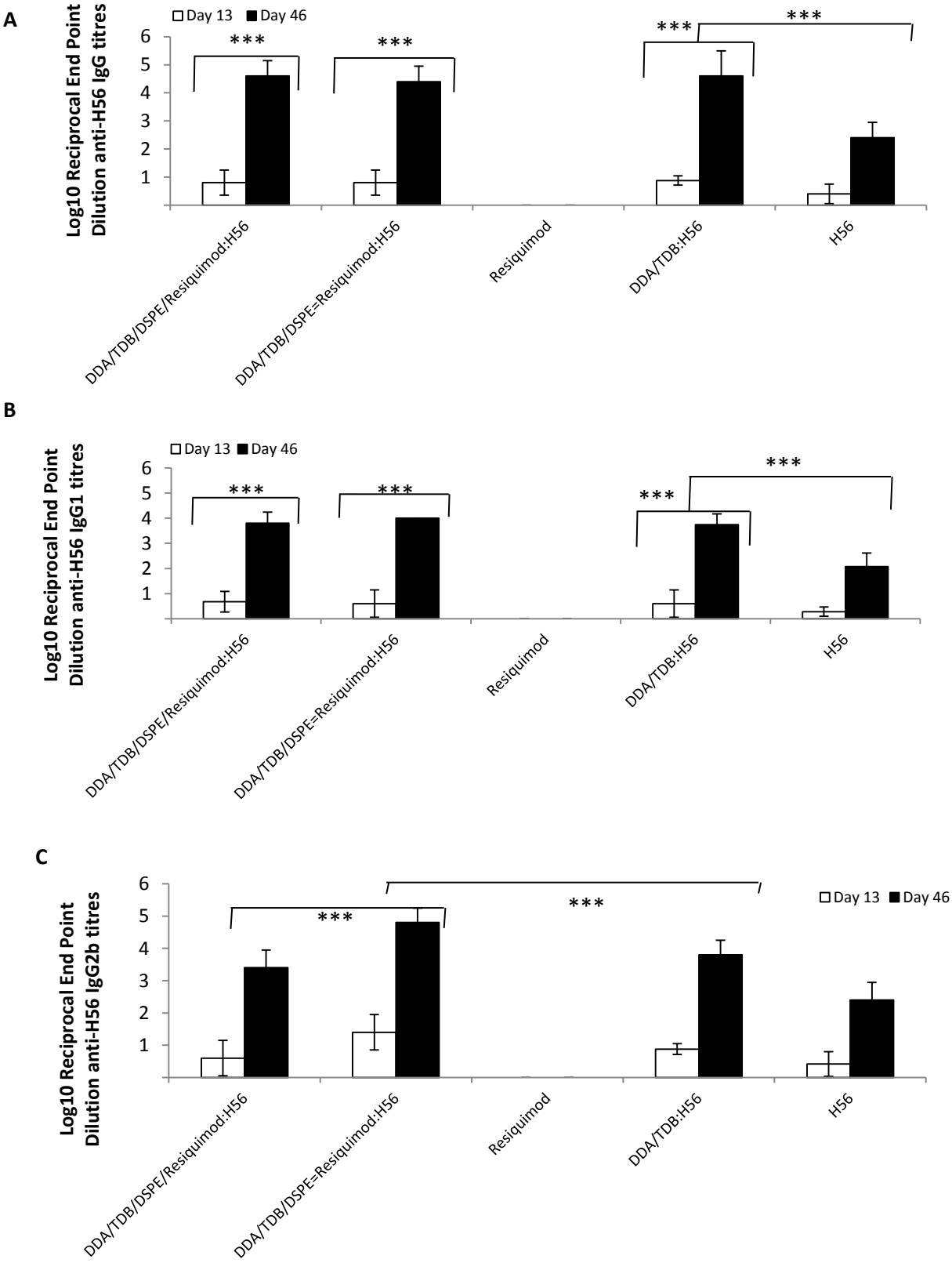


Figure 7-22. H56-antigen specific antibody responses in the blood sera [A: IgG; B: IgG1; C: IgG2b]. Blood was collected at days 13 and 46. Results are the mean of five mice ± SD. Significance between experimental groups and time points was measured by one-way ANOVA (*** $p<0.001$). For A and B, significance was measured compared to the H56-immunised group. For C, significance was measured compared to all other formulations tested.

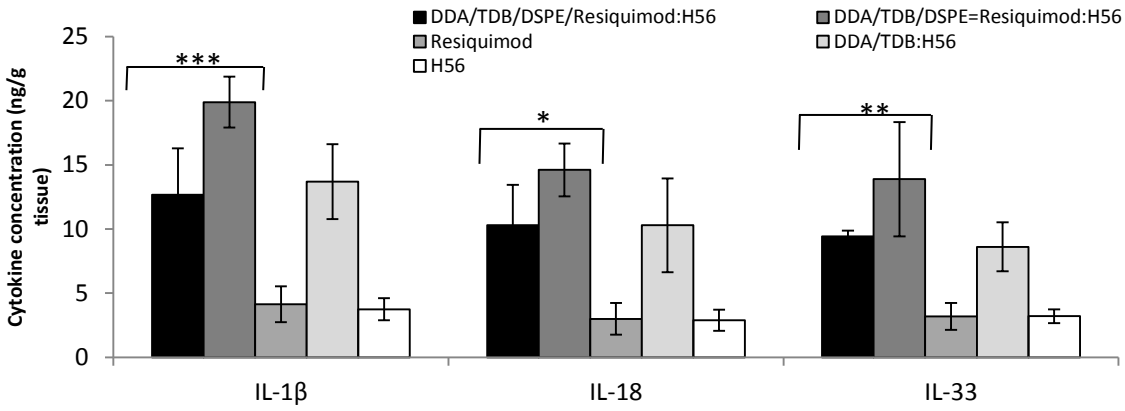


Figure 7-23. IL-1β, IL18 and IL33 production (ng/g tissue) from excised leg muscle from the SOI derived from mice immunised with H56 in combination with either DDA/TDB/DSPE/Resiquimod, DDA/TDB/DSPE=Resiquimod or DDA/TDB. As negative controls, resiquimod and H56 antigen were injected alone. Mice received 3 injections with 2-week intervals; muscle from the SOI was collected at day 49 upon termination of the study. Cytokines were measured from muscle supernatants using sandwich ELISAs. Results represent the mean of 5 mice per group ± SD. Significance between experimental groups was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

7.3.7.3. Cytokine Analysis from *ex vivo* restimulated splenocytes

The supernatants of restimulated splenocytes were assayed for the presence of cytokines IFN-γ, IL-17, IL-2, IL-6, IL-5 and IL-10. H56 antigen was applied to cells at concentrations of 0.05, 0.5 and 5 µg/mL, in addition to ConA (2 µg/mL) stimulation as a positive control and media only as a negative control. Figure 7.24 gave a conclusive overview of the results derived from each experimental group, following restimulation at 5 µg/mL H56. Whilst individual cytokine production in response to media (negative control), ConA (positive control) and also H56 (0.05, 0.5 and 5 µg/mL) was shown in the Appendix (Figure 5).

Very low or no responses were measured in the absence of splenocyte restimulation (for all cytokines tested in this study; Appendix Figure 5, A to F), which suggested that cytokine production was highly-dependent on the activation of splenocytes with previously encountered antigen. Also, as a positive control, splenocytes were restimulated with ConA, which caused significantly higher cytokine production (for all further cytokines tested) which highlighted the ability of ConA to restimulate splenocytes irrespective of the vaccine used to immunise mice (Appendix Figure 5, A to F).

The first cytokine investigated was IFN-γ which is a commonly used marker to determine the efficacy of tuberculosis vaccines (Agger and Andersen, 2001). Upon re-stimulation of splenocytes with H56,

there were significant increases in IFN-γ production for all liposome-injected mice (Figure 7.24); resiquimod and free H56 immunised mice only responded in low levels to H56 at any of the concentrations added. As discussed previously in Chapter 5, IFN-γ production from splenocytes for H56-adsorbing liposomal vaccines was significantly higher than any other cytokine studied during this investigation. For liposome-adjuvanted vaccines on average around 13000-18000 pg/mL IFN-γ was detected following splenocyte restimulation. Interestingly, mice immunised with cationic liposomes with bilayer-conjugated resiquimod gave rise to significantly increased ($P < 0.01$) IFN-γ cytokine production (Figure 7.24) in comparison to cationic DDA/TDB (with or without surface-adsorbed resiquimod). The presence of increased amounts of resiquimod within the formulation resulted in enhanced Th1 immune responses as has been discussed previously for resiquimod and its derivatives upon local application (Bishop et al., 2000, Tomai et al., 2000, Wille-Reece et al., 2005, Smirnov et al., 2011). However, there were no significant differences between the other two cationic DDA/TDB liposome formulations in terms of the production of all cytokines tested, therefore this showed that as resiquimod only was only lowly retained by the delivery system (along with the inclusion of DSPE), this had no effect on downstream cytokine production. Therefore vaccine design in formulating resiquimod to remain at the SOI had important effects for optimal adjuvant activity (Tomai et al., 2007).

The following cytokine investigated was interleukin-17 (IL-17), which is able to act as a potent mediator in delayed-type reactions by increasing chemokine and cytokine production in order to recruit neutrophils and monocytes to the site of inflammation. IL-17 production is stimulated by the action of IL-23 on Th17 cells (Cooke, 2006, Schoenen et al., 2010, Kamath et al., 2012). There was a significant trend noticed in IL-17 production upon immunisation with H56 antigen-adsorbing liposomal vaccines, in comparison to both control groups (free resiquimod and H56); upon restimulation of splenocytes at all concentrations tested this leads to enhanced cytokine production. As described with IFN-γ, peak IL-17 production (around 6000 pg/mL) was noted by splenocytes derived from mice immunised with bilayer-conjugated resiquimod within cationic DDA/TDB liposomes ($P < 0.05$), in comparison to other cationic liposome formulations (Figure 7.24), therefore showing the potential of resiquimod to enhance Th17 responses upon inclusion within the formulation.

Interleukin-2 (IL-2) is important in the regulation of the activities of white blood T-cells (leukocytes) and also in the maturation of regulatory T-cells (Treg) which modulate the immune system and maintain tolerance to self-antigens. In combination with IFN-γ, IL-2 acts in the induction of central memory T-cells which possess multifunctional abilities. As noted previously, immunisation with H56

antigen-adsorbing liposomal vaccines, in comparison to both control groups (free resiquimod and H56) led to enhanced IL-2 production upon restimulation of splenocytes with previously re-encountered H56 antigen (5 µg/mL). As described previously with IFN-γ and IL-17, peak IL-2 production (around 2000 pg/mL) was noted by splenocytes derived from mice immunised with (Figure 7.24) cationic DDA/TDB with resiquimod conjugated to the bilayer of vesicles (Figure 7.24), in comparison to other liposome formulations ($P < 0.01$). This suggested that resiquimod enhanced Th1 immune responses and the combination of liposome, antigen and agonist was important in this induction.

Interleukin-6 (IL-6) is an important cytokine for B cell differentiation towards antibody secreting plasma cells. IL-6 also plays a role in the differentiation of T cells to a Th1 or Th2 phenotype so is therefore an important cytokine for detection of humoural immune responses as well as cell-mediated immunity. This pro-inflammatory cytokine is also secreted by T-cells and macrophages in order to stimulate the immune response, and IL-6 is also enhanced upon activation of macrophages by Toll-like receptor (TLR) agonists within the vaccine formulations. When analysing the results for IL-6 production it was observed that splenocytes derived from mice immunised with liposome-adjuvanted vaccines displayed increased IL-6 cytokine release as has been shown for all other cytokines tested (Figure 7.24). Upon splenocyte restimulation with H56 antigen, cationic DDA/TDB with bilayer-conjugated resiquimod (DDA/TDB/DSPE=Resiquimod:H56) resulted in significant increases in cytokine production, when compared to both other cationic formulations ($P < 0.01$).

Interleukin-5 (IL-5) plays a crucial role in activating naïve T cells towards a Th2 phenotype. IL-5 is also one of the principle cytokines used to detect immune responses after experimental administration of mineral salt adjuvants, such as alum (Noe et al., 2010, Hutchison et al., 2012), due to their Th2 polarising abilities. When analysing the data for IL-5 cytokine release for H56-liposome adjuvanted vaccines it was interesting to note that bilayer conjugation of resiquimod within the formulation down-regulated the production of this cytokine upon splenocyte restimulation with H56 antigen (Figure 7.24), in comparison to other cationic formulations tested ($P < 0.001$). In line with this finding, work in previous literature has demonstrated that upon inclusion of resiquimod within vaccine formulations, this has been shown to inhibit Th2 immune responses (Wagner et al., 1999). However levels of IL-5 were relatively low in comparison to other cytokines tested during this study, which showed the strong Th1 polarising abilities of these cationic liposomal vaccines (Davidsen et al., 2005, Perrie et al., 2013).

The final cytokine of the interleukin family investigated during this immunisation study was interleukin-10 (IL-10) which is also known to have dual Th1/Th2 differentiating abilities as well as

being involved in establishing humoural immune responses. However this cytokine also down-regulates the activities of Th1 cytokines, IL2 and IFN-γ (Fiorentino et al., 1989), as well as inhibiting cell proliferation (Mocellin et al., 2004). The bilayer conjugation of resiquimod within the cationic DDA/TDB (DDA/TDB/DSPE=Resiquimod:H56) led to no difference in the production of IL-10 (Figure 7.24), compared to both other cationic formulations tested.

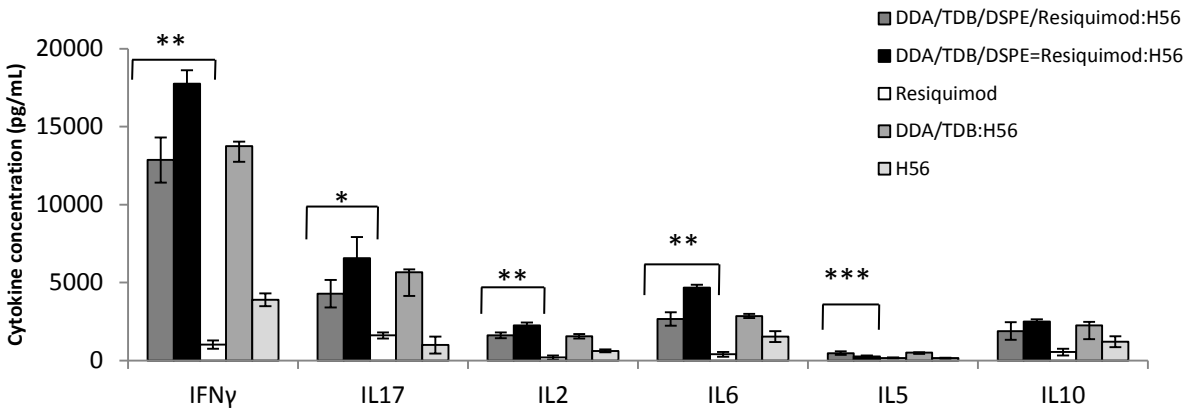


Figure 7-24. Cytokine production [IFN-γ, IL-17, IL-2, IL-5, IL-6 and IL-10] from cultured restimulated splenocytes derived from mice immunised with resiquimod alone (negative control), H56, either alone or combined with DDA/TDB, DDA/TDB/DSPE/Resiquimod (surface attached resiquimod) or DDA/TDB/DSPE=Resiquimod (bilayer-incorporated resiquimod). Mice received 3 injections with 2-week intervals and splenocytes were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 72 hrs in the presence of H56 (at 5 μg/mL). Cytokines were detected from splenocyte supernatants and measured using sandwich ELISAs. Significance between experimental groups was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

7.3.7.4. Cytokine Analysis from *ex vivo* restimulated popliteal lymph node cells

The supernatants of restimulated popliteal lymph node cells were also assayed for the presence of cytokines IFN-γ, IL-17, IL-2, IL-6, IL-5 and IL-10 following restimulation of these cells with H56 antigen (Ag85B-ESAT6-Rv2660c) at concentrations of 0.05, 0.5 and 5 μg/mL, in addition to ConA (2 μg/mL) stimulation as a positive control. Also as a negative control, cytokine production was measured in the absence of splenocyte restimulation (media only). Figure 7.25 gave a conclusive overview of the results derived from each experimental group, following restimulation at 5 μg/mL H56. Whilst individual cytokine production in response to media (negative control), ConA (positive control) and also H56 (0.05, 0.5 and 5 μg/mL) was shown in the Appendix (Figure 6).

The main results from these studies showed that unstimulated PLN gave rise to background levels of all cytokines tested (< 100 pg/mL) during this investigation, which suggested that cytokine production was highly-dependent on the activation of splenocytes with previously encountered

antigen. Also the restimulation of PLN cells with ConA led to high positive responses of all cytokines tested with no hugely significant differences measured between formulation groups (Appendix Figure 6, A to F). Also liposome-adjuvanted vaccines gave rise to significantly higher ($P < 0.001$) cytokine production than negative control groups (H56 and resiquimod respectively; Figure 7.25).

For H56-antigen restimulated splenocytes, IFN-γ production peaked following immunisation with DDA/TDB/DSPE=Resiquimod:H56, which was significantly higher than other liposome formulations tested ($P < 0.05$) and negative control groups ($P < 0.001$). As was noted during *in vivo* biodistribution studies (Section 7.3.6), including DSPE-conjugated resiquimod within the formulation (DDA/TDB/DSPE=Resiquimod:H56) resulted in increased retention and controlled release drainage to the LN, so higher amounts of TLR agonist drained to the PLN (in combination with liposome delivery system), leading to increased cytokine production following PLN restimulation. Similar results were described for IL-17 and IL-6 cytokine production (Figure 7.25), in which the general trend that cationic liposome formulations with bilayer-conjugated resiquimod enhanced the levels of production of these cytokines following PLN restimulation with previously encountered H56 antigen.

Upon PLN restimulation with H56 antigen (5 μg/mL), this resulted in inhibition of Th2 cytokines following immunisation of mice with DDA/TDB/DSPE=Resiquimod:H56 compared to other cationic liposomes tested. This suggested that vaccine design to formulate resiquimod to remain at the SOI and subsequently drain to the lymph node led to the down-regulation of Th2 cytokine production (Tomai et al., 2007). As described previously for IL-5 responses at the spleen, these responses at the PLN are also the lowest of all cytokines tested with levels of < 400 pg/mL being measured (Figure 7.25). As at the spleen, there were no significant differences between the liposome-adjuvanted vaccines in terms of the release of IL-10 at the PLN (Figure 7.25) therefore this suggested that resiquimod inclusion within the formulation does not affect these responses both at the spleen and PLN.

Therefore, the main findings from the results, in terms of immune responses at the spleen and PLN, were that bilayer inclusion of resiquimod (resiquimod conjugation) within the formulation (DDA/TDB/DSPE=Resiquimod:H56) resulted in enhanced production of Th1/Th17 cytokines and down-regulation of Th2 cytokines from restimulated splenocytes. These results were in comparison to other cationic liposome vaccines tested, DDA/TDB/DSPE/Resiquimod:H56 (TLR agonist added post-lipid film hydration) and DDA/TDB:H56. Similar trends at both of these sites showed that vaccine antigen (in association with liposomal adjuvant) drained to different areas of the body as is shown for the biodistribution of these tested vaccine formulations (Section 7.3.6), which was especially important for T-cell rich sites associated with the initiation of the immune response.

Also all vaccines formulated within liposomal adjuvants gave rise to significantly higher cytokine production (for all cytokines tested) than the negative control groups (H56 and resiquimod respectively). This showed the importance of formulating sub-unit vaccines, such as H56, with delivery system in order to achieve optimal immune responses (Henriksen-Lacey et al., 2010a).

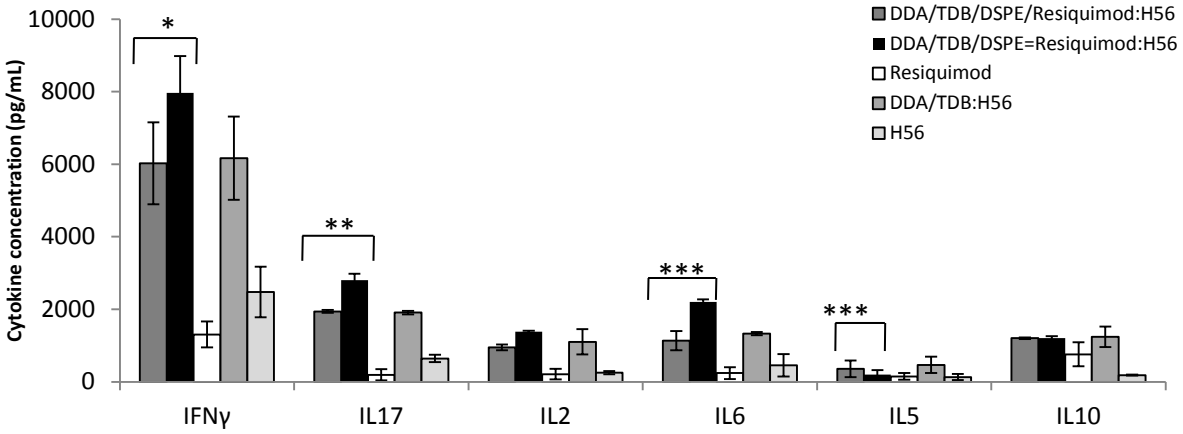


Figure 7-25. Cytokine production [IFN-γ, IL-17, IL-2, IL-5, IL-6 and IL-10] from cultured restimulated popliteal lymph node cell derived from mice immunised with resiquimod alone (negative control), H56 (Ag85B-ESAT6-Rv2660c), either alone or combined with DDA/TDB, DDA/TDB/DSPE/Resiquimod (surface attached resiquimod) or DDA/TDB/DSPE=Resiquimod (bilayer-incorporated resiquimod). Mice received 3 injections with 2-week intervals and splenocytes were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 72 hrs in the presence of H56 (at 5 µg/mL). Cytokines were detected from splenocyte supernatants and measured using sandwich ELISAs. Significance between experimental groups was measured by one-way ANOVA (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

7.4. Conclusions

During these studies, we have been able to design and synthesise a novel conjugate between lipid and TLR agonist, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and resiquimod, respectively. This was a two-step reaction, involving succinylation and esterification, which was confirmed by TLC, IR and NMR. Cationic DDA/TDB liposomes, with the inclusion of this DSPE=Resiquimod conjugate structure, displayed a vesicle size in the region of 450-500 nm and a zeta potential of 65 mV which was similar to previously characterised DDA/TDB (which was shown throughout this thesis). Addition of the H56 subunit vaccine antigen led to no further changes in vesicle characteristics. It was suggested that the intercalation of the lipid tail of the lipid-TLR conjugate into the bilayer of the delivery system resulted in the displaying of the TLR agonist at the liposome surface as has been described previously in the literature (Smirnov et al., 2011).

The high association of resiquimod (when conjugated to DSPE) and/or antigen within the cationic liposome formulation, DDA/TDB was confirmed following radiolabelling experiments. As a negative

control, resiquimod was added to pre-formed DDA/TDB/DSPE liposomes (also with or without H56 antigen). There were no measured differences in the vesicle characteristics; however, there was a significant ($P < 0.001$) reduction in resiquimod loading by the liposome formulation thus showing that resiquimod was unable to bind to the delivery system through electrostatics and instead required to be associated by other means as has been shown through inclusion within the bilayer.

The biodistribution of these formulations was investigated following dual radiolabelling of the liposome delivery system and TLR agonist respectively. The bilayer incorporation of DSPE-conjugated resiquimod into DDA/TDB leads to 'depot formation' of TLR agonist at the SOI. This was also shown to lead to the subsequent controlled release and drainage of resiquimod to the draining popliteal lymph node (PLN). *In vivo* immunisation studies using the latent TB vaccine, H56, concluded that bilayer incorporation of lipid-TLR conjugate within the DDA/TDB vaccine formulation resulted in a significant increase in release of Th1 cytokines including IFN-γ, IL-2, IL-1β and IL-18 both at the spleen, popliteal lymph node and site of injection respectively. Whereas, this formulation was shown to down-regulate the production of Th2 cytokines such as IL-5 at both the spleen and PLN.

In terms of antibody responses in the blood sera, all cationic formulations displayed similar levels of H56-specific IgG and IgG1 titres. However, inclusion of resiquimod within the bilayer of the liposome adjuvant led to increased anti-H56 IgG2b antibody titres, therefore this showed that resiquimod inclusion within the adjuvant strongly enhanced the generated Th1 immune response. Therefore, these studies showed that this novel formulation, with resiquimod conjugated within the bilayer of the vesicles was able to be delivered with DDA/TDB which has subsequently helped increase the bias further towards Th1 immunity.

Chapter 8: General Discussion

8.1. Optimisation and formulation of liposomal adjuvants

During initial studies we sought to optimise and formulate previously characterised cationic DDA/TDB liposomes. This liposome system is currently in phase I clinical trials in combination with the TB Ag85B-ESAT6 fusion protein, otherwise known as Hybrid 1 (NCT no: NCT00922363). To determine the role of charge in the initial development and formulation of liposomes, the cationic lipid DDA was substituted with DSPS therefore giving a high anionic surface charge to the liposomes due to the anionic nature of DSPS. These novel liposomes exhibited a fairly similar size to DDA/TDB, being in the region of 400 nm, however display a highly anionic surface charge.

The effect of hydration buffer and dilution buffer was investigated, in which PBS-hydrated liposomes tended to aggregate (Yan and Huang, 2009) with this effect being deemed greater for cationic DDA/TDB compared to anionic DSPS/TDB liposomes. Upon liposome characterisation, dilution buffer also had an effect on vesicle characteristics with this effect being higher for PBS-hydrated liposomes. Increasing the concentration of PBS dilution buffer led to slight neutralisation of the surface charge. However for Tris-hydrated liposomes, vesicle characteristics remained fairly constant both for vesicle size and surface charge which has been addressed during short term stability studies. Therefore as a product specification the ideal hydration buffer for formulation of liposomes during these studies was Tris (10 mM; pH 7.4) whilst using a weak dilution (of the same buffer) for characterisation

Further modulation of the liposome surface charge was displayed by increasing the content of the neutral lipid DSPC within cationic DDA/TDB and anionic DSPS/TDB liposomes respectively. Through increase in the concentration of DSPC within the formulation this resulted in neutralisation of the surface charge, with complete DSPC replacement also leading to a significant increase in the vesicle size. During this thesis the latency-associated TB subunit vaccine H56 (Ag85B-ESAT6-Rv2660c) has been used in initial characterisation and loading studies, as well as immunisation studies. Due to the anionic charge of this subunit vaccine, high adsorption was noted for cationic DDA/TDB liposomes with observed changes in vesicle characteristics measured.

8.2. The role of liposome surface charge and antigen charge for the depot effect and subsequent ability to induce immune responses

It has been hypothesised that cationic liposomes interact with negatively charged serum proteins at the injection site, therefore resulting in the formation of a depot of liposome and co-administered

antigen (Henriksen-Lacey *et al.*, 2010a). Cationic DDA/TDB and anionic DSPS/TDB liposomes were formulated with the H56 subunit vaccine or the cationic model protein lysozyme during initial studies. Upon formulation between oppositely charged liposome and antigen components this resulted in high antigen loading, due to electrostatic binding at the liposome surface (as shown by trypsinisation studies) which led to observed changes in vesicle characteristics. The delivery of anionic DSPS/TDB, and co-administered antigen, led to rapid drainage from the injection site. These non-depot forming liposome vaccines appeared to drain quickly to the popliteal lymph node which was disadvantageous for the generation of Th1 immune responses. In contrast, cationic DDA/TDB liposomes (and associated antigen) were efficiently retained at the injection site and displayed more controlled release and drainage to the local lymph node. This displayed retention and drainage characteristics of cationic DDA/TDB appear to be beneficial in the induction of Th1 cytokines (Perrie *et al.*, 2013) at the spleen (IFN-γ and IL-2) and injection site (IL-1β and IL-18) as well as eliciting enhanced antibody responses. In terms of splenocyte proliferation, these studies suggested that liposome charge had an important role to play in terms of the ability of liposome vaccine formulations to deliver antigen to APCs in the spleen with DDA/TDB liposomes being able to induce a population of T-cells that could respond upon re-exposure to the disease antigen. The induction of cytokine production at the injection site suggested a link with the inflammasome in the generation of enhanced immune responses, upon injection of liposome adjuvants such as CAF01 (Sharp et al., 2009, Schweneker et al., 2013).

8.3. The effect of liposome preparation method; does antigen entrapment play a role in the generated immune response?

As it was previously established that a positive (cationic) charge, along with antigen adsorption played a role in the biodistribution of liposomal adjuvants, the role of liposome preparation method was also investigated. Liposomes have been prepared by the dehydration-rehydration vesicle (DRV) method and the double emulsion (DE) method (Nordly *et al.*, 2011c), with the aim of entrapping the antigen within the delivery system. As described previously both cationic DDA/TDB and anionic DSPS/TDB were co-formulated with either the H56 subunit vaccine or model protein lysozyme. DE formulations displayed a reduction in vesicle size, and exhibited lower initial levels of antigen loading, in comparison to their respective DRV formulations. However antigen loading was still enhanced through addition of oppositely charged vaccine components therefore this showed that charge-mediated electrostatics still played a role in this interaction. Trypsin digestion studies have

shown that antigen was efficiently entrapped within DRV-formulations, whereas for DE formulations antigen was partly entrapped and also surface-adsorbed to the delivery system.

A similar general trend was observed, in which irrespective of preparation method, anionic DSPS/TDB liposomes were removed faster from the injection site compared to their respective cationic DDA/TDB formulations. However as described previously the interaction between oppositely charged liposome and antigen leads to the formation of an antigen depot at the injection site, whereas similarly charged vaccine components lead to low adsorption efficiency and subsequent removal from the injection site. In terms of vaccine drainage to the popliteal lymph node, non-depot forming anionic DSPS/TDB drained faster to this site irrespective of preparation method. Whereas as described in Section 8.2, cationic DDA/TDB (formulated as DRV and DE) have a more controlled release drainage pattern which was important for the generation of an immune response when formulated with the H56 subunit vaccine. In general, DRV and DE DDA/TDB:H56 formulations gave rise to similar high release of IFN-γ and IL-17, as well as low release of IL-5, at both the spleen and PLN (upon restimulation with previously encountered antigen) thus showing the high Th1/Th17 profile of these vaccine adjuvants. However, anionic DRV DSPS/TDB (and not DE) liposomes enhanced IL-5 release at both these sites thus this demonstrated the change in bias towards Th2-like responses. In terms of antibody production, in general cationic DDA/TDB formulations induced higher responses than their anionic counterparts. DRV-formulated vaccines enhanced the responses for DDA/TDB:H56 liposomes, when compared to their DE-counterparts, with increased IgG2 (a and b) responses noted. Whilst for IgG1, similar responses were measured irrespective of the preparation method. These studies have shown that liposome preparation method plays a role in antigen presentation to APCs and the subsequent immune response, as characterised by cytokine production and antibody responses.

8.4. The inclusion of immunostimulatory TLR agonists within cationic liposome adjuvants

The versatile nature of liposomes allows for the inclusion of immunostimulatory molecules within the delivery system. With this in mind the TLR3 agonist, polyI:C, was chosen as a candidate molecule to include within cationic liposome vaccine adjuvants with the aim of further optimisation of these vesicle systems (Milicic *et al.*, 2012). The inclusion of this TLR agonist within liposome vaccine adjuvants was shown to increase $CD8^+$ T-cell responses due to the cross-presentation of antigen (Zaks et al., 2006, Nordly et al., 2011c). Due to opposing charges, the binding between the cationic

liposome surface (of MLV and SUV) and the anionic TLR agonist was mediated through charge-mediated electrostatic interactions, which at the whole dose concentration was also shown to lead to significant dose-dependent changes in vesicle characteristics such as vesicle aggregation. However variation in liposome preparation was also used to entrap TLR agonist within the delivery system through the use of the DRV method, with less significant changes in vesicle characteristics being noted. Through incorporation of antigen within liposome-TLR agonist formulations (DDA/TDB/polyI:C) this resulted in no significant changes in initial antigen loading.

8.5. Optimisation of cationic liposome adjuvants with incorporation of TLR agonists; stepwise incorporation and high shear mixing

In terms of combined liposome-TLR agonist formulations it was important to maintain constant vesicle size and surface charge characteristics in terms of their *in vivo* mode of action. With this in mind, the polyI:C TLR agonist dose could be incorporated step-wise within the formulation, followed by either vortexing or high shear mixing. Through stepwise incorporation of polyI:C within the formulation (prepared as MLV) this led to slower incorporation and high loading of TLR agonist and hence no measured significant changes in vesicle characteristics. Whereas initial formulation of cationic DDA/TDB through high shear mixing (HSM), led to a significant reduction in vesicle size with no such changes noted for the zeta potential of these systems. Through the introduction of high energy forces using HSM this led to the breakdown of the outer layers of the delivery system in order to reduce the size of liposomal adjuvants. This process was also used upon stepwise incorporation of increasing concentrations of polyI:C, followed by HSM, which led to no further significant changes in vesicle characteristics. Therefore through HSM, cationic liposomal adjuvants can be formulated with reduced vesicle size which could still incorporate and load high levels of TLR agonist, up to the *in vivo* dose level.

8.6. Investigating the stability of liposome systems

The stability of liposomes was a crucial issue for the use of these vesicles as vaccine delivery systems. The storage and handling of these liposomes was extremely important in order to maintain their ability to act as vaccine adjuvants and also in being able to transport them to other countries for use in vaccination. The handling of these liposomes is very difficult due to their fragility and

delicate nature. Liposomes in solution offer a weak chemical and physical stability – therefore explaining the reason for only a relatively small number of liposomal-based vaccines being available, with several currently in clinical trials. Since most vaccines were thermally unstable, there was an important need to create stable vaccine formulations which can be transported to third world countries. The distribution of vaccines required "cold-chains" with functional freezers, as well as reliable transport links (Christensen *et al.*, 2007a) therefore making vaccine stability a key criteria in the development of these delivery systems. Throughout this thesis, the stability of these liposome vaccine adjuvants (with and without antigen) was investigated in a number of short 28-day studies, as well as longer term 6 month stability trials. These studies were carried out to show the effect of hydration buffer, liposome surface charge, liposome preparation method as well as the inclusion of immunostimulatory TLR agonists on the stability of the delivery system in terms of measured vesicle characteristics.

Throughout this thesis, vesicles were demonstrated to be relatively stable during short term studies (28 days) with storage at the 'cold chain' (4 °C) leading to the maintenance of constant vesicle characteristics. Storage at 25 °C leads to more slight changes in vesicle characteristics, however vesicles remain relatively stable both without and with subunit vaccine antigen (H56). Longer term stability trials were carried out for MLV and HSM formulations with the inclusion of immunostimulatory polyI:C within the liposome system. Storage of liposomes in accelerated conditions (40 °C) led to a general trend for more significant changes in liposome characteristics, with liposomes increasing in size due to vesicle aggregation. This suggested vesicle aggregation is temperature-dependent, as liposome storage at 4 and 25 °C resulted in lesser changes in characteristics for all formulations tested. Therefore, the storage of these liposomes under lower temperatures will potentially increase the shelf-life and stability of these vesicles. During liposome formulation, the optimisation of the preparation process including the temperature, ionic strength of the buffer as well as the addition of stabilising components within the bilayer (including cholesterol and immunostimulatory lipids) could lead to increased stability of the delivery system also (Grit and Crommelin, 1993, Zuidam et al., 1995, Davidsen et al., 2005).

8.7. The inclusion of immunostimulatory resiquimod within cationic liposomes; does conjugation with lipid and inclusion within the membrane bilayer affect the biodistribution and subsequent immune response?

Resiquimod is an agonist that activates the endosome-located TLR7 receptor. This TLR agonist has had mixed success within vaccine formulations due to its ability to very quickly distribute throughout the body rather than remain at the site of injection (Tomai *et al.*, 2007). This systemic distribution is disadvantageous for the initiation of immune responses. Therefore the formulation of resiquimod to remain at the injection site, along with antigen and delivery system, was an important strategy in order to overcome this issue. This can be achieved through dermal or topical application, as well as formulation within liposome vaccine adjuvants (Tomai et al., 2007, Smirnov et al., 2011). In order to incorporate resiquimod within these liposomes, it was hypothesised to formulate a conjugate between DSPE lipid and this TLR agonist. This lipid-TLR agonist conjugate was successfully incorporated within the bilayer of cationic DDA/TDB liposomes. This has been proven using radiolabelling and biodistribution studies. This novel cationic DDA/TDB liposome formulation (with bilayer incorporated resiquimod) was strongly retained at the injection site following intramuscular administration. In terms of drainage to the PLN, both delivery system and TLR agonist display controlled release kinetics (when resiquimod was conjugated to the bilayer of vesicles) in terms of movement from the injection site which was also important for increased immune responses due to the simultaneous delivery of antigen, agonist and adjuvant (Henriksen-Lacey et al., 2010a, Kamath et al., 2012). This was correlated by enhanced Th1/Th17 immune responses both at the spleen and PLN as characterised by increased production of IFN-γ, IL-2 and IL-17 as well as the down-regulation of Th2 responses (as characterised by a reduction in IL-5 release). In terms of cytokine analysis at the SOI, efficient inclusion of resiquimod within the formulation resulted in increased production of IL-1β, IL-18 and IL-33 which suggested a further role for this liposome formulation (and associated TLR agonist) in the activation of the inflammasome in the downstream induction of these immune responses.

8.8. Final conclusions

To summarise, in relation to initial aims and objectives set in Chapter 1 of the thesis, these studies have shown that;

1. A novel anionic liposome system comprising DSPS and TDB was formulated.
2. Cationic DDA/TDB liposomes have an advantage over their anionic DSPS/TDB counterparts in terms of their ability to promote Th1 immune response, due to the formation of a strong 'antigen depot' at the injection site as well as controlled drainage to the lymph nodes.
3. Formulation of liposomal adjuvants by the DRV method resulted in higher levels of antigen entrapment within the system compared to their respective DE counterparts. This led to no significant effect on the Th1-inducing effects of cationic DDA/TDB, however caused elevation in the Th2-inducing effects of anionic DSPS/TDB liposomes
4. The TLR3 agonist, polyI:C, could be efficiently loaded by cationic DDA/TDB liposomes with resulting changes in vesicle characteristics being measured including vesicle aggregation, neutralisation of the surface charge and greater antigen release however this varied depending on the liposome preparation method used.
5. The vesicle characteristics (size and zeta potential) of polyI:C containing DDA/TDB liposomal adjuvants could be maintained through stepwise incorporation of TLR agonist within the delivery system, and be further modified through the use of the high shear mixing.
6. A novel conjugate structure has been synthesised between the TLR7 agonist resiquimod and DSPE lipid, which was confirmed through chemical analysis methods.
7. This conjugate structure was formulated within cationic DDA/TDB liposomes, with this novel vaccine adjuvant being shown to significantly increase Th1 responses and down-regulate Th2 responses (compared to DDA/TDB) at the SOI, spleen and PLN as well as leading to enhanced IgG2b antibody titres in the blood sera.

8.9. Future work

With regards to future experiments, the immunogenicity of the non-disease antigen lysozyme could be determined following co-formulation with DDA/TDB and DSPS/TDB liposomes prepared by a variety of methods. This could be in order to show whether liposome surface charge, liposome-antigen interactions or the method of antigen attachment plays a more crucial role in stimulating the immune response. Part of the mechanism for the mode of action of liposomal adjuvants is termed

the ‘depot effect’ however it may be interesting to elucidate if further factors played a role in this process, such as the activation of downstream immune responses. Further optimisation of cationic DDA/TDB including TLR agonists and other immunostimulatory molecules might also be an interesting route to further understand how these liposomal vaccines function. Future work may also focus on the effect of preparation method on the biodistribution of TLR agonists. Also further TLR agonists could be included within liposome formulations to see what effect this exerts on the mode of action of the vaccine in terms of the immune response. In order to further understand the immunogenicity of the formulations studied in this thesis, TB challenge studies could be carried out and also larger animal models could be used.

To finalise, cationic DDA/TDB liposomes have been further described as an ideal platform for the delivery of subunit vaccines. The versatile and flexible nature of this vaccine adjuvant system allows for the further modification and optimisation with the aim of further stimulating immune responses. The results presented within this thesis will further bring forward our understanding of the way cationic vaccine adjuvants exert their mode of action.

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Appendix

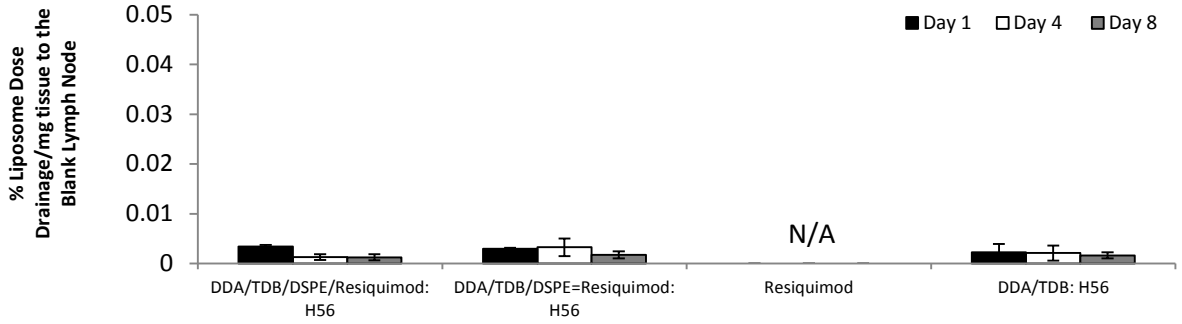


Figure A-1. DDA/TDB/DSPE/Resiquimod, DDA/TDB/DSPE=Resiquimod and DDA/TDB, all adsorbing H56 antigen. The proportion of ^{3}H radionucleotide at the blank lymph node (BLN) as a percentage of the initial dose was calculated. Results represent the mean ± SD of four mice.

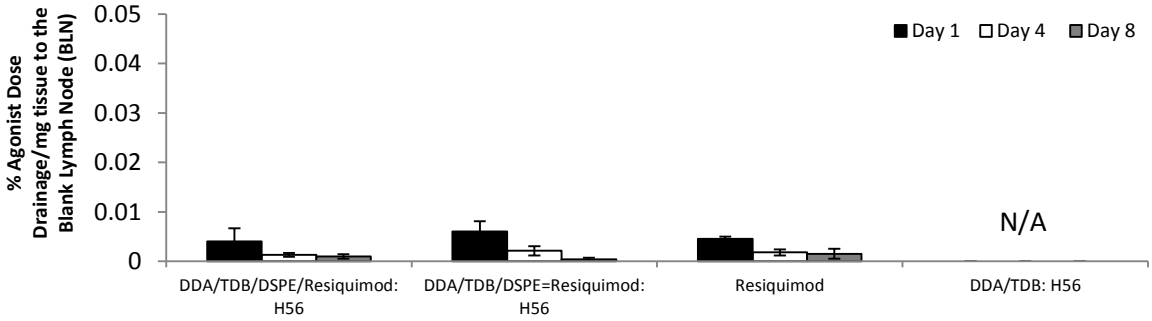
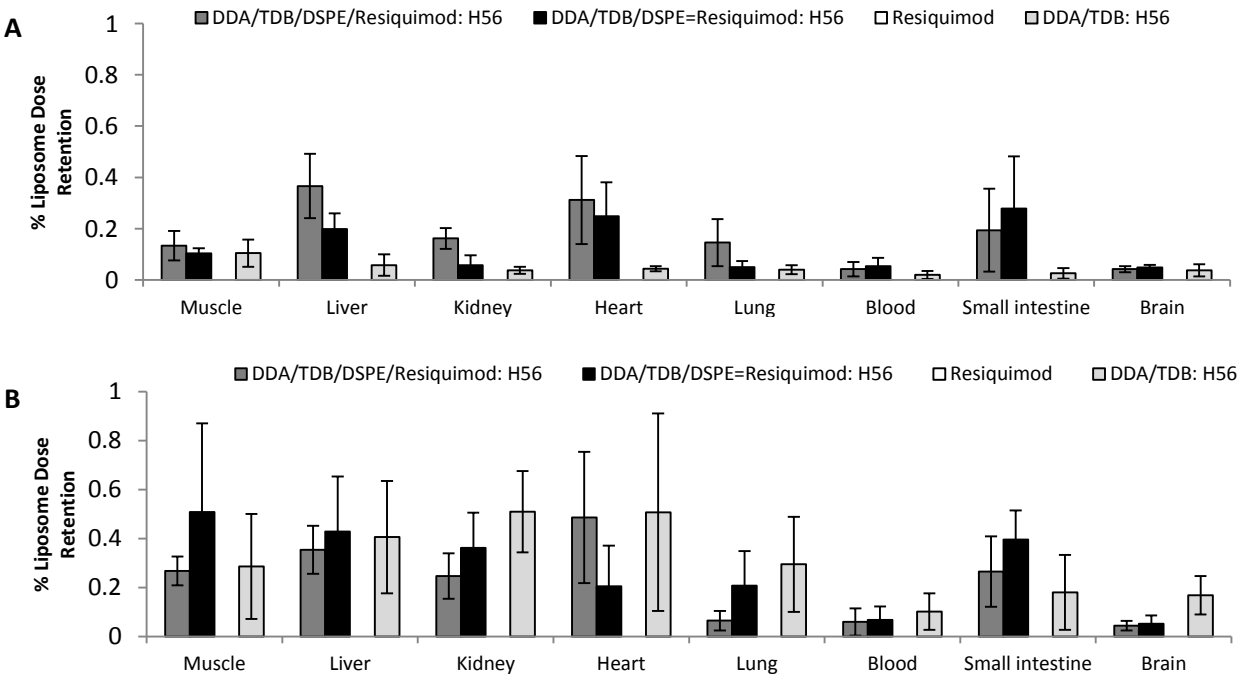


Figure A-2. DDA/TDB/DSPE/Resiquimod, DDA/TDB/DSPE=Resiquimod, Resiquimod and DDA/TDB, all adsorbing H56 antigen apart from free resiquimod. The proportion of ^{125}I radionucleotide at the blank lymph node (BLN) as a percentage of the initial dose was calculated. Results represent the mean ± SD of four mice.



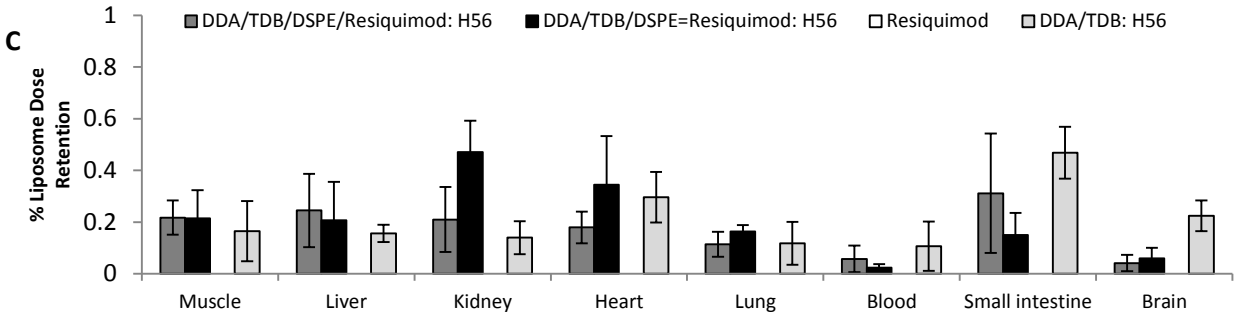


Figure A-3. A range of tissues were collected at (A) one (B), four and (C) eight days p.i and processed to determine the proportion of ^{3}H-labelled liposomes. Results represent the mean ± SD of four mice.

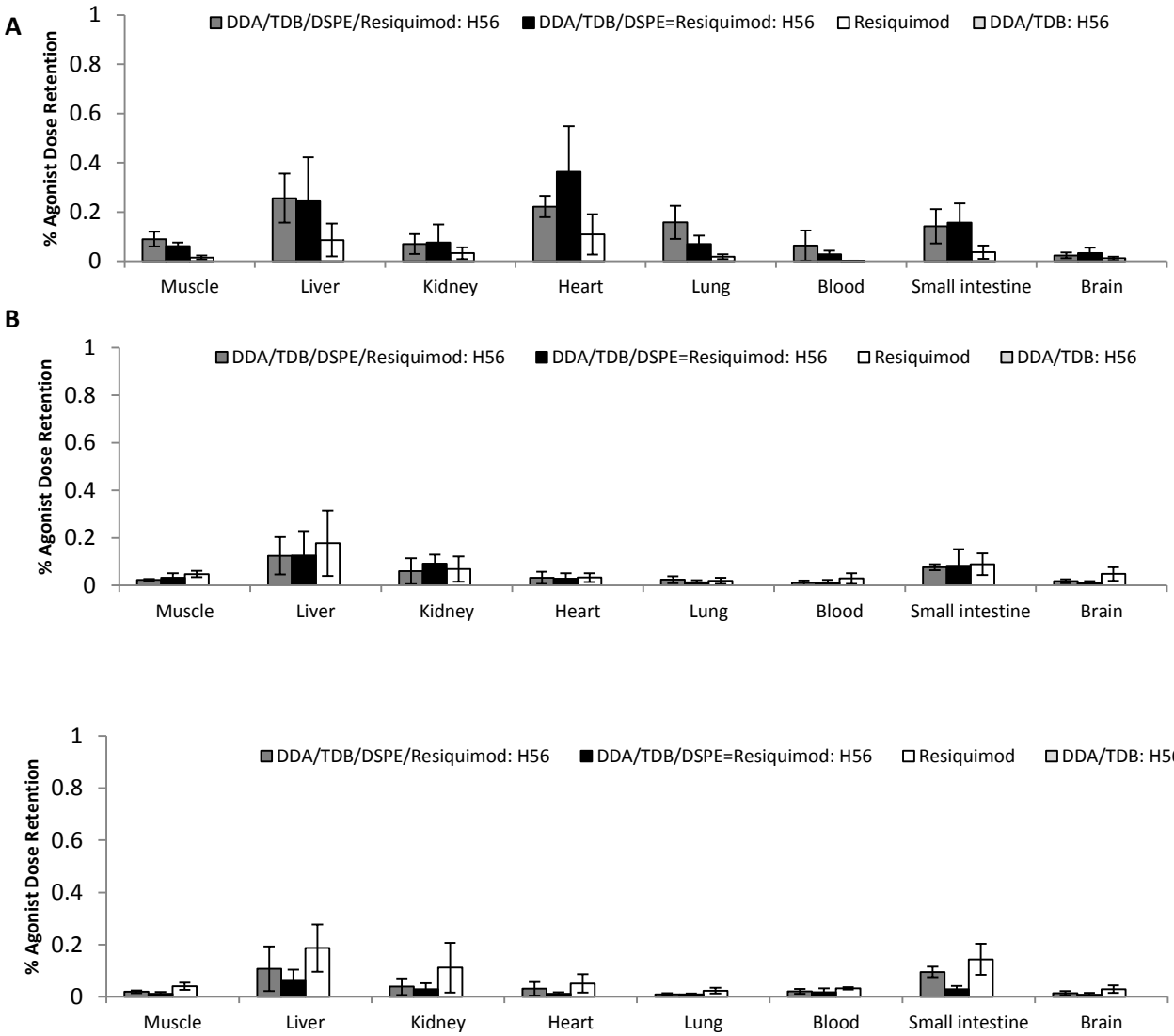
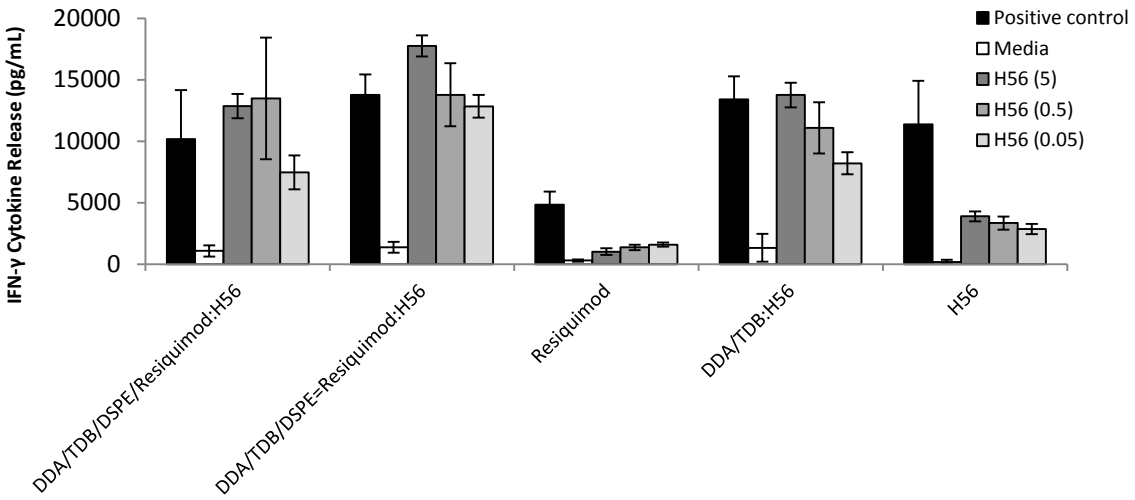
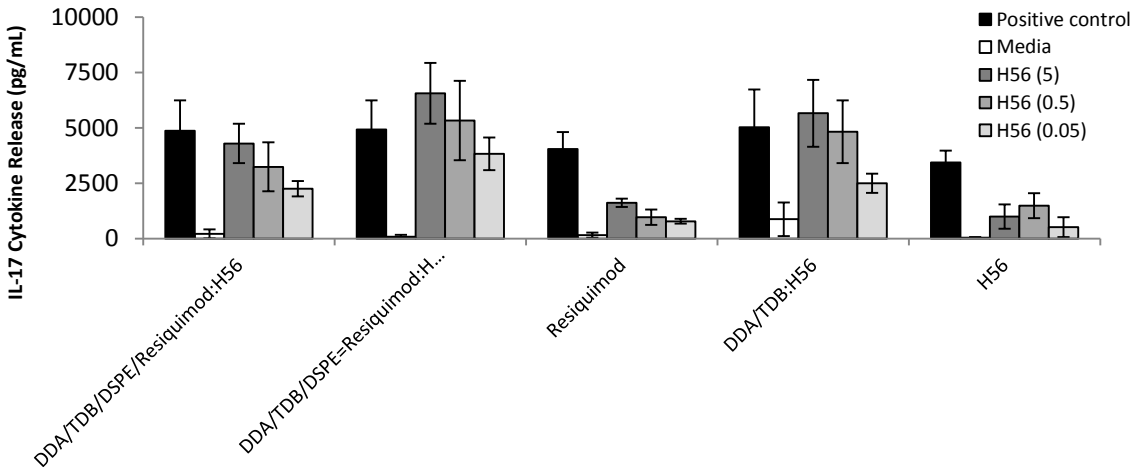


Figure A-4. A range of tissues were collected at (A) one (B), four and (C) eight days p.i and processed to determine the proportion of ^{125}I-labelled resiquimod. Results represent the mean ± SD of four mice.

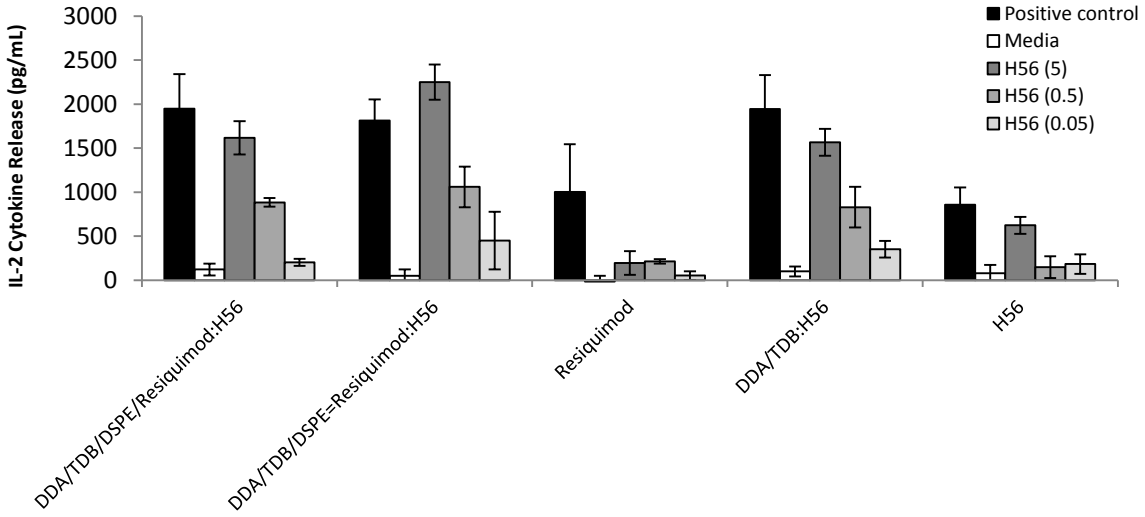
A


IFN-γ Cytokine Release (pg/mL)
20000
15000
10000
5000
0
Positive control
Media
H56 (5)
H56 (0.5)
H56 (0.05)
DDA/TDB/DSPE/Resiquimod:H56
DDA/TDB/DSPE=Resiquimod:H56
Resiquimod
DDA/TDB:H56
H56

B


IL-17 Cytokine Release (pg/mL)
10000
7500
5000
2500
0
Positive control
Media
H56 (5)
H56 (0.5)
H56 (0.05)
DDA/TDB/DSPE/Resiquimod:H56
DDA/TDB/DSPE=Resiquimod:H...
Resiquimod
DDA/TDB:H56
H56

C


IL-2 Cytokine Release (pg/mL)
3000
2500
2000
1500
1000
500
0
Positive control
Media
H56 (5)
H56 (0.5)
H56 (0.05)
DDA/TDB/DSPE/Resiquimod:H56
DDA/TDB/DSPE=Resiquimod:H56
Resiquimod
DDA/TDB:H56
H56

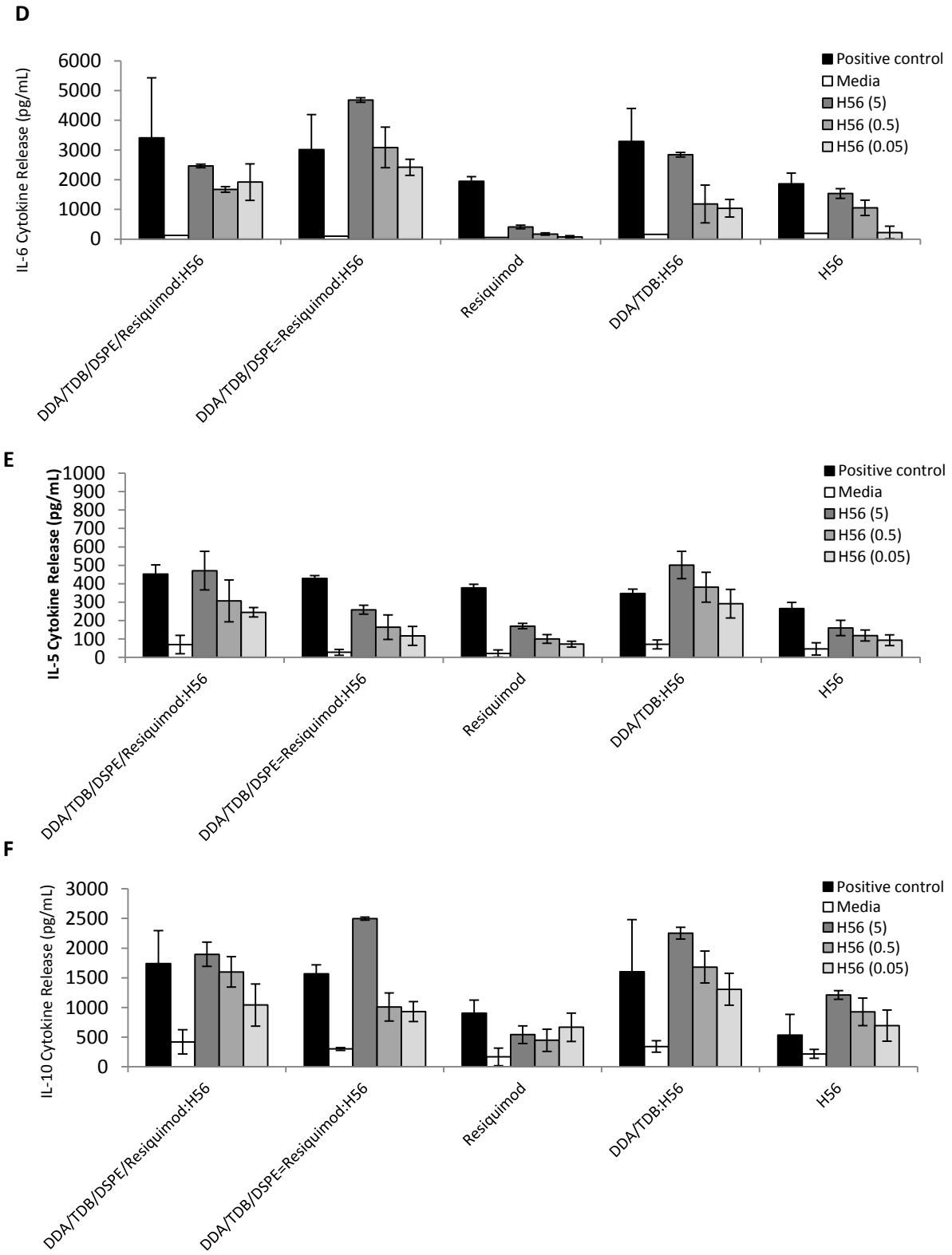
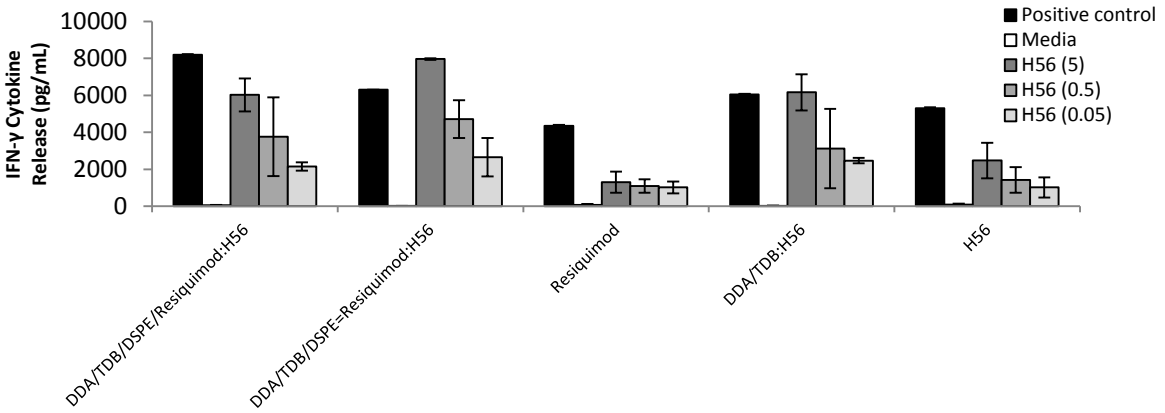
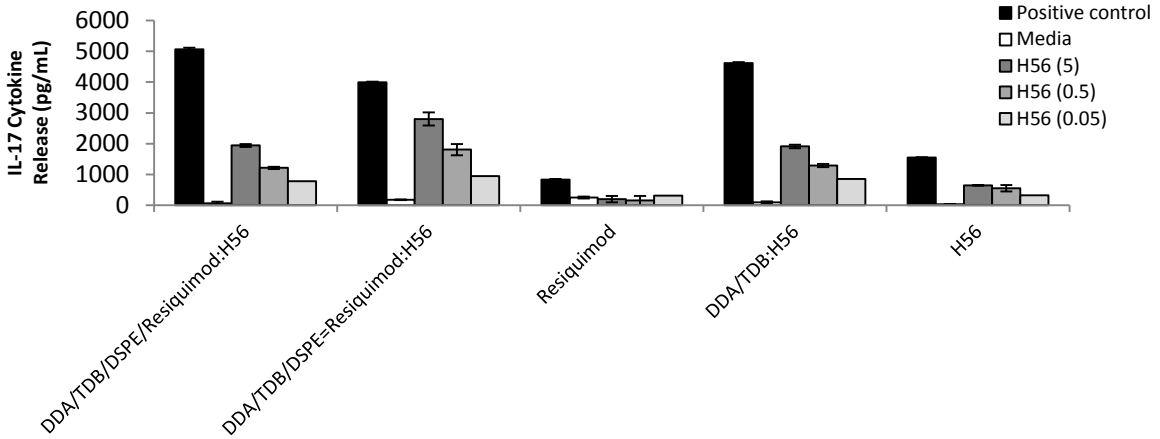


Figure A-5. Cytokine production [A: IFN-γ; B: IL-17; C: IL-2; D: IL-5; E: IL-6; F: IL-10] from cultured restimulated splenocytes derived from mice immunised with resiquimod alone (negative control), H56 (Ag85B-ESAT6-Rv2660c), either alone or combined with DDA/TDB, DDA/TDB/DSPE/Resiquimod (surface attached resiquimod) or DDA/TDB/DSPE=Resiquimod (bilayer-incorporated resiquimod). Mice received 3 injections with 2-week intervals and splenocytes were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 72 hrs in the presence of media alone, H56 at 0.05 µg/mL, 0.5 µg/mL or 5 µg/mL, or in the presence of 2 µg/mL ConA to act as a positive control. Cytokines were detected from splenocyte supernatants and measured using sandwich ELISAs.

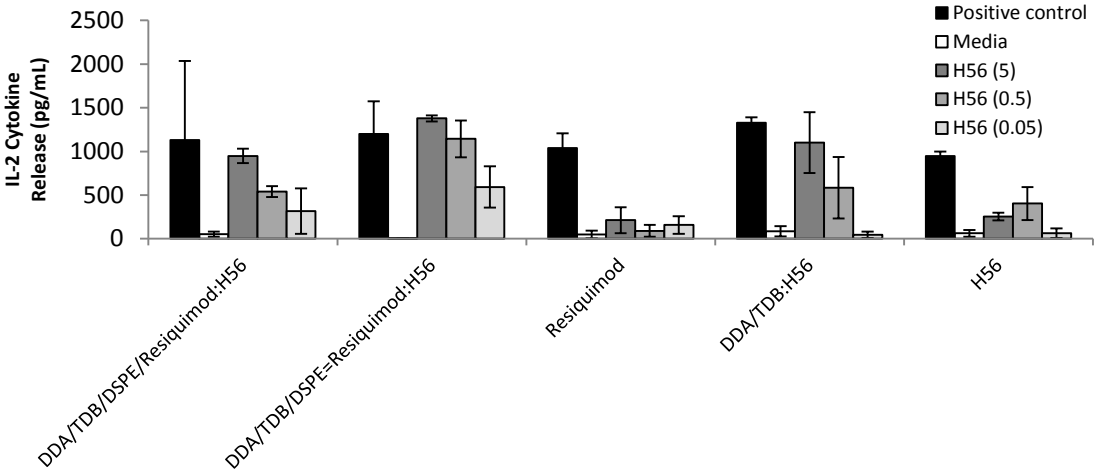
A


IFN-γ Cytokine Release (pg/mL)
10000
8000
6000
4000
2000
0
Positive control
Media
H56 (5)
H56 (0.5)
H56 (0.05)
DDA/TDB/DSPE/Resiquimod:H56
DDA/TDB/DSPE=Resiquimod:H56
Resiquimod
DDA/TDB:H56
H56

B


IL-17 Cytokine Release (pg/mL)
6000
5000
4000
3000
2000
1000
0
Positive control
Media
H56 (5)
H56 (0.5)
H56 (0.05)
DDA/TDB/DSPE/Resiquimod:H56
DDA/TDB/DSPE=Resiquimod:H56
Resiquimod
DDA/TDB:H56
H56

C


IL-2 Cytokine Release (pg/mL)
2500
2000
1500
1000
500
0
Positive control
Media
H56 (5)
H56 (0.5)
H56 (0.05)
DDA/TDB/DSPE/Resiquimod:H56
DDA/TDB/DSPE=Resiquimod:H56
Resiquimod
DDA/TDB:H56
H56

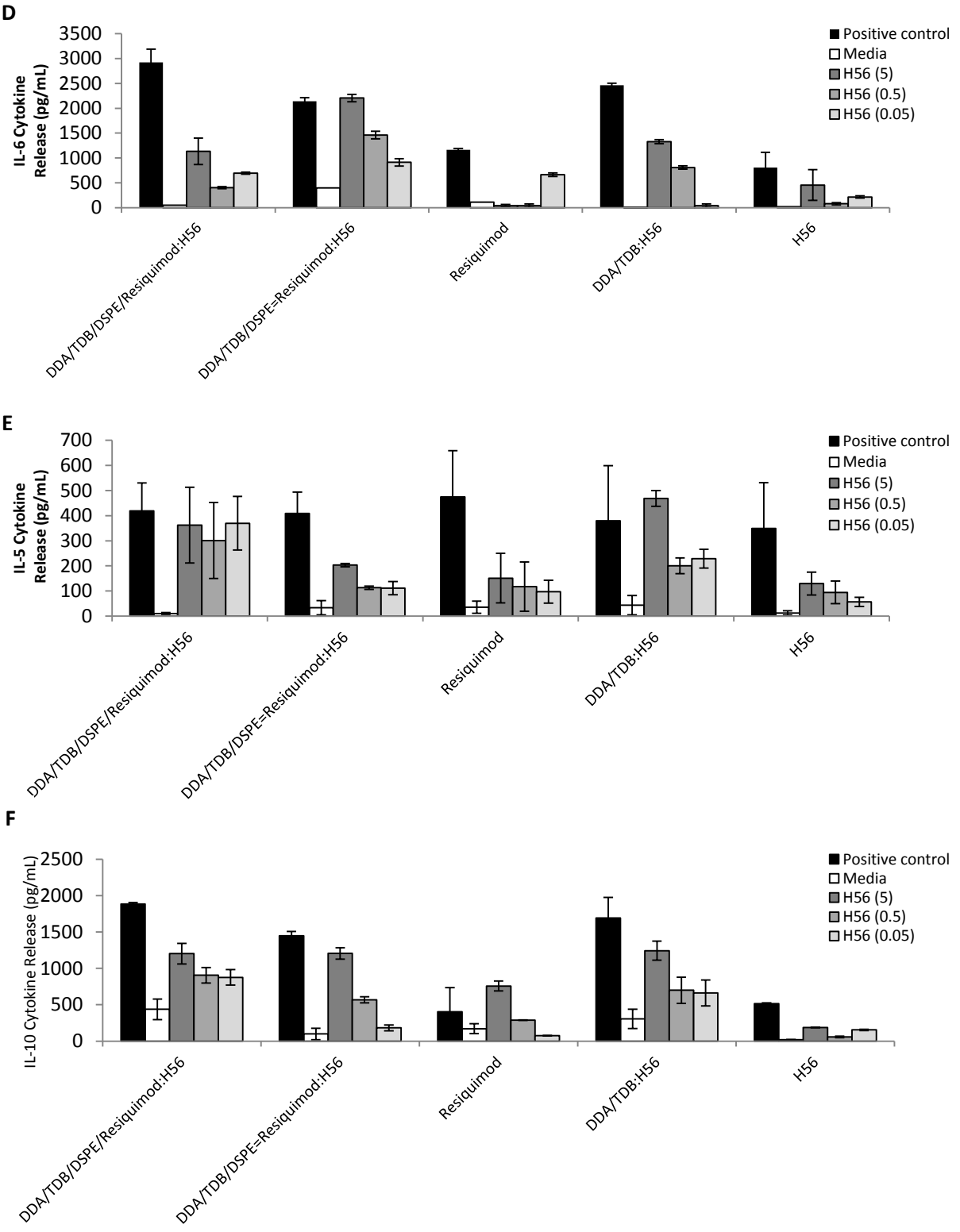


Figure A-6. Cytokine production [A: IFN-γ; B: IL-17; C: IL-2; D: IL5; E: IL-6; F: IL-10] from cultured restimulated popliteal lymph node cells derived from mice immunised with resiquimod alone (negative control), H56 (Ag85B-ESAT6-Rv2660c), either alone or combined with DDA:TDB, DDA/TDB/DSPE/Resiquimod (surface attached resiquimod) or DDA/TDB/DSPE=Resiquimod (bilayer-incorporated resiquimod). Mice received 3 injections with 2-week intervals and splenocytes were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 72 hrs in the presence of media alone, H56 at 0.05 µg/mL, 0.5 µg/mL or 5 µg/mL, or in the presence of 2 µg/mL ConA to act as a positive control. Cytokines were detected from splenocyte supernatants and measured using sandwich ELISAs.