IMMUNO-POTENTIATING ACTIVITY OF SURFACTANT VESICLES IN DNA AND SUBUNIT VACCINATION

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Summary

Enhanced immune responses for DNA and subunit vaccines potentiated by surfactant vesicle based delivery systems outlined in the present study, provides proof of principle for the beneficial aspects of vesicle mediated vaccination.

The dehydration-rehydration technique was used to entrap plasmid DNA or subunit antigens into lipid-based (liposomes) or non-ionic surfactant-based (niosomes) dehydration-rehydration vesicles (DRV). Using this procedure, it was shown that both these types of antigens can be effectively entrapped in DRV liposomes and DRV niosomes. The vesicle size of DRV niosomes was shown to be twice the diameter (~2μm) of that of their liposome counterparts. Incorporation of cryoprotectants such as sucrose in the DRV procedure resulted in reduced vesicle sizes while retaining high DNA incorporation efficiency (~95%). Transfection studies in COS 7 cells demonstrated that the choice of cationic lipid, the helper lipid, and the method of preparation, all influenced transfection efficiency indicating a strong interdependency of these factors. This phenomenon has been further reinforced when 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE): cholesteryl 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol)/DNA complexes were supplemented with non-ionic surfactants. Morphological analysis of these complexes using transmission electron microscopy and environmental scanning electron microscopy (ESEM) revealed the presence of heterogeneous structures which may be essential for an efficient transfection in addition to the fusogenic properties of DOPE. *In vivo* evaluation of these DNA incorporated vesicle systems in BALB/c mice showed a weak antibody and cell-mediated immune (CMI) responses. Subsequent mock challenge with hepatitis B antigen demonstrated that, 1-monopalmitoyl glycerol (MP) based DRV, a more promising DNA vaccine adjuvant. Studying these DRV systems as adjuvants for the Hepatitis B subunit antigen (HBsAg) revealed a balanced antibody/CMI respone profile on the basis of the HBsAg specific antibody and cytokine responses which were higher than unadjuvated antigen. The effect of addition of MP, cholesterol and trehalose 6,6'-dibehenate (TDB) on the stability and immuno-efficacy of dimethyldioctadecylammonium bromide (DDA) vesicles was investigated. Differential scanning calorimetry showed a reduction in transition temperature of DDA vesicles by ~12°C when incorporated with surfactants. ESEM of MP based DRV system indicated an increased vesicle stability upon incorporation of antigen. Adjuvant activity of these systems tested in C57BL/6j mice against three subunit antigens i.e., mycobacterial fusion protein- Ag85B-ESAT-6, and two malarial antigens- merozoite surface protein-1, (MSP1), and glutamate rich protein, (GLURP) revealed that while MP and DDA based systems induced comparable antibody responses, DDA based systems induced powerful CMI responses.

Key words: Niosome, Liposome, Adjuvant, Antigen, Immunisation, DNA immunisation.

Dedicated to my lordships

Sri Sri **Radha-Madhava**

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Abbreviations

APC – Antigen presenting cell

bp – Base pair

CD – Cluster of differentiation

Chol – Cholesterol

CMC – Critical micellar concentration

CPP – Critical packing parameter

CTL – Cytotoxic T lymphocyte

DC-Chol – 3β-[N-(N',N'-Dimethylaminoethane)-carbamoyl]Cholesterol

DCP – Dicetylphosphatidylcholine

DDA – Dimethyldioctadecylammonium bromide

DMPC – 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine

DMPG – 1,2-Dimyristoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)]

DNA – Deoxyribonucleic acid

DRV – Dehydration-rehydration vesicles

DRV(DNA) – DNA encapsulated in DRV

DRV-DNA – DNA adsorbed on to DRV

dsDNA – Double stranded DNA

EDTA – Ethylenediaminetetraacetic acid

HLB – Hydrophilic-lipophilic balance

IL – Interleukin

kb – Kilo base

kDa – KiloDalton

LB – Luria bertini

LUV – Large unilamellar vesicles

MHC – Major histo-compatibility complex

MLV – Multilamellar vesicles

PC – Phosphatidylcholine

pRc/CMV-HBS – Plasmid recombinant/cyto-megalo virus-hepatitis B surface antigen

pRc/CMV-luc – Plasmid recombinant/cyto-megalo virus-luciferase

RES	–	Reticulo endothelial system
RNA	–	Ribonucleic acid
rpm	–	revolutions per minute
SA	–	Stearyl amine
SDS	–	Sodium dodecyl sulphate
SOC	–	SOB (Super optimal broth) SOC had the 'B' in SOB changed to 'C' for catabolite repression, reflective of the added glucose in the medium.
SUV	–	Small unilamellar vesicles
TBE	–	Tris-boric acid-EDTA (buffer)
T_c	–	Phase-transition temperature
TDB	–	α, α'-trehalose 6,6'-dibehenate
TE	–	Tris-EDTA (buffer)
T_g	–	Glass-transition temperature
Th1/ Th2	–	T helper type 1/ T helper type 2
UV	–	Ultra-violet

Chapter One

General Introduction

1.1 GENERAL INTRODUCTION

1.1.1 Surfactant vesicles – A brief review

Liposomes and non-ionic surfactant vesicles (niosomes) together can be conveniently termed as surfactant vesicles. These are unilamellar or multilamellar, highly ordered concentric bilayer structures alternating with aqueous compartments (Fig.1.1). The former usually made up of phospholipids and the latter with non-ionic surfactants.

Liposomes were first identified by Bangham *et al.* (1965) and were initially used as a model to study the effect of narcotics on lipid bilayer membranes. Since these early experiments, liposomes have become an established carrier and delivery system in the field of pharmaco- and immuno-therapy with more advanced formulations (e.g. PEGylated 'stealth' liposomes) been developed and their superior properties demonstrated with regard to stability, pharmacokinetics, biodistribution and toxicity (Allen, 1994). Encapsulation of drugs into liposomes can reduce the toxic effects related to high systemic concentrations of the drug when in its free form (Gabizon *et al.*, 1998). Liposomal targeting through the interaction of specific ligands and a mechanism of controlled drug release results in the accumulation of the drug at the site of action. Specific conditions found in the target tissues (e.g. a low pH in inflammatory tissues and tumours) can be used as a trigger for controlled release by the integration of pH-sensitive components into the liposomal bilayer. A similar mechanism, pH-dependent release via membrane fusion, can be used to escape endolysosomal degradation after endocytosis and to deliver active molecules or antigens to the cytoplasm of target cells (Drummond *et al.*, 2000). Although only four liposomal formulations are available in the market for the delivery of drugs (excluding vaccines

which are discussed in section 1.1.8.1), liposomal formulations of several substances such as antibiotics, photosensitizers, enzymes, hormones, cytokines and nucleic acids are currently in preclinical and clinical development with encouraging results (Felnerova *et al.*, 2004).

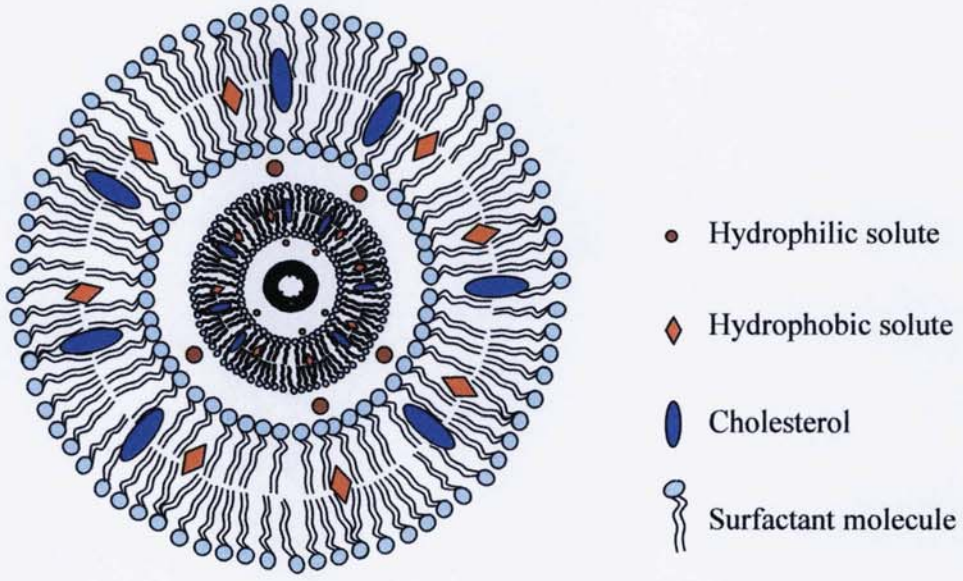


Fig. 1.1 Schematic representation of a typical multilamellar surfactant vesicle. The hydrophilic solute is encapsulated into the aqueous core between the bilayers while the hydrophobic solute and cholesterol are embedded within the bilayers.

Niosomes were among the first alternative systems to liposomes and although they were initially reported as a feature of the cosmetic industry (Vanlerberghe *et al.*, 1973; Handajani – vila *et al.*, 1979) they have since been studied as drug delivery systems. Electron micrographs and fluorescence anisotropy studies of these systems (e.g. Santucci *et al.* 1996) have demonstrated niosomes (in this case containing a combination of Tween 20 and cholesterol) form bilayer vesicles similar in structure to that of liposomes, and like

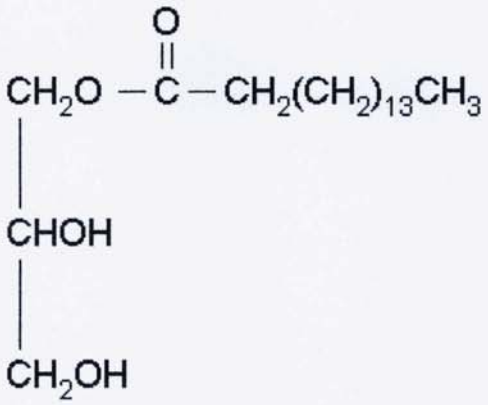
liposomes, these vesicles can be loaded with hydrophilic and hydrophobic solutes with efficiencies comparable to those of phospholipid based vesicles (Florence and Baillie, 1989). A number of non-ionic surfactants including polyglycerol alkyl ethers, glucosyl dialkyl ethers, crown ethers, ester-linked surfactants, poly oxyethylene alkyl ethers, and a series of Brij, Spans and Tweens have been used along with cholesterol to prepare niosomes. Further research has also shown that many synthetic amphiphiles form vesicles but as most of them are ionic and relatively toxic, they are generally unsuitable for use as drug carriers (Fedler, 1982).

Niosomes are osmotically active and exhibit flexibility in their structural characteristics, i.e., composition, fluidity and size, and as carrier systems they possess certain advantages over liposomes such as chemical stability, low cost and purity. Phospholipids used in preparation of liposomes are prone to chemical degradation such as lipid peroxidation and hydrolysis (Lichtenberg and Barenholz, 1988), and hence are stored and handled in nitrogen atmosphere. Variable purity of natural phospholipids obtained is another limitation. The low cost, greater stability, purity and resultant ease of storage of non-ionic surfactants (Florence, 1993) has lead to the exploitation of these compounds as alternatives to phospholipids which could also initiate a wider study on the influence of chemical composition with respect to the biological fate of these vesicles.

Amphiphilic moieties (including phospholipids and non-ionic surfactants) capable of forming vesicles possess tripartite chemical structure consisting of a lipophilic aliphatic fatty acid double or single chain linked to a hydrophilic head group generally by ether or

ester group. An example structure of a non-ionic surfactant molecule, 1-monopalmitoyl glycerol is shown in Fig. 1.2.

(A)



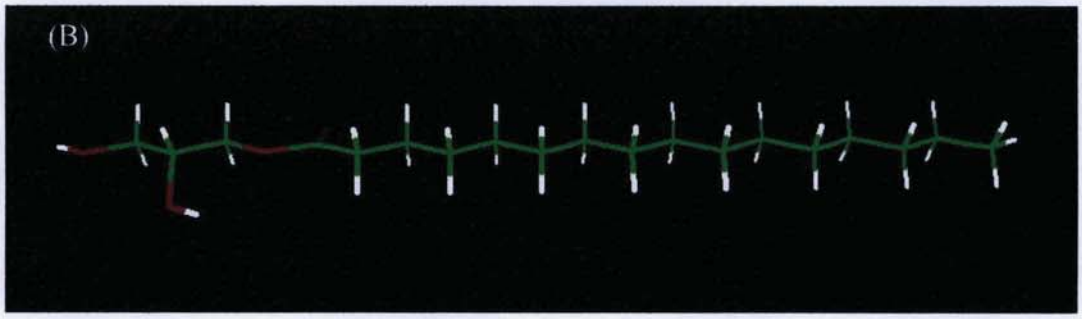


Fig. 1.2 Chemical structure of a non-ionic surfactant, 1-monopalmitoyl glycerol (MP) (C16:0) whose glycerol head group is linked to 16 carbon, saturated single alkyl chain by an ester group (A); 3-D image of MP molecule (B) constructed using *Work space cache*® software with red, white and green regions representing oxygen, hydrogen and carbon atoms respectively.

Most of the physico-chemical properties of niosomes are described in similar terms as that of liposomes such as hydrophilic–lipophilic balance (HLB) value of the non-ionic surfactant, bilayer rigidity based on phase transition temperature (T_c), the nature of the non-ionic surfactant and method of vesicle preparation (Szoka and Papahadjopoulos, 1980). In

addition, the vesicle composition, surface charge and effective mean diameter are important pharmacokinetic variables (Abra and Hunt, 1981). *In vivo* niosomes have been found equi-active to liposomes in their ability to improve the therapeutic performance of entrapped solute (Hunter *et al.*, 1988) and their distribution in body follows the pattern of any other colloidal carrier systems such as microspheres, polymer based systems etc. Although the tissues of extravasation (liver, lung, spleen and bone marrow) are responsible for the disposal of niosomes, their level in liver is always significantly higher than the remaining tissues due to the natural vectoring process (Baillie, 1986; Hunter *et al.*, 1988; Azmin *et al.*, 1985). It also appears that, like liposomes, niosomes are taken up intact by liver, after which they are broken down to release the solute which eventually re-enters the circulation and maintains the drug plasma levels (Azmin *et al.*, 1985). In addition, like liposomes, niosomes are able to reduce the toxic side effects of many entrapped solutes including anti-cancer drugs like doxorubicin (Uchegbu and Florence, 1995) and its analogue, daunorubicin (Balasubramaniam *et al.*, 2002) due to their passive targeting to the tumour site. Given the potency and toxicity of these solute drugs, tissue selectivity becomes an important consideration where the phenomenon of passive targeting could be exploited because of the unique structural changes that occur within a given vascular pathophysiology, for example, vesicles that escape from the circulation are normally directed to the sites where the capillaries have open fenestrations as in the sinus endothelium of the liver or when the integrity of the endothelial barrier is perturbed by inflammatory process or even by a growing tumour (Moghimi *et al.*, 2001) thus restricting the toxicity of an encapsulated drug to the confined regions in the body such as the tumours.

1.1.2 Theory of vesicle formation

As noted earlier, the dual chemical nature of vesicle forming surfactants i.e., their hydrophilic head groups and hydrophobic alkyl chains enable them to possess an affinity towards both aqueous as well as non-aqueous environments (Lasic, 1988). In general the head group, due to its hydrophilic nature, will weakly bind with water molecules resulting in their preference for the aqueous phase. The alkyl chains on the other hand, interact via hydrophobic interactions and will have a tendency to avoid interaction with the aqueous phase. Some of the terms that are considered for understanding this phenomenon in quantitative terms are the hydrophilic-lipophilic balance (HLB) number and critical micelle concentration (CMC) of surfactant in water. HLB number of a surfactant indicates its hydrophilicity (or lipophilicity) based on the contribution from its head group or alkyl chain. The CMC is defined as the concentration of the surfactant in water above which monomers will self associate into larger aggregates, most commonly micelles, rather than remaining in solution as monomers. Unfortunately, the prediction of vesicle forming ability is not only a matter of HLB number, CMC, and chemical structure, but various other factors also come into play. It is generally accepted that the parameters for self-assembly can be predicted by considering the overall molecular structure of a surfactant as outlined by Israelachvili (1985) in which a critical packing parameter (CPP) of a molecule is defined as:

$$\text{CPP} = v / l_c.a_0 \quad \ldots\ldots \quad \textit{Eqn 1.1}$$

Where v = hydrophobic group volume, l_c = the critical hydrophobic group length and a_0 = the area of the hydrophilic head group. A CPP between 0.5 and 1 indicates that the

surfactant is likely to form vesicles (Israelachvili, 1985) while a CPP of below 0.5 (indicating a large contribution from the hydrophilic head group area) is said to give spherical micelles and a CPP of above 1 (indicating a large contribution from the hydrophobic group volume) should produce inverted micelles, the latter presumably only in an oil phase, or otherwise precipitation would likely to occur. It is understood that in cases where a mixture of surfactants and additives such as cholesterol are used to prepare vesicles, the operational CPP values will be those of the entire components (Uchegbu and Vyas, 1998). It is clear from the thermodynamic consideration that bilayer structures do not exist as such in the absence of water because it is water that provides the driving force for surfactant molecules to assume a bilayer configuration. Unlike micelles, the assembly into closed bilayers is rarely spontaneous and usually involves input of energy such as physical agitation or heat (Lasic, 1990). The excess free energy change between water and a hydrophobic environment explains the overwhelming preference of typical surfactants to assemble into bilayer structures, excluding water as much as possible from their hydrophobic core, in order to achieve the lowest free energy level and consequently the highest stability for the aggregate structure.

The bilayer membrane being an ordered structure could exist in a gel state or in a liquid crystalline state. The difference between these two states (or phases) is the degree of conformational and translational order, with the gel state being a more ordered structure than the liquid crystalline state (Fig. 1.3). In the gel state the alkyl chains are crystallised or otherwise less mobile (Fig. 1.3a) where as in the liquid crystalline state there is lateral diffusion of bilayer material (Fig. 1.3b).

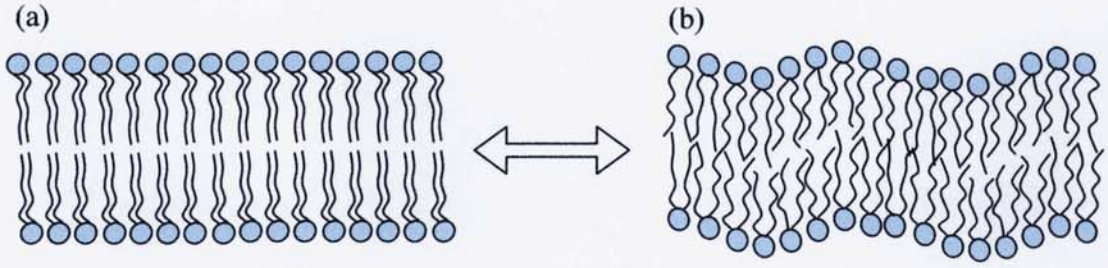


Fig. 1.3 Schematic representation of thermodynamics of a bilayer membrane. In gel phase (a) there is both lipid chain conformational and translational order while in the liquid-crystalline phase (b) there exists a conformational and translational disorder with later diffusibility.

For any system, the liquid crystalline state exists at a higher temperature than the gel state. The increase in the transition temperature (T_c), although yielding an increase in the enthalpy term (ΔH), also results in an increase in entropy (ΔS) and thus a lowering of the free energy (ΔG) of the system occurs and it is the application of heat that is the driving force for this transition (New, 1990).

1.1.3 Methods of preparation of surfactant vesicles

Depending on the method of preparation, surfactant vesicles consist of one or more bilayer membranes (lamellae) and are known as small unilamellar vesicles (SUV) with a diameter of about 75 to 100 nm, large unilamellar vesicles (LUV) which can be several microns large, and multilamellar vesicles (MLV) varying in size from anywhere above 100 nm to several microns (Fig. 1.4).

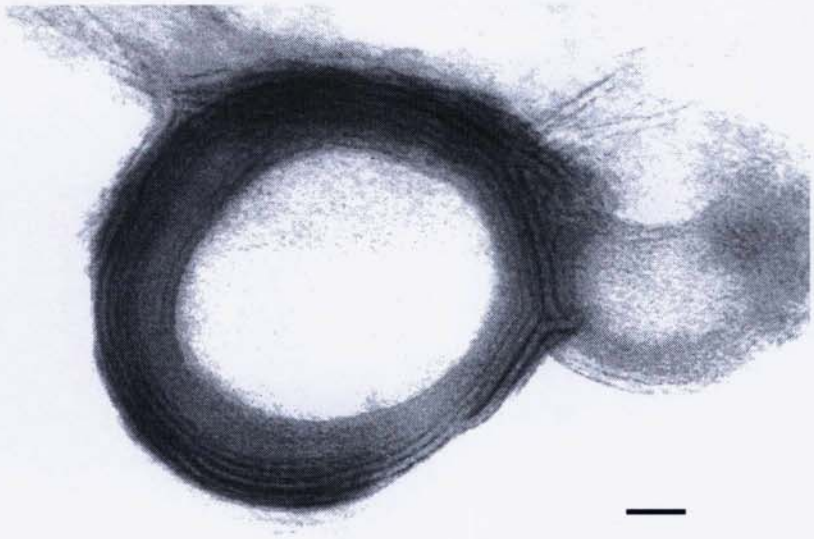


Fig. 1.4 Transmission electron micrograph of surfactant vesicle composed of 1-monopalmitoyl glycerol, cholesterol, DDA, TDB (16:16:4:0.5 μmoles) showing multilamellar bilayers. Bar = 100 nm. (unpublished picture, detailed discussion in chapter 6).

In the process of their formation, and depending on the method used, surfactant vesicles passively entrap water and solutes contained in it, e.g. small drugs such as anticancer and microbial agents, enzymes, cytokines and other proteins, vaccines, nucleic acids and even viruses or bacteria (Gregoriadis, 2003). On the other hand, lipid soluble drug molecules (e.g. steroids) can be incorporated into the lipid phase (bilayers) of vesicles by combining such drugs with the lipid/surfactant mixture used for their preparation. The efficient encapsulation of a given compound is a prerequisite for the successful application of vesicular delivery, whose physico-chemical characteristics could vary considerably not only with the vesicle composition and properties of the active ingredient but also by the method of preparation, which are described in Table 1.1

Table 1.1 Brief description of techniques used in preparing surfactant vesicles.

Vesicles	Method	Procedure
Multilamellar vesicles (MLV)	a. Lipid-hydration method	In this method (Bangham *et al*, 1965), the organic solvent consisting a mixture of vesicle-forming ingredients, is evaporated under reduced pressure on a rotavapor, leaving a thin film on the wall of round bottom flask which is hydrated by shaking the film in the presence of water that contains the solute to be incorporated, above the phase-transition temperature (T_c) of the surfactant/lipid until a good dispersion of multilamellar vesicles are formed.
	b. Dehydration-rehydration method	This method (Kirby and Gregoriadis, 1984) involves freeze-drying of preformed SUV mixed with the solute to be entrapped and rehydrated under controlled conditions giving multilamellar vesicles with high entrapment.
Large unilamellar vesicles (LUV)	a. Reverse phase evaporation method	LUV can be prepared (Pidgeon *et al.*, 1987) by forming water-in-oil emulsion of surfactant/lipid and buffer in excess organic phase followed by removal of organic phase under reduced pressure.
	b. Calcium induced fusion method	This method (Papahadjopoulos *et al.*, 1975) takes advantage of the fact that small vesicles aggregate in the presence of calcium and subsequently fuse. Large vesicles could be produced upon addition of EDTA.
	c. Detergent removal method	Removal of detergent (Wacker and Schubert, 1998) from mixed micelles formed by solubilization of dried surfactant mixtures or preformed vesicles with a detergent containing aqueous phase, results in the formation of unilamellar vesicles. Detergents can be removed by dialysis or by gel filtration chromatography.
Small unilamellar vesicles (SUV)	a. Sonication	Multilamellar vesicles are sonicated (Baillie *et al.*, 1985) either with a probe sonicator or a bath type sonicator leading to rapid size reduction, forming SUV.

	b. French press method	A French pressure cell is used to reduce the size of MLV by extrusion under high pressure (Berenholtz *et al.*, 1979). This technique yields homogenous SUV with sizes depending upon the pressure used and the membrane pore size.
	c. Ether injection method	Typically, the lipid mixture dissolved in diethyl ether is injected into an aqueous medium containing the material to be encapsulated (using a syringe-type infusion pump) at a temperature of 60°C or under reduced pressure, subsequent vaporization of the residual ether leads to formation of unilamellar vesicles (Deamer and Bangham, 1976).
	d. Ethanol injection method	This method (Batzri and Korn, 1973), employs a procedure where surfactant/lipids are dissolved in ethanol and rapidly injected into a vast excess of buffer solution through a fine needle yielding a high proportion of small unilamellar vesicles.
	e. Microfluidization method	This method (Suzuki, 1988), is based on submerged jet principle in which two fluidised streams interact at ultra high velocities (up to 1700 ft/sec) in precisely defined micro channels within the interaction chamber. The impingement of thin liquid sheets along a common front is arranged such that the energy applied to the system remains within the area of vesicle formation. This procedure gives small vesicles with uniform size distribution.
	f. Homogenization	Homogenization of MLV (Gamble, 1988) by commercially available high shear homogenisers produces small unilamellar vesicles. The size of the vesicles depends on the speed of the probe, duration of homoginisation and also on the surfactant/lipid composition.

1.1.4 Factors affecting size, incorporation and release characteristics

The major factors that affect vesicle size, incorporation efficiency and release characteristics of surfactant vesicles include:

A) Solute. Most of the time nature of the solute incorporated has a strong influence on vesicle size, for example, an increase in vesicle size by a micron was observed (Mohammed *et al.*, 2004) in PC:Chol:SA based liposomes when hydrophobic drug, ibuprofen was incorporated. Interestingly, elsewhere, a reduction in the vesicle size by 50% was also achieved in PC:Chol:DCP based liposomes when an amphiphilic drug, tretinoin was incorporated (Manconi *et al.*, 2002) and was presumed that the intercalation of the amphiphilic drug into the lipid bilayer lead to an increased cohesion among the hydrophilic portions of the bilayer, causing a reduction in the vesicle diameter. This phenomenon was also seen in Brij 30:Chol:DCP based niosomes where the vesicle size reduction was reported to be significant (Manconi *et al.*, 2002).

B) Surfactant characteristics and concentration. The mean size of vesicles increases with increase in the surfactant's hydrophilic-lipophilic balance (HLB) since surface free energy decreases with the increase in hydrophobicity (Yoshioka *et al.*, 1994). For example, using the lower HLB (1.8) surfactant, Sorbitan tri-oleate, (Span 85) resulted in vesicles 5 times smaller than those prepared with sorbitan monolaurate (Span 20) possessing higher HLB (8.6). Within this study Yoshioka *et al.,* (1994) also demonstrated a linear correlation between concentration of surfactant and entrapment efficiency. The phase transition temperature (T_c) of surfactant also affected entrapment efficiency, for example, niosomes

prepared using Span 60 with a higher T_c (50°C) yielded high entrapment values for carboxyfluorescein than those prepared from surfactants with lower T_c (e.g. Span 20; Yoshioka *et al.*, 1994).

C) Cholesterol content and surface charge. Most non-ionic surfactants are not capable of forming stable bilayer structures without the inclusion of additional surfactants such as cholesterol or a charge lipid such as dicetylphosphate (Uchegbu and Florence, 1995). Inclusion of cholesterol in the vesicle composition has been shown to increase the vesicle's hydrodynamic diameter and entrapment efficiency (Yoshioka *et al.*, 1994) and reduce solute permeability in case of both niosomes (Rogerson *et al.*, 1988) and liposomes (Sada *et al.*, 1990) which is attributed mainly to the space filling action by its accommodation in the bilayers compared to cholesterol free membranes. One of the main explanations for an improved membrane stabilisation of cholesterol is its ability to alter the fluidity of the bilayer chains and when present in sufficient concentration (50 mole%) was reported to abolish the gel to liquid phase transition endotherm of the surfactant bilayers (Cable, 1989). Thus, cholesterol aids in the formation of vesicle structures even in case of lipids/surfactants that melt at a higher temperature by increasing their bilayer chain fluidity (Bouwstra and Hofland, 1994). Presence of charge in vesicular dispersions is important, as in isotonic salt solutions, aggregation of vesicles occurs when prepared without the inclusion of a charged molecule within the bilayers. This aggregation is a result of the shielding of the vesicle surface charge by ions in solution, reducing electrostatic repulsion (Lawrence *et al.*, 1996). Vesicle surface charge is estimated by measurements of particle

electrophoretic mobility and expressed as zeta potential which may be calculated using the Henry equation:

$$\zeta = \mu E 4\pi\eta / \varepsilon \quad \ldots\ldots \textit{Eqn 2}$$

where ζ = zeta potential, μE = electrophoretic mobility, η = viscosity of medium and ε = dielectric constant (Florence and Attwood, 1988).

D) Method of preparation. As noted in section 1.1.3, vesicle size and lamellarity are affected based on the method of preparation. The lipid hydration method, for example, forms multilamellar vesicles (MLV) with larger diameters (0.5-15 μm; Carter *et al.*, 1989) than vesicles prepared by the ether injection method, which were in the range of 50-1000 nm (Kandare *et al.*, 1994). Sonication of MLV prepared by above methods form small unilamellar vesicles (75-100nm).

E) Osmotic effect. In a hypertonic medium, surfactant vesicles show a reduction in the vesicle diameter with concomitant water efflux and vesicle contents (Baillie *et al.*, 1985) where as in hypotonic medium, there is a slow release of solute initially due to cross-flow of the medium into the vesicle resulting in slight swelling of the vesicle probably due to inhibition of eluting fluid followed by faster release, which may be due to mechanical loosening of vesicle bilayer due to osmotic stress. In some systems osmotic activity may not be observed because there is initial aggregation of the vesicles due to the shielding effect of electrostatic charge on the vesicle surface by the surrounding ions as noted earlier. However, incorporation of steric stabilisers in the vesicle membrane such as

polyoxyethylene compounds (e.g. solulan C24) prevents this aggregation (Arunothayanun *et al.*, 1996).

1.1.5 Methods for controlling vesicle size

The ideal vesicle size from biological point of view has yet to be fully characterised and studies designed to systematically define the size requirements for certain pharmacodynamic objectives are very much needed. However, it is suggested that, submicron (colloidal) sizes for intravenous and transdermal, up to 10 μm for intraperitoneal and intramuscular and >10 μm for ophthalmic delivery may be suitable (Uchegbu and Vyas, 1998). Vesicles of size greater than 200 nm diameter are reported to be cleared rapidly from the blood circulation after intravenous injection, ending up in the macrophages of the reticuloendothelial system (RES), namely the liver, spleen and bone marrow (Allen *et al.*, 1995). Therefore, the size of surfactant vesicle systems employed in pharmaceutical applications will have to be controlled within reasonable and verifiable limits. A few possible approaches (described in Table 1.2) have been explored in the past for achieving defined particle size distribution which includes: a) fractionation b) homogenisation c) extrusion and d) modified DRV method incorporating carbohydrates.

1.1.6 Improving efficacy of vaccines through vesicular delivery

As mentioned, liposomes and niosomes have been investigated for their ability to deliver a wide range of pharmacological agents including vaccines. The use of vaccines for the prevention of infectious diseases is a preferred alternative to treatment. For instance, vaccination has been applied successfully in the eradication of smallpox and is also used

Table 1.2 Different methods of controlling surfactant vesicle size.

1. Fractionation	**a. Centrifugation** Vesicles sediment in a centrifugal field at a rate that relates to their size and density. Large vesicles composed of high amount of surfactant and other adjuvants can easily be pelleted at fairly low centrifugal forces in conventional centrifuge. These differences in sedimentation rates can be exploited to separate differently sized vesicle populations. Sucrose gradients can also be used to further enhance the fractionation.
	b. Size-exclusion chromatography This method is particularly useful for separating small unilamellar vesicles from larger ones. Typically a column of sepharose 48 is used (Huang, 1969). As the column is run with buffer, larger vesicles retain in the void volume, while small unilamellar vesicles are eluted. Large pore size chromatographic media have been used in a similar fashion to fractionate populations of larger particles (Nozakki *et al.*, 1982). In general such chromatographic separations are quite limited as the volumes and throughput must be carried out in batches, resulting in significant dilution of the product and require strict controls to ensure that no microbial contamination of gel occurs.
2. Homogenisation	When fairly small particles are desirable, homogenisation has proven to be a useful approach. The average particle size of vesicle dispersion can be reduced by high pressure homogenization. For example a microfluidizer was used to generate vesicles in the 50 – 200 nm size range (Mayhew *et al.*, 1984). Conventional homogenisers and high sheer mixers have also been reported to be useful for down sizing vesicles (Redziniak *et al.*, 1986).

3. Extrusion	**a. Polycarbonate membrane extrusion** This technique, which gained wide acceptance for the production of vesicles of defined size and narrow size distribution, is based on the extrusion of a heterogeneous population of fairly large vesicles through polycarbonate membranes under pressure (Olson *et al.*, 1979). This single technique can reduce a heterogeneous population of multilamellar vesicles to a much more homogenous suspension of vesicles exhibiting a mean particle size that approaches the diameter of the pores through which they are extruded.
	b. Ceramic extrusion If the size of the vesicles is greater than the membrane pore diameter, the pores of the membrane tend to clog. The clogged membranes can not easily be cleared because the filter housing configuration does not allow back - flushing. One approach that overcomes this limitation is by the use of ceramic membranes (Lichtenberg and Barenholz, 1988). Extruded vesicles yield a relatively narrow size distribution. The suspension may be alternatively passed through the membrane in an outside to inside direction to maintain the membrane in an unclogged condition.
4. DRV in the presence of carbohydrates	A modified dehydration-rehydration procedure (Zadi and Gregoriadis, 2000) in which dehydration of preformed "empty" SUV (60-80 nm) in the presence of solute that is to be entrapped and defined concentrations of sugars leads in the formation of small sized MLV upon subsequent rehydration exhibiting high entrapment values. This procedure is relatively simple, mild and applicable to solutes of a wide range.

effectively against tetanus, diphtheria, whooping cough, polio, and measles, thus preventing millions of deaths around the world each year. Indeed, it has been shown that vaccines have saved more lives than drugs (Powell, 1996). Early vaccines are categorised into three main groups that include, live attenuated vaccines, eg., Bacillus Calmette-Guerin (BCG) and influenza virus vaccine (Wareing and Tannock, 2001), killed/inactivated vaccines, eg., whole cell pertussis vaccine (Fletcher *et al.*, 2001) and subunit vaccines, eg., tetanus and diphtheria toxoid vaccines (Makela, 2000). Within the latter group, the first recombinant vaccine intended for human use was the hepatitis B vaccine (Valenzuela *et al.*, 1982). Other currently available vaccines against viral diseases in humans include live attenuated measles, mumps, rubella, varicella and yellow fever vaccines however there remains a clear need for effective vaccines against many diseases including the 3 main 'global killers' malaria, HIV and TB .

An ideal vaccine should consist of only the key antigenic determinants which will elicit a potent, specific immune response against the target pathogen apart from being safe and efficacious for all ages and high risk groups, preferably in single dose with early onset of life-long protection, administrable without needle and syringe and stable (without the need for cold chain). The production of such a vaccine requires the availability of either highly purified subunit antigens or synthetic determinants (Shek and Barber, 1986). The clear advantage of synthetic vaccines is that they are devoid of unrelated antigenic determinants inherently present in vaccine extracts obtained from microbes. Although contaminant-free synthetic peptide antigens do not induce undesirable side-effects seen in some conventional vaccines, they do usually suffer from the drawback of being rather weak immunogens,

primarily because of their relatively low molecular weight. Thus, the successful production of a potent, efficacious synthetic vaccine requires the association of the synthetic antigenic moiety with an appropriate vaccine adjuvant.

The demand for more effective vaccines is clearly apparent, with the emergence of increasingly diverse and novel vaccination strategies and an urgent need to protect living beings from more than twenty major diseases, including major killers like AIDS, tuberculosis, hepatitis B and C, and malaria (Hilleman, 2000) with AIDS, tuberculosis, and malaria responsible for an estimated 2.3, 1.5, and 1.1 million deaths reported worldwide per year, respectively (Wenger, 2001) and more than 350 million people world wide are now chronically infected with hepatitis B virus (Akbar *et al.*, 2004). Whilst vaccines are already available for some of these diseases (e.g. TB and Hepatitis B) they are hindered by poor efficacy. For example, the anti-hepatitis B response to hepatitis B immunisation is reported to be bimodal, with 4.2% of healthy people producing no antibody response and an additional 10% being hypo-responders (Alper, 1995). This non-response is thought to be due to an inherited defect in helper T cells (Alper, 1995; Salazar *et al.*, 1995). Extensive analysis of immune responses by hepatitis B vaccine non-responders and their response to tetanus toxoid show that non-responders are unable to generate cytokine responses to the hepatitis B antigen (Larsen *et al.*, 2000). Post-exposure immunisation in infants at risk of maternal-infant transmission of hepatitis B has also highlighted the need for more effective vaccination strategies (He *et al.*, 1998). For tuberculosis, the current vaccine, BCG has also demonstrated variable efficacy (for 0 to 80%) in a number of clinical trials (Hart and

Sutherland, 1977) and a more robust vaccine strategy to either replace or supplement BCG is also required.

1.1.6.1 The DNA vaccine

A series of publications from 1992 onwards established the ability of plasmid DNA to induce an immune (antibody) response to the encoded foreign protein (Tang *et al.*, 1992) and then, in experiments with DNA encoding influenza nucleoprotein, that immunity was both humoural and cell-mediated and also protective in mice challenged with the virus (Ulmer *et al.*, 1993; Fynan *et al.*, 1993). This was the first demonstration of an experimental DNA vaccine. Shortly after, humoural and cell-mediated immunity against HIV-1 using plasmids encoding the HIV rev and env proteins was reported (Wang *et al.*, 1993) and similar results were obtained a little later with a gene for the hepatitis B surface antigen (HBsAg) (Davis *et al.*, 1994). It also appeared that DNA immunisation could be applied in cancer treatment: injection of plasmids encoding tumour antigens resulted in the induction of immune responses (Conry *et al.*, 1995; Bright *et al.*, 1996) which were protective in an animal model (Manickan *et al.*, 1997). Following these pioneering studies, the concept of DNA immunisation has now been adopted by vaccinologists worldwide using an ever increasing number of plasmids encoding immunogens from bacterial, viral and parasitic pathogens and a variety of tumours (Gregoriadis, 1998; Lewis and Babiuk, 1999). In many of these studies genetic immunisation led to the protection of animals from infection (Davis *et al.*, 1993a; Manickan *et al.*, 1997; Gregoriadis, 1998). Several clinical trials for the therapy of or prophylaxis against a variety of infections are in progress (Zhang *et al.*, 2003; Wu *et al.*, 2003; Locher *et al.*, 2004).

DNA vaccines could be more efficient than conventional vaccines (Davis and McCluskie, 1999) in a number of ways such as:

1) Ease of production. Different protein antigens exhibit a wide variation in physicochemical properties, and as such require different and complex methods of production and purification. In contrast, plasmid DNA possesses essentially the same physicochemical properties regardless of the antigen they code, and can be purified by a single method.

2) Increased stability. Plasmid DNA vaccines are relatively more stable on storage than conventional protein vaccines, and their cost of storage and transport could potentially be lower than most current vaccines.

3) Ease of manipulation. Development of molecular techniques such as cloning allows the rapid construction and alteration of DNA vaccine plasmid vectors. It is possible for a single plasmid to be designed to express more than one antigen, and to restrict the expression of the transgene to certain cell types (Sasaki *et al.*, 2003). In addition, other genes encoding adjuvants such as cytokines may be included (Egan and Israel, 2002).

4) Enhanced purity. DNA vaccines share the advantage of subunit vaccines over their live and killed counterparts in that they permit the administration of a single antigen, and as such can be used to focus the immune response to an antigen or epitopes known to confer protective immunity. In addition, as with subunit vaccines (Hui *et al.*, 1999), plasmid DNA vaccines have the potential for the delivery of chimeric antigen (Schirmbeck *et al.*, 2001) or fusion proteins based on multiple antigenic determinants.

5) Increased safety. Similar to recombinant subunit vaccines, they can be manufactured to high standards of purity and, as a result, safety. In addition, they do not suffer from the risks involved with the use of live vaccines in terms of reversion to virulence, or side effects associated with these and killed whole cell vaccines.

The plasmid construct (Fig. 1.5) usually supercoiled (Manickan *et al.*, 1997) can be divided into two main elements based on the sequences expressed in mammalian (eukaryotic) and bacterial (prokaryotic) cells: (1) the unit that drives antigen synthesis incorporating a promoter and enhancer region, introns, polyadenylation signals, and the antigenic gene of interest; and (2) the unit that mediates the propogation and amplification of the construct in the bacterial system, comprising the replication origin, multiple cloning sites, and a selectable marker (Azevedo *et al.*, 1999). A promoter sequence often derived from the cytomegalovirus (CMV) or rous sarcoma virus (RSV) is used to drive the transcription of the antigen gene insert. Generally, the CMV promoter is perceived to be effective and to offer high rates of expression in a broad range of tissues (Feltquate, 1998). An mRNA stability polyadenylation region at the 3' end of the insert, to ensure translation, the plasminogen activator gene which controls the secretion of the recombinant product and ancillary signals. Origin of replication serves the function of amplification of the plasmid in bacteria and a gene for antibiotic resistance to select the transformed bacteria. As only one or two representative viral genes are selected for insertion in the DNA plasmid and not the full length viral genetic information, there is no danger of genetic recombination with superinfection by natural viral isolates.

Recent developments in genetic vaccination technology have led to a common set of operational procedures (Chattergoon *et al.*, 1997). In brief, after its construction, the plasmid is amplified in *Escherichia coli*. A large set of different *E. Coli* host strains have been studied to identify those producing large amounts of DNA per cell of the highest quality. The produced plasmid is then purified from the lysed cells by gel chromatography or density gradient centrifugation, followed by phenol extraction. It is essential that plasmids made for *in vivo* use are highly homogeneous, sterile and free of all contamination, particularly endotoxins, RNA, protein and genomic DNA. To that end, the QIAGEN procedure has been approved by European countries and the USA (Scheef *et al.*, 1996).

Fig. 1.5 Schematic diagram of plasmid DNA encoding hepatitis B surface antigen (pRc/CMV-HBS, 5618 base pairs) (Image source: aldevron web page).

Vaccination with naked DNA by intramuscular route relies on the ability of myocytes to engulf the plasmid. Some of the DNA may also be endocytosed by APCs infiltrating the site of injection or in the lymph nodes following its migration to the lymphatics. The extent of DNA degradation by extracellular deoxyribonucleases is unknown but, depending on the

time of residence interstitially, degradation could be considerable. In the gut milieu, naked DNA has failed to elicit an immune response, probably because of its anticipated complete degradation (Manickan *et al.*, 1997). It follows that approaches to protect DNA from the extracellular biological milieu, introduce it into cells more efficiently or target it to immunologically relevant cells should contribute to optimal DNA vaccine design.

1.1.6.2 The subunit vaccine

Subunit vaccines have gained credence in their potential to ameliorate safety concerns; vaccines based on subunits lack the extraneous materials often present in heat or chemically inactivated preparations and, hence, are often far less reactogenic and thus better tolerated by vaccinees (Alpar *et al.*, 2005). The downside of using the subunit approach is their relatively low immunogenicity as compared with attenuated and killed organisms, evoking the need for effective adjuvants (Bramwell and Perrie, 2005a). Safety has been particularly improved with the advent of recombinant technology, which allowed the production of safer and less expensive subunit vaccines. However, with many pathogens it is not possible to identify an antigen or antigens that are capable of inducing protective immunity (Makela, 2000).

Earlier work with the protein subunit vaccines (Horwitz *et al.*, 1995) built on observations that only the 30 kDa protein (Ag85B) was able to confer protection when administered alone. They achieved effective results using this antigen alone and in combination with other extracellular proteins, showing protection against weight loss, death, and growth of *M. tuberculosis* in the lungs and spleens of their guinea pig model. In a different study

(Baldwin *et al.*, 1999), it was found that mycobacterial antigen, Ag85A was protective in mice only when secreted and not when targeted to the cytoplasm. The secreted form engendered increased levels of transgene specific antibody titers in addition to enhanced antigen-specific lymphoproliferation and CTL-mediated lytic activity.

In an antibody response, the immune system responds to a foreign antigen by invoking the involvement of three cell types, namely antigen presenting cells (APC, e.g. macrophages and dendritic cells), T cells, and B cells. These three cell types need to interact with one another, directly or indirectly, prior to the successful production of specific effector molecules, i.e., antibodies. In the scheme of events shown in Fig. 1.6 resulting in antibody production, the macrophage represents an APC which engulfs protein antigens; processes the antigens (possibly by lysosomal proteolysis) into immunogenic peptides; couples the immunogenic peptides to class II MHC molecules; and expresses the peptide-MHC II complex on the cell surface for presentation to T cells (Harding and Unanue, 1990; Harding *et al.*, 1991; Weinberger *et al.*, 1981).

The helper T cells ($CD4^+$), through the idiotypic specificity of the T cell receptor (TCR), can only recognise and interact with antigens, presented in the context of class II MHC on the surfaces of macrophages or other APC, whereby a putative activation signal is delivered. To become fully activated, however, it appears that $CD4^+$ T cells require the stimulation by a second signal, e.g., in the form of interleukin-1 (IL-1) released by macrophages (Mizel and Ben-Zvi, 1980; Scala and Oppenheim, 1983). The activated T cells, being triggered to release IL-2 and other cytokines such as IL-4, IL-6 etc., in the

process, can then synergize with B cells resulting in their proliferation and differentiation to plasma cells, which produce and secrete specific antibodies (Fig. 1.6). Most of the peptide antigens are T-cell dependent antigens which also require macrophage participation in engendering an antibody response. Only a limited number of non-protein antigens, e.g., lipopolysaccharide, dextran, and ficoll, which bear repeating polymeric units, are B-cell antigens, independent of T-cell and APC involvement (Anderson *et al.*, 1972).

Fig. 1.6 Illustration of the interactions and cell types involved in a normal antibody response to a polypeptide antigen (adapted from Shek and Sabiston, 1995).

1.1.7 Features of vesicle mediated DNA and protein subunit vaccination

Present conventional vaccines have certain limitations. If a dead organism or product is used as a vaccine to stimulate our protective immune response, the degree and longevity of

the protective response may not only vary among individuals, but also not lead to life-long protection (Alpar and Bramwell, 2002). Also, if a live, attenuated form of virus, for example is used to prepare a vaccine, the virus itself may revert to the wild-type - strong form - of the virus and cause disease. Indeed due to this small but plausible risk, the UK has recently changed its vaccination programme, replacing the live form of Polio (OPV) with the inactivated form (IPV; Perrie, 2006). For certain immuno-compromised individuals, even injection with an attenuated form of virus that is safe for the majority of individuals, can cause disease and even death. On the other hand, although the extra-cellular localisation of killed virus vaccines and their subsequent phagocytosis by professional antigen-presenting cells (APCs) or antigen specific B cells lead to MHC class II restricted presentation and to T helper cell and humoural immunity, they do not elicit significant cytotoxic T lymphocyte (CTL) responses which can be overcome by the use of DNA vaccines. Screening of effective delivery systems for DNA vaccines will certainly have implications on the delivery of other nucleic acid moieties such as antisense oligonucleotides and small interfering RNA as they are likely to share similar barriers to effective delivery (Demeneix *et al.*, 2004).

Subunit vaccines tend to induce a mostly antibody-mediated immune response, which is generally more effective in combating extracellular microbes and toxin neutralisation. This is because of the presence of the antigen in the extracellular environment, which suggests to the immune system the presence of an extra cellular pathogen. Such a humoural response is often not sufficient to protect from intracellular pathogens that tend to require a predominantly cell-mediated immune response. DNA vaccines, due to the production of

their antigen within host cells, mimic intracellular pathogens in that the antigen is endogenously expressed, and as a result they are more efficient at eliciting the cell-mediated immunity (Gregoriadis, 1998).

However, with reference to regulatory concerns, the possibility of germ-line alteration, expression of the antigen in inappropriate tissue sites, inappropriate immune responses and immunopathology, longevity of antigen expression, and the induction of tolerance or autoimmunity as well as the generation of acute or chronic inflammatory responses, autoimmune sequelae and destruction of normal tissues potentially associated with the aberrant expression of some proteins have all been raised as issues of concern (Alpar and Bramwell, 2002). In addition, the co-expression of cytokine genes may require the assessment of unintended adverse consequences, such as generalised immunosupression, chronic inflammation, autoimmunity or other immunopathology.

Potentially, DNA vaccines are capable of providing the immunogenicity of live attenuated vaccines, with the safety of a recombinant subunit vaccine. Since a DNA vaccine leads to synthesis of foreign proteins (from a virus or bacterium) inside (usually) the muscle cell, the immune system recognizes this event much more precisely. A small percentage all of the protein molecules synthesized within a cell break down into small fragments (peptides). These protein fragments ultimately appear on the surface of the cell in association with special proteins known as MHC Class I proteins (major histocompatibility protein class I). Our immune system's cells which constantly survey the surface of our cells for the presence of foreign protein fragments, special cells of the immune system known as cytotoxic

(killer) T-cells molecularly recognize these foreign shapes and respond to them by killing them. Other kinds of T cells are also activated that lead to the activation of antibody-producing cells known as B cells. Thus, an infection or a DNA vaccine, lead to a very similar complete activation of the immune system - the generation of killer T cells as well as the generation of soluble, circulating antibodies that react with the foreign material. Therefore, if we are actually infected by a live organism at a later time, our immune system responds quickly and more efficiently, having been previously primed to respond, because of a previous infection or the DNA vaccine. However, despite their possible advantages, DNA vaccines have yet to fulfil their promise (Adams *et al.*, 2004) with their success generally being limited to small animal models. Further relatively high and multiple doses are required for DNA vaccines to be effective in comparison to protein vaccines (Donnelly *et al.*, 2003). This may be due to a combination of the rapid degradation of DNA by exonucleases *in vivo* and the poor antigen presenting ability of muscle cells (Gregoriadis, 1998).

Alternatively subunit vaccines do offer many of the safety advantages that DNA vaccines but unfortunately, subunit and peptide vaccines produced recombinantly or synthetically, while considered safe, are weak immunogens and often unable to induce appropriate immune responses and these antigens generally require adjuvants to improve their efficacy. There are a great variety of experimental immunological adjuvants ('*adjuvare*' in latin means, '*to help*') which include saponins and other surface active agents, vesicle delivery systems such as liposomes and immunostimulating complexes (ISCOMS), freund's complete and incomplete formulations (killed mycobacteria in mineral oil), lipid A derived

adjuvants such as monophosphoryl lipid A (MPL), muramyl dipeptides (MDP), cationic agents such as DDA, oils such as squalane and squalene, immunostimulants such as CpG motifs and stimulatory cytokines (Orme, 2006, Gregoriadis *et al.*, 1993). However, besides aluminium based compounds (generically called alum), the only adjuvants that are approved for use in humans are liposomes (Gregoriadis, 1990), a squalene based oil-water emulsion (MF59) (Powell and Newman, 1995) and virosomes for use in influenza vaccines (Singh and O'Hagan, 1999). Unfortunately, Alum generally promotes weak humoural Th2 type immune responses to protein subunits, and elicits insufficient cell-mediated Th1 immune responses (Singh and O'Hagan, 1999; Gupta, 1998). In particular, the failure of alum to stimulate a cell mediated Th1 immune response, required for protective immunity against cancers and infectious diseases has led to the search for more potent vaccine adjuvants that not only enhance the level of immune activation but also selectively induce cell-mediated immune responses. For example, the use of the cationic lipid DC-Chol as an adjuvant has been shown to overcome non-response to the hepatitis B vaccine in mice (Brunel *et al.*, 1999).

1.1.8 Surfactant vesicles as immunological adjuvants

Surfactant vesicles aids in the protection of DNA and antigen degradation allowing its administration by a variety of routes, provides the ability to sustain the antigen release over an extended period of time, promotes intracellular delivery of antigen contributing to cytotoxic T cell stimulation, and targeting the antigen system into APCs. Hence, with the aim of eliciting broad immune response, especially with a strong cellular component, the trend has been to formulate vaccines to include adjuvants (some recent studies outlined in Table 1.3) in order to achieve depot formation, recruitment, targeting and activation or

conditioning of APCs in the presence of desired antigen. This way the particulate adjuvants could play a fundamental role, alone or as part of a formulation, acting as carrier to target immune cells and as depot (Sasaki *et al.*, 2003).

1.1.8.1 Liposomes as adjuvants

The immunological adjuvant property of liposomes was first established (Allison and Gregoriadis, 1974) when strong humoural immune responses to diphtheria toxoid (encapsulated in liposomes) were obtained after injection into mice. Since then, a large variety of bacterial, viral, protozoan, tumour, and other antigens (Alving, 1991; Gregoriadis, 1990) have been successfully encapsulated in liposomes, and in such a form shown to elicit the adjuvanticity without any side effects that are observed in other adjuvants. Moreover, liposomes composed of appropriate phospholipid (e.g., egg phosphatidylcholine) are not immunogenic by themselves and do not generate antibodies against their phospholipid components (Alving, 1991). These amenable features of liposomes led many formulations to preclinical and clinical trials with the first related vaccines, Epaxal™-Berna against hepatitis A and Inflexal™ against influenza licensed for use in humans. Epaxal™ and Inflexal™ are both based on liposomes (virosomes) produced from the lipids of influenza virus (Gluck *et al.*, 1999). These so called virosomes are liposomal formulations that have viral envelope proteins anchored in their lipid membrane which produces virus-like particles which have been proven to be effective immunogens with unique adjuvant properties (Moser *et al.*, 2003). In addition, promising data has also been generated with other lipid-based carrier/adjuvant systems, such as transferosomes (liposomes consisting of phosphatidylcholine and cholate with enhanced skin penetrating

properties for cutaneous administration), archaesomes (liposomes prepared using archaeobacteria membrane lipids that are reported to induce superior immune responses comparable to immunisation with complete freund's adjuvant), cochleates (formed from non-vesicular bilayer sheets consisting of phosphatidylethanolamine, phosphatidylserine and cholesterol intercalated by calcium ions) or proteosomes (prepared by reconstitution of the bacterial outer membrane and comprised mainly from proteins; they provide high immunogenicity) (Kersten and Crommelin, 2003). For the delivery of plasmid DNA based vaccines cationic liposomes have been widely reported as an efficient carrier systems (Wong *et al.*, 2001; Gregoriadis *et al.*, 2002; Perrie *et al.*, 2003).

1.1.8.2 Niosomes

Initial investigations into the adjuvanticity of niosomes prepared from 1-monopalmitoyl glycerol, cholesterol and dicetyl phosphate (5:4:1 molar ratio), has demonstrated in mice, on subcutaneous administration of bovine serum albumin (Brewer and Alexander, 1992), ovalbumin (Brewer *et al.*, 1998), or a synthetic peptide containing a known T-cell epitope (Brewer *et al.*, 1996) that niosomes were generally better stimulators of IgG2a than the potent freund's complete adjuvant, but weak stimulators for IgG1, and that the adjuvant activity of niosomes was wholly dependent on the model antigen being entrapped within the vesicles while mixing free antigen with the preformed vesicles was not effective (Brewer and Alexander, 1992). The same niosome system has also been shown to act as a vaccine adjuvant when administered intraperitoneally to severe combined immunodeficiency mice reconstituted with peripheral blood lymphocytes (PBL-SCID

mice) (Walker *et al.*, 1996). This mouse model was designed to mimic the human response to an antigen challenge (Walker *et al.*, 1996). Niosomal systems though possess a promising scope, have been relatively less used for the purposes of gene delivery. Some recent work evaluating 1-monopalmitoyl glycerol-based niosomes formulated by the DRV method has shown to display an increased stability and increased plasmid DNA retention in the presence of competitive anions when compared to similarly formulated PC-based liposomes (Obrenovic *et al.*, 1998). In addition, these systems showed good promise as agents for DNA vaccine delivery, engendering transgene-specific immune responses comparable with their liposomal counterparts (Obrenovic *et al.*, 1998; Perrie *et al.*, 2002; Perrie *et al.*, 2004). A similar trend depicting the superior features of 1-monopalmitoyl glycerol-based niosomes over DDA-based liposomes employing several subunit vaccines is demonstrated in this present work.

Novasome™ adjuvants, also referred to as non-phospholipid liposomes (Chambers *et al.*, 2004), are multicomponent adjuvant systems made up of dioxyethylene cetyl ether, cholesterol, and oleic acid. These systems have been licensed for veterinary application (for the immunisation of fowl against Newcastle virus disease and avian rheovirus). Recent work using Novasome™ technology has shown protection of guinea pigs against aerosol challenge with virulent *M. bovis*. This Novasome™ formulation was made with glycerol monostearate and butyl alcohol and included the potent adjuvant monophosphoryl lipid A (Chambers *et al.*, 2004).

Table 1.3 Recent *in vivo* studies in mice employing DNA and subunit antigen based vaccines formulated through different surfactant vesicle delivery systems.

Composition	Antigen/plasmid	Route/dose/ regimen	Key observations/reference
Liposomes consisting PC:Chol and niosomes consisting Span 85:Chol (both 7:3 molar ratio).	pRc/CMV-HBS	Topical/ 100 µg/ 2 inj. at two week intervals.	Topical niosomes elicited a comparable serum antibody titers and endogenous cytokines levels with respect to intramuscular recombinant HBsAg and topical liposomes. The study signifies the potential of niosomes as DNA vaccine carriers for effective topical immunization. The proposed system is simple, stable and cost effective compared to liposomes (Vyas *et al.*, 2005).
Niosomes consisting mannosylated Span 60:Chol:SA (6:3:1 molar ratio).	pRc/CMV-HBS	Oral/100 µg/ 2 inj. at three week intervals.	The serum anti-HBsAg titers obtained after oral administration of o-palmitoyl mannan (OPM) coated niosomal formulations was although less as compared to that elicited by naked DNA and pure HBsAg administered intramuscularly, the mice were seroprotective within 2 weeks and antibody level well above the clinically protective limit for humans was achieved (Jain *et al.*, 2005).
Polymer coated liposome/DNA complexes.	pRc/CMV-HBS	Intramuscular/Subcutaneous/ 10, 20, 20 µg/ 3 inj. at 1 month intervals.	Polymer (poly(D,L-lactic acid)) coated DNA lipoplexes were more effective than uncoated lipoplexes or naked DNA in eliciting transgene-specific immune responses via subcutaneous route. The polymer coated DNA lipoplexes by this route were also more effective than naked DNA delivered via intramuscular route in inducing antibody reponses (Bramwell *et al.*, 2002).
1-monopalmitoyl glycerol and PC based niosomes and liposomes.	pI.18Sfi/NP	Subcutaneous/ 10 µg/ 2 inj. 28 days apart.	No significant difference was observed between the PC and 1-monopalmitoyl glycerol based DRV vesicles in inducing humoural responses, both systems elicited significantly higher IgG, IgG1 and IgG2 responses compared to naked DNA (Perrie *et al.*, 2004).

DDA/Chol (1:1 molar ratio) based Liposome/DNA complexes.	pcDNAIE180	Intravenous/ 50 μg/ 2 inj. 2 weeks apart Intramuscular/ 50 μg/ 3 inj. 2 weeks apart.	High rate of seroconversion has resulted in C3H/HeJ mice when compared to that induced by the naked DNA vaccine, with 40 and 25% of mice vaccinated via i.v. and i.m. route respectively being protected against challenge. Data also support co-administration of the pcDNAIE180 with IL-2 gene which increased its immunogenicity (Bu *et al.*, 2003).
DC-Chol based preparations.	HBsAg	Subcutaneous/1 μg/ 2 inj. at 3 week intervals.	DC-Chol increased IgG1 and IgG2a and cellular immune responses inducing a balanced Th1/Th2 response overcoming the inherited unresponsiveness to HBsAg using alum-adjuvated vaccine, thus have implications for vaccination against hepatitis B virus, as well as for enhancing weak immunogenicity of other recombinant subunit antigens (Brunel *et al.*, 1999).
Sorbitan monostearate niosomes contained in organogel.	Haemagglutinin (HA)	Intramuscular/ 0.1 μg/ 2 inj. 1 month apart.	Vesicle-in-water-in-oil (v/w/o) gel was observed to possess immunoadjuvant propeties and enhance the primary and secondary IgG, IgG1, IgG2a, IgG2b titers to Haemagglutinin antigen (Murdan *et al.*, 1999).
DMPC:DMPG:Chol based liposomes (8:2:2 molar ratio).	Opa B128 and Opa J129 proteins	Intranasal/ 5μg/ 3 inj. 2 weeks apart.	Antigens were combined with adjuvants such as purified meningococcal LPS, monophosphoryl lipid A (MPL) or the B-subunit of *Escherichia coli* heat-labile enterotoxin. Anti-Opa IgA antibodies were found in nasal lavages. Of the different adjuvants used, meningococcal LPS gave the strongest overall immune response. Non-adjuvated liposomal Opa formulations were poorly immunogenic (de Jonge *et al.*, 2004).
DDA:TDB based cationic liposomes (4:0.5 molar ratio).	Ag85B-ESAT6	Subcutaneous/2 μg/ 3 inj. 2 weeks apart.	Induced strong, specific Th1 response characterised by substantial production of the IFN-γ cytokine and high levels of IgG2b antibodies was observed (Davidsen *et al.*, 2005).

1.1.9 Morphological investigation of surfactant vesicles

In addition to standard characterisation studies, this present work reports morphological and structural analysis of surfactant vesicles using Environmental Scanning Electron Microscopy (ESEM). This is the first time such studies have been reported for niosomal systems. Morphological studies are usually performed using various techniques such as scanning electron microscopy (SEM) (Lopez *et al.*, 2001), freeze fracture analysis (Egelhaaf *et al.*, 2003), scanning tunnelling microscopy (STM) (Zareie *et al.*, 1997) etc. However, most of these techniques require samples to be dried, fixed, frozen and/or coated with conducting materials before imaging, resulting in potential changes in the morphology of vesicles due to the mechanical stresses encountered during specimen preparation. As a result these images can be poor representative of the systems in real time conditions. Alternatively transmission electron microscopy (TEM) and in particular ESEM can overcome these problems with their ability to image wet systems with no prior elaborate specimen preparation. Though TEM has been extensively used in imaging surfactant vesicles (e.g. Darwish and Uchegbu, 1997, Arunothayanun *et al.*, 2000), ESEM is a new technique as far as imaging surfactant vesicles is concerned (Mohammed *et al.*, 2004). The underpinning superior feature of ESEM compared to other microscopy methods is that it allows altering the sample environment dynamically through a range of pressures, temperatures and gas compositions enabling hydration and dehydration processes to be followed as they happen in the sample chamber. Indeed ESEM has already proved valuable for analysis of hydrated samples such as thermo-responsive microspheres (D'Emanuele and Dinarvand, 1995), polymeric surfactant micelles (Cao and Li, 2002), and stability analysis of hydrophobic drug encapsulated liposomes (Mohammed *et al.*, 2004). Real time

morphological studies using TEM and ESEM techniques investigating the structural features of vesicles and monitoring the resistance of vesicles to coalescence during dehydration process respectively are discussed with ESEM technique providing as an alternative assay to study the vesicle formulation/stability relationships as previously reported for drug encapsulated liposomes (Mohammed *et al.*, 2004).

1.2 Aims and Objectives

Through continued characterisation of successful surfactant vesicle systems as immunological adjuvants in plasmid DNA and protein subunit delivery for major infectious diseases such as hepatitis B, tuberculosis and malaria, a much safer alternative to viral vectors can be reached. Keeping this as the central aim, the work presented here primarily focuses on development and screening of various cationic surfactant vesicle systems that are effective in increasing the magnitude of immune responses to the respective antigens in mice. The main objectives were to:

1. Investigate the physico-chemical parameters of niosomal-DNA vectors incorporating hepatitis B surface antigen encoded plasmid DNA (pRc/CMV-HBS) and compare them with their liposomal counter parts.

2. Study and evaluate various factors pertaining to vesicle composition on transfection efficiency *in vitro*.

3. Carry out immunisation experiments in mice employing various plasmid DNA incorporated surfactant vesicle systems following their *in vitro* study in order to assess their adjuvant properties.

4. Characterise and evaluate various surfactant vesicle systems for subunit vaccination in mice.

Chapter Two

Encapsulation of Plasmid DNA in DRV: Initial Characterisation Studies

2.1 INTRODUCTION

2.1.1 Surfactant vesicles in DNA delivery

It has been shown that naked, antigen-encoding plasmid DNA when injected, usually through the intramuscular route leads to humoural and cell-mediated immune responses against the antigen (e.g. Lewis and Babiuk, 1999). This happens when DNA is taken up by muscle cells followed by the DNA expression and extracellular release of the generated antigen, which in turn is taken up by antigen presenting cells (APCs). The disadvantage is that the DNA is taken up only by a minor fraction of muscle cells thus necessitating the use of relatively large quantities of DNA. Secondly, naked DNA is prone to deoxyribonuclease attack in the interstitial fluid. Previous results have shown that such problems could be overcome by encapsulating plasmid DNA into liposomes (Gregoriadis, 1998; Gregoriadis *et al.*, 1997). This approach would eliminate involvement of muscle cells and facilitate, instead, uptake of DNA by APCs infiltrating the site of injection or in the lymphatics (Gregoriadis, 1995; Gregoriadis 1990) at the same time the encapsulated DNA is protected from nuclease attack (Gregoriadis *et al.*, 1996a). In addition, transfection of APCs with encapsulated DNA could be enhanced by optimising the vesicle surface charge, size and surfactant/lipid composition. It has already been shown (Gregoriadis, 1998; Gregoriadis *et al.*, 1997; McCormack and Gregoriadis, 1998; Perrie *et al.*, 2001; Obrenovic *et al.*, 1998) that immunization of mice by various routes with cationic liposomal or niosomal DNA leads to humoural and cell-mediated immune responses much greater than those obtained with naked DNA. These features have formed the basis for the use of surfactant vesicles as immunological adjuvants for protein and peptide and DNA vaccines (Gregoriadis 1990).

2.1.2 Aims and Objectives

In the preliminary studies as a prerequisite to any *in vivo* investigation, physico-chemical characteristics of the surfactant vesicle systems incorporating plasmid DNA (pRc/CMV-HBS) are to be optimised. To this end, surfactant vesicles formulated using dehydration-rehydration method employing a single alkyl chain non-ionic surfactant, 1-monopalmitoyl glycerol (MP) (16:0) and egg phosphatidylcholine (PC), along with DOPE, cholesterol and different cationic lipids such as DC-Chol and DOTAP were characterised to assess the effect of surfactant/lipid composition on the vesicle size, surface charge, incorporation efficiency and stability.

2.2 MATERIALS AND METHODS

2.2.1 Materials

2.2.1.1 Chemicals

Agarose, ethidium bromide (EtBr), sucrose, trehalose, triton X-100, glycerol, ethylene diaminetetraacetic acid (EDTA), disodium EDTA, Tris-chloride, magnesium chloride, deoxyribonuclease I (DNase I), ampicillin, luria bertani (LB) agar, LB broth, sodium chloride and phosphate buffer saline tablets (PBS) were purchased from Sigma-Aldrich, Poole, Dorset, UK. Sodium dodecyl sulphate was purchased from Bio-Rad Laboratories, USA. Library efficiency® DH5α™ Competent cells and S.O.C medium were obtained from Gibco-Invitrogen, Carlsbad, CA. Endotoxin-free plasmid purification kits were from Qiagen® Ltd, West Sussex, UK. Restriction enzyme (*Hin*d III), Restriction enzyme buffer, acetylated-bovine serum albumin (BSA), supercoiled DNA ladder (2-10 kb), *Eco*R I ladder DNA (3.530 - 21.226 bp) and loading buffer were products of Promega, Madison, WI, USA. Tris, boric acid were purchased from ICN

Biomedicals, Inc. Ohio, USA. PicoGreen dsDNA quantitation reagent was purchased from Molecular Probes-Invitrogen, UK. All other reagents and solvents were of analytical grade.

2.2.1.2 Surfactants and Lipids

1-monopalmitoyl glycerol (C16:0) (MP), cholesterol (Chol), cholesteryl 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) were purchased from Sigma-Aldrich, Poole, Dorset, UK. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) were purchased from Avanti® Lipids, Alabaster, AL, USA. Egg phosphatidylcholine (PC) was purchased from Lipid Products, Nutfield, Surrey, UK.

2.2.1.3 Plasmid DNA

Plasmid DNA (pRc/CMV-HBS, 5.6 kb) encoding the Hepatitis B surface antigen (HbsAg) was purchased from Aldevron, Fargo, USA.

2.2.2 Methods

2.2.2.1 Amplification and purification of plasmid DNA

2.2.2.1.1 Transformation

The competent cells of *E. coli* DH5α were removed from –70°C freezer, thawed in hand and gently mixed, 100 µl of cells were aliquoted into 15 ml pre-sterilised, chilled *falcon* tubes using chilled tips. 5 µl (20 ng) of pRc/CMV-HBS plasmid DNA was added and mixed thoroughly by tapping and incubated on ice for 30 min. Later this tube was placed in water bath at 42°C for 45 seconds subjecting it to heat shock. Shaking of the tube during this stage was avoided. The tube was placed in ice bath for 2 min. To this

tube, 900 µl of S.O.C. medium was added at room temperature and incubated in water bath at 37°C with shaking (225 rpm) for 1 hour. 10 µl and 100 µl of this growth medium was spread on ampicillin containing agar plates separately and further incubated by placing them inverted for 12-16 hours (overnight) at 37°C.

2.2.2.1.2 Amplification of plasmid DNA (transformants)

Plasmid DNA was amplified in the host strain *E.coli* DH5α (Sambrook, *et al.*, 1989). A single colony from a freshly streaked agar plate (prepared as described in section 2.2.2.1.1) was picked with a sterile loop and inoculated the flask with starter culture of 20 ml LB medium containing ampicillin (100 µg/ml) and incubated for 8 hours at 37°C in an incubator shaker (300 rpm). After 8 hours the starter culture was diluted 1 in 500 into 500 ml of sterile, selective LB medium and was grown at 37°C with vigorous shaking (300 rpm) for 12-16 hours (overnight). The culture reaches a cell density of approximately 3-4 × 10^9 cells ml^{-1} corresponding to the wet weight of pellet, approx. 1.5 g /500 ml medium.

2.2.2.1.3 Purification of plasmid DNA

Plasmid DNA that was quantitatively amplified in as described in section, 2.2.2.1.2 was extracted from the *E.coli* and purified using the Qiagen® kit according to alkaline lysis method which involves harvesting of the bacterial cells by centrifugation at 6000 × *g* for 15 min at 4°C. The pellet obtained was re-suspended in buffer containing RNase, the bacterial cells were lysed in NaOH-SDS buffer which was mixed gently by inverting 4-6 times and incubated at room temperature for 5 min. SDS solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents. NaOH denatures the chromosomal and plasmid DNA, as well as proteins. The lysate was neutralized by the addition of chilled acidic potassium acetate buffer, mixed

gently by inverting 4-6 times and incubated on ice for 30 min. The high salt concentration causes potassium dodecyl sulfate to precipitate, the denatured proteins, chromosomal DNA and cellular debris become trapped in salt-detergent complexes. Plasmid DNA being smaller and covalently closed, re-natures correctly and remains in solution. RNase which was added in the beginning digests the liberated RNA efficiently during alkaline lysis. The precipitated debris was removed by centrifugation at 20,000 × *g* for 30 min at 4°C producing a clear lysate, if any suspended material from the pellet was found in the lysate, it was filtered using Whatman filter paper in order to avoid clogging of the Qiagen® tip. The lysate was then loaded onto a pre-equilibrated Qiagen® tip consisting of anion-exchange resin and eluted by gravity flow. The salt and pH conditions of the lysate and precise selectivity of the resin makes plasmid DNA to bind, while degraded RNA, cellular proteins, and metabolites flow through the resin. The Qiagen® tip was then washed with medium-salt buffer which completely removes any remaining contaminants, such as traces of RNA and protein without affecting the bound plasmid DNA. The plasmid DNA was then eluted from the Qiagen® tip with high-salt buffer. The eluted plasmid DNA was desalted and concentrated by isopropanol precipitation. Precipitation was carried out at room temperature to minimize co-precipitation of salt. This mixture was centrifuged at 15,000 × *g* for 30 min at 4°C. Following the centrifugation, the DNA pellet was washed with 70% ethanol to remove residual salt and also to replace the isopropanol with ethanol, which was then air-dried for about 15 min and re-dissolved in 1 ml of endotoxin-free TE buffer, pH 8.0 and stored between 2 – 8°C for experimental use.

2.2.2.2 Characterisation of plasmid DNA using restriction enzyme digestion

Restriction enzymes, also referred as restriction endonucleases, are enzymes which recognise short, specific DNA base sequences. They cleave double-stranded DNA at

specific sites within or adjacent to their recognition sequences. Restriction enzyme digestion was performed on purified pRc/CMV-HBS plasmid to confirm its structure, size and purity. The restriction enzyme *Hind* III was used to cut open the plasmid at a single, specific site and hence linearise it to give a single band which moves slower than the closed circular plasmid DNA on the agarose gel. The digestion was performed in a sterile microfuge tube by adding 16.3 µl of sterile, deionised water, 2 µl of 10 × restriction (RE) buffer, 0.2 µl of acetylated bovine serum albumin (BSA), 1 µg of plasmid DNA. After mixing the contents, 0.5 µl of *Hind* III restriction enzyme (10 units/ µl) was added. Addition of restriction enzyme was omitted in the control. The final volume was adjusted to 20 µl with sterile, deionised water. All the ingredients were gently mixed by pipetting, the tube was closed and centrifuged for a few seconds in a microcentrifuge to collect the contents in the bottom of the tube and incubated at 37°C for 2 hours after which the digested and undigested DNA were subjected to agarose gel electrophoresis for a period of 2 hours on a 0.8 % agarose gel at 80 V as described in section 2.2.2.3 using supercoiled ladder DNA (2 –10 kb) and lambda DNA/ *Eco*R I (3,530 bp – 21,226 bp) as markers to confirm the sizes of undigested supercoiled plasmid DNA and the digested, linearised plasmid DNA respectively.

2.2.2.3 Agarose gel electrophoresis

The integrity of plasmid DNA and its nature of binding (entrapped or surface-complexed) on the surfactant vesicles was determined by subjecting the samples to agarose gel electrophoresis and analysing the retention of DNA by the vesicles under various conditions. In brief, 8 µl (1.6-2.0 µg DNA) of DRV or SUV suspension were mixed with gel loading buffer (sucrose, 40% w/v; EDTA, 0.1 M pH 8) and subjected to agarose gel electrophoresis at 25°C in the presence of ethidium bromide (0.5 µg/ml) for

1 h at 80 V. In some experiments the gel loading buffer was supplemented with sodium dodecyl sulphate (SDS) to a concentration below the critical micelle concentration (0.05-0.1% w/v; Florence and Attwood, 1998) to measure the effect of anionic competition on the liposome formulations (Perrie and Gregoriadis, 2000). Agarose gel (0.8% w/v) was prepared in 0.5 × TBE buffer including ethidium bromide at a concentration of 0.5 μg/ml. The DNA bands were visualized by placing the gel under UV light and imaged on Gene Flash (Syngene, UK).

2.2.2.4 Preparation of surfactant vesicles

2.2.2.4.1 Multilamellar vesicles (MLV)

Multilamellar vesicles (MLV) were prepared using a technique based on the established lipid hydration method (Bangham *et al.*, 1965). Briefly, the chosen lipids/surfactants were dissolved in a 9:1 (v/v) solvent mixture of chloroform and methanol and the solvent was evaporated on a rotary evaporator to obtain a thin dry film. The film was flushed with nitrogen and the film was hydrated with 2 ml of aqueous buffer or double distilled water above the liquid-crystalline temperature (T_c) of the surfactants and the suspension was allowed to stand for about 30 min above T_c during which multilamellar vesicles (MLV) of various sizes were formed. A typical cationic niosome or liposome preparation consists of MP or PC, Cholesterol, DOPE, DC-Chol; 16:8:4:4 μmoles respectively.

2.2.2.4.2 Small unilamellar vesicles (SUV)

The 2 ml suspension of MLV was sonicated (MSE probe sonicator) above T_c of the lipid using a titanium probe for 2 min at amplitude of 5 microns. The milky white dispersion of MLV converted into a clear to slightly hazy transparent dispersion of

small unilamellar vesicles (SUV) ranging in the size of 75 - 100 nm in diameter. The haze is presumed to occur due to light scattering induced by residual large vesicles remaining in the suspension.

2.2.2.4.3 Dehydration-rehydration vesicles (DRV)

The dehydration-rehydration procedure (Kirby and Gregoriadis, 1984; Perrie and Gregoriadis, 2000) was used for the incorporation of plasmid DNA (pRc/CMV-HBS; 100 μg) into surfactant vesicles. In brief, 2 ml of small unilamellar vesicles (SUV) prepared by sonication and composed of 16 μmol PC or MP, 8 μmol of DOPE, 4 μmol of Chol and 4 μmol of either the cationic lipid DC-Chol or DOTAP. These SUV were mixed with 100 μg of plasmid DNA, frozen at –70°C for 30 min and freeze dried over night. Controlled rehydration (Kirby and Gregoriadis, 1984) of the dry powders led to the formation of DNA-containing multilamellar dehydration-rehydration vesicles (DRV(DNA)). DRV preparations were then centrifuged twice at 25000 × *g* for 40 min to remove non-entrapped or non–complexed DNA and re-suspended in 0.01 M sodium phosphate containing 0.15 M NaCl (phosphate-buffered saline; PBS, pH 7.4) to the required volume.

2.2.2.4.4 Small dehydration-rehydration vesicles (DRV)

The preformed SUV (section 2.2.2.4.2) were subjected to freeze drying in the presence of a carbohydrate (either sucrose or trehalose) at concentrations which are below those known (Crowe and Crowe, 1993) to preserve the stability of the vesicles during dehydration process. At these low concentrations, the SUV may be destabilized just enough to allow the entry of solute present upon rehydration while they reform either to their original state or slightly larger vesicles (Zadi and Gregoriadis, 2000). In this

modified procedure of DRV preparation, the SUV are mixed with 100 μg of plasmid DNA and varying quantities of carbohydrate based on lipid mass ratio, samples were mixed and frozen at –70°C for 30 min and freeze dried at –40°C (below the glass transition temperature (T_g) of carbohydrate, for example, sucrose has a T_g of -32°C) for 72 hours. Controlled rehydration of the dried cake was performed similar to the procedure described in section 2.2.2.4.3.

2.2.2.4.5 Determination of vesicle size

The z-average vesicle diameter of surfactant vesicles was measured on *ZetaPlus* (Brookhaven Instruments, USA) using photon correlation spectroscopy (PCS) technique, at 25°C by diluting 50 μl of the dispersion to the appropriate volume with doubly-filtered (0.22 μm pore size) distilled water.

2.2.2.4.6 Determination of zeta potential

Zeta potential (an indirect measurement of the vesicle surface charge) of various vesicle preparations was also measured on *ZetaPlus* (Brookhaven Instruments, USA). 50 μl of the dispersion was diluted in 4 ml of 0.001 M PBS and proceeded for the measurement at a temperature of 25°C.

2.2.2.4.7 Removal of un-encapsulated DNA

Un-encapsulated DNA was removed from the vesicle dispersions by centrifuging (15000 rpm) for 30 min at a temperature of 4°C using JA 20 rotor (BECKMAN Centrifuge, USA). The supernatent was separated and the pellet obtained was dispersed to appropriate volume using PBS. Small DRV(DNA) were subjected to ultra-centrifugation at 100000 × g for 1 hour at 4°C.

2.2.2.4.8 Evaluation of DNA incorporation

DNA incorporation with in the various vesicle preparations was estimated on the basis of PicoGreen analysis of plasmid DNA recovered in the suspended pellets and the supernatants after treatment with 0.1% Triton X-100 (Fenske *et al.*, 2002). PicoGreen is a fluorescent, intercalating cyanine dye which selectively binds to the double stranded plasmid DNA with a linear detection range extending over more than four orders of magnitude of DNA concentration and therefore can be effectively used to determine niosomal or liposomal DNA loading. The brightness of this reagent is due to high quantum yield and large molar extinction coefficient (Fenske *et al.*, 2002).

2.2.2.4.8.1 Preparation of standard curve

0.2 ml of 10 µg/ml plasmid DNA was diluted in 1.8 ml of 0.01 M PBS, pH. 7.4. This stock solution was used to prepare serial aliquots of 0.01 to 0.50 µg/ml DNA. To each 1 ml aliquot 2 µl PicoGreen reagent was added. Containers were wrapped with aluminium foil to avoid exposure to light as PicoGreen reagent is susceptible to photo degradation. Samples were incubated for 2-5 min at room temperature after which 200 µl volume from each sample was pipetted into a black flat-bottomed 96 well plate and fluorescence was measured using a spectrofluorimeter (Spectra Max Gemini XPS from Molecular Devices, USA) at excitation and emission wavelengths of 495 nm and 525 nm respectively (Fig. in appendices). For sample assay, samples were diluted 1 in 40 (i.e., 25 µl of sample + 975 µl of PBS). Further 1 in 400 dilutions of the samples were made (i.e., 100 µl of the first dilution + 900 µl of PBS). To each diluted 1 ml sample 10 µl of 10% triton x-100 was added followed by the addition of 2 µl PicoGreen reagent and measured for the fluorescence at similar wavelengths as described above for the standards.

2.2.2.4.9 Statistical analysis

Where appropriate in all the chapters, results were statistically evaluated using one-way ANOVA followed by a multiple comparison Tukey test at a significance level of $P<0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 Purification and Characterisation of plasmid DNA

Plasmid DNA administered *in vivo* is intended to be sterile, highly homogeneous, free from contaminants, particularly endotoxins or lipo-polysaccharides (Raetz, 1990; Rietschel and Brade, 1992), RNA, proteins and genomic DNA since such molecules could reduce the efficiency and contribute to the damage of non-viral gene delivery systems apart from being toxic to host cells (Cotten *et al.*, 1994) if the isolation of DNA is not carried out stringently. Isolation and purification of plasmid DNA in these studies was performed using kits and optimised protocols supplied by Qiagen® patented technology.

2.3.1.1 Plasmid purification

The plasmid DNA used in these studies was transformed into and amplified in *E. coli* host strain Library efficiency DH5α™ Competent Cells and later was extracted and purified according to the alkaline lysis method (Birnboim and Doly, 1979) described in section 2.2.2.1.3. Samples were collected at different stages of plasmid purification process and a gel electrophoresis was performed to determine the efficiency of the entire procedure.

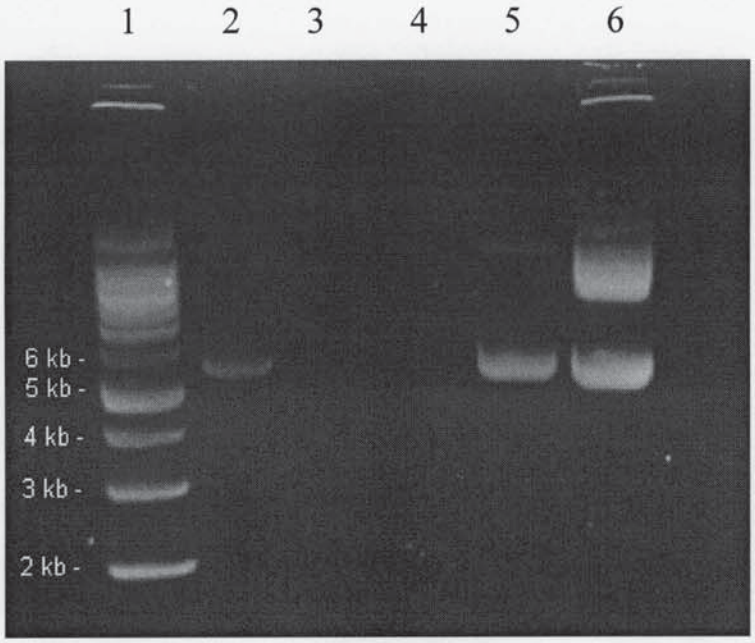


Fig. 2.1 Gel electrophoresis of samples collected at different stages of the plasmid (pRc/CMV-HBS) purification procedure. Lane 1: super coiled DNA marker (2-10 kb), lane 2: lysate, lane 3: flow through fraction, lane 4: wash fraction, lane 5: eluted pure plasmid DNA (pRc/CMV-HBS), lane 6: control plasmid DNA (pRc/CMV-HBS).

Fig. 2.1 shows a distinct band in lane 2 pertaining to supercoiled DNA in clear lysate collected during the purification procedure which also contains very small amounts of open circular DNA and degraded RNA (not visible in the figure). Lane 3 consists flow through fraction that has cellular proteins, metabolites, RNA fragments which do not bind to the resin and pass out as unbound material separating from the supercoiled DNA, lane 4 has the wash fraction which consists of remaining traces of RNA and protein and in lane 5, the elute consisting of pure supercoiled DNA free from contamination with other nucleic acids and matching with the control DNA in lane 6 was seen which lies between 5 and 6 kb of the super coiled marker DNA spanning between 2-10 kb in lane 1, as expected for the pRc/CMV-HBS plasmid which is 5618 bp in size. The control DNA in lane 6 was observed to have both open circular DNA and supercoiled DNA where the former usually is observed to run slower than the later.

2.3.1.2 Determination of plasmid DNA yield

20 µl of purified plasmid DNA was diluted to 1 ml with T.E buffer and the absorbance was read (Table 2.1) using UV spectrophotometer (JENWAY Ltd, UK) at a wavelength of 260 nm, using T.E buffer as blank. 50 µg/ml of control DNA gives an absorbance of 1, correspondingly, the concentration of the sample was calculated by multiplying A_{260} with 50 µg taking dilution factor, 50 into consideration. Hence, the final concentration obtained for the freshly amplified and purified plasmid DNA was 1.6 mg /ml. The ratio of A_{260}/A_{280}, signifying the purity of plasmid DNA was 1.86 (Table 2.1), which was greater than the desired 1.85 value. A_{230} values which signify the organic solvent contamination complied by not exceeding A_{260} values, A_{325} values which signify the presence of particulate matter when they are near to zero and were observed to comply in all the batches of plasmid purification.

Wave length (nm)	Absorbance
260	0.630
280	0.338
260/280	1.864
230	0.283
325	0.008

Table 2.1 Typical UV absorbance values obtained from a plasmid (pRc/CMV-HBS) DNA sample after purification.

2.3.1.3 Characterisation of plasmid DNA using restriction enzyme digestion

Restriction enzymes, also known as restriction endonucleases are used to identify specific base sequence in plasmid DNA by cleaving the plasmid at specific site (for example, *Eco*R I obtained from the bacteria *Escherichia coli* cuts at *GAATTC* sequence of the lambda bacteriophage DNA yielding fragments of predictable size often used as a marker). Digestion of purified plasmid pRc/CMV-HBS was performed as described in

section 2.2.2.2 with selective restriction enzyme, *Hin*d III which cleaves the DNA at specific base sequence and enables to confirm its original structure, size and homogeneity. Restriction enzyme cuts the circular plasmid DNA at a single site and converts it into a linearised plasmid monomer which is represented as a single band on gel electrophoresis and runs slower than the closed circular plasmid DNA.

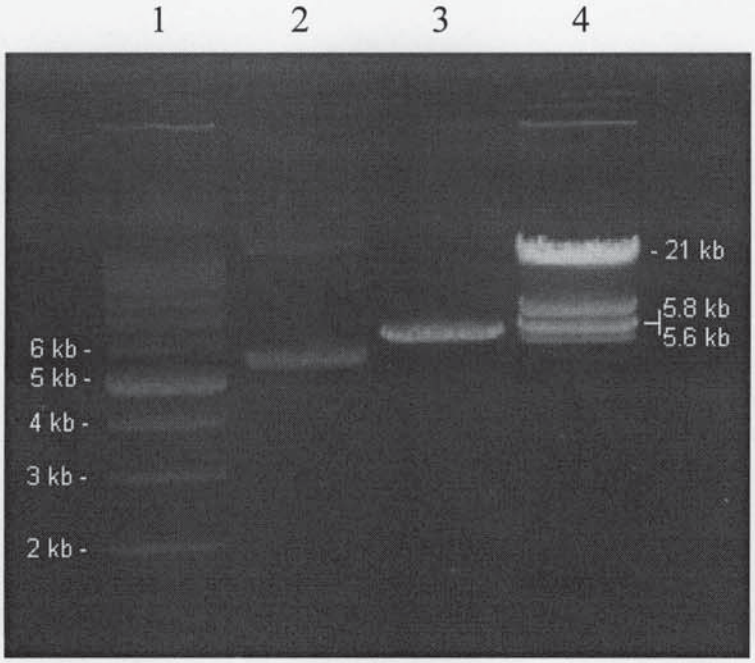


Fig. 2.2 Gel electrophoresis of plasmid DNA (pRc/CMV-HBS) before and after restriction enzyme digestion. Lane 1: super coiled DNA marker (2-10 kb), lane 2: plasmid DNA incubated in the absence of restriction enzyme, lane 3: plasmid DNA digested with *Hin*d III restriction enzyme, lane 4: lambda DNA/*Eco*R I marker (3.5 – 21 kb).

Fig. 2.2 describes the quality of the purified, supercoiled pRc/CMV-HBS plasmid DNA before and after digestion. As anticipated, in lane 2, the band of plasmid DNA (actual size 5.6 kb), incubated in the absence of *Hin*d III restriction enzyme, was observed to be between the bands of 5 and 6 kb of supercoiled DNA marker (2 – 10 kb) in lane 1. After subjecting the plasmid to digestion with *Hin*d III restriction enzyme, a linearised plasmid monomer was obtained which was represented as a single band indicating the absence of any impurities such as genomic DNA in the plasmid DNA solution, whose

molecular size was again seen to be between 5 and 6 kb in lane 3 although it is known that linearised DNA moves slower on the gel than the circular DNA (lane 2), which was measured against the bands of lambda DNA/*Eco*R I marker (3.5 – 21 kb) in lane 4.

2.3.2 Optimisation of surfactant vesicle preparation method encapsulating plasmid DNA

To assess the appropriate stage for addition of plasmid DNA during the dehydration-rehydration protocol surfactant vesicles composed of MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles) were prepared by adding the plasmid DNA at different stages of the protocol (described in section 2.2.2.4.3). MP is a synthetic non-ionic surfactant which was previously shown to efficiently mediate DNA immunisation (Obrenovic *et al.*, 1998). In the first instance, addition of DNA to the SUV solution was omitted prior to freeze-drying in order to produce "empty" water filled DRV while on the other hand, DNA was mixed with SUV prior to freeze-drying to produce DRV(DNA) wherein the DNA is assumed to be encapsulated with in the vesicles. Subsequent preparations were made by rehydrating the freeze-dried surfactant/lipid powder with a solution of plasmid DNA (100 μg; DRV-DNA_{reh}). To form DRV-DNA complexes, "empty" DRV prepared in the absence of DNA, were incubated with 100 μg plasmid DNA at 20°C for 30 min (DRV-DNA_{inc}).

2.3.3 The effect of preparation method on vesicle size and zeta potential

The method of surfactant vesicle preparation was shown to have a significant effect on the resultant vesicle size (z-average diameter, Table 2.2). It was notable that the measured vesicle size of the SUV doubled after addition of DNA, from approximately 100 nm to 200 nm (Table 2.2) presumably due to the formation of SUV-DNA complexes. Results for the various DRV preparations, however, indicate that, at the cationic lipid to DNA ratio employed the presence of entrapped DNA does not

influence vesicle size with the measured z-average diameter of 'empty' DRV and DRV(DNA) being in the similar size range (~1500-1700 nm; Table 2.2). In contrast, the size of the complexes formed on mixing preformed cationic DRV with 100 μg of DNA (DRV-DNA_{inc}) resulted in the formation of larger aggregates (2700 nm; Table 2.2). Interestingly, rehydration of freeze-dried SUV with DNA (DRV-DNA_{reh}) resulted in the formation of vesicles with a similar mean vesicle size (~2800 nm; Table 2.2) suggesting that these vesicles have similar characteristics between the two preparations. This is further supported by zeta potential studies. Values of the zeta potential of surfactant vesicles indirectly reflect vesicle net surface charge and can therefore be used to evaluate the extent of interaction of the vesicle surface cationic charges with the anionic charges of the DNA. On this basis, the characteristics of entrapped versus complexed DNA in the DRV preparations was investigated. Results (Table 2.2) showed no significant variation ($P>0.05$) in zeta potential among all the DRV preparations with a cationic charge being approximately ~45 mV. Interestingly, these results are in contrast to those in agarose gel electrophoresis studies (Fig. 2.3b), where in, more DNA displacement from the vesicle surface was seen in complexes but the neutralising effect by the anionic DNA on cationic DC-Chol head groups was not observed in case of DRV preparations unlike SUV-DNA complexes (~35 mV). Moreover, dehydration and rehydration of SUV appear beneficial with DRV having a higher measured zeta potential (~45 mV, Table 2.2).

DNA incorporation (% of used) was measured based on PicoGreen quantification of plasmid DNA, all the preparations tested gave a high (90-97%; Table 2.2) incorporation values which were at par with the lipid based DRV formulations previously reported (Gregoriadis *et al.*, 1996b; Perrie and Gregoriadis, 2000; Perrie *et al.*, 2001). PicoGreen

is an intercalating cyanine dye which selectively binds to double stranded plasmid DNA with a linear detection range extending over more than four orders of magnitude of

Formulation	Vesicle size (nm)	Zeta potential (mV)	Incorporation (% of used)
"Empty" SUV	95.8 ± 18.0	51.2 ± 1.8	-
SUV-DNA	217.4 ± 42.0	34.8 ± 3.1	91.6 ± 4.8
"Empty" DRV	1525.4 ± 79.1	46.8 ± 2.2	-
DRV(DNA)	1624.2 ± 150.8	45.3 ± 4.5	95.4 ± 2.6
DRV-DNA$_{reh}$	2794.0 ± 118.6	46.0 ± 3.6	96.9 ± 1.9
DRV-DNA$_{inc}$	2739.2 ± 86.6	46.6 ± 1.7	94.7 ± 3.3

Table 2.2 The effect of DRV preparation method on vesicle size, zeta potential and entrapment. DRV niosomes (composed of MP:DOPE:Chol:DC:Chol;16:8:4:4 μmoles) were prepared by various methods. SUV with no DNA added produced "empty" water-filled SUV and subsequent dehydration and rehydration of similar preparation resulted in formation of "empty" water-filled DRV. Incubation of "empty" DRV with DNA solution (100 μg) for 30 min resulted in formation of DRV with surface complexed DRV (DRV-DNA$_{inc}$) while dehydration and rehydration of SUV mixed with DNA (SUV-DNA) produced DRV(DNA) and rehydration of dry freeze-dried powder with DNA solution (100 μg) yielded DRV-DNA$_{reh}$. Results denote means ± S.D., n=3.

DNA concentration and therefore can be effectively used to determine DNA loading in vesicle preparations. Surface complexation of plasmid DNA with preformed cationic SUV (SUV-DNA) or DRV (DRV-DNA) resulted in similarly high DNA incorporation values (92-95%; Table 2.2), presumably due to the cationic lipid concentration at the surface of the vesicles being sufficiently high to complex most of the DNA present. Similarly, rehydration of freeze-dried SUV with a DNA solution (DRV-DNA$_{reh}$) also resulted in the formation of vesicles with high DNA incorporation values (97%; Table 2.2) suggesting all preparation methods tested give similarly high plasmid incorporation values. However, studies (Felgner *et al.*, 1987) have previously shown that surface loading of DNA to preformed cationic lipid vesicles can result in the formation of DNA vectors which display poor stability and transfection efficiency in the presence of sera

suggests that the spatial location of DNA in these preparations may play an important role in their ability to protect and deliver the DNA *in vivo*. To investigate this DNA spatial location within the various cationic vesicles, the preparations were subjected to gel electrophoresis in the presence of 1% SDS. It has previously been shown (Perrie and Gregoriadis, 2000) that the anionic SDS at this concentration, although unable to solubilise the vesicle forming surfactants, is able to electrostatically compete with the DNA bound to the cationic surface charges and release it into the medium. Released DNA would then be expected to migrate to the cathode, with DNA unavailable (presumably entrapped) to SDS displacement remaining at the site of application.

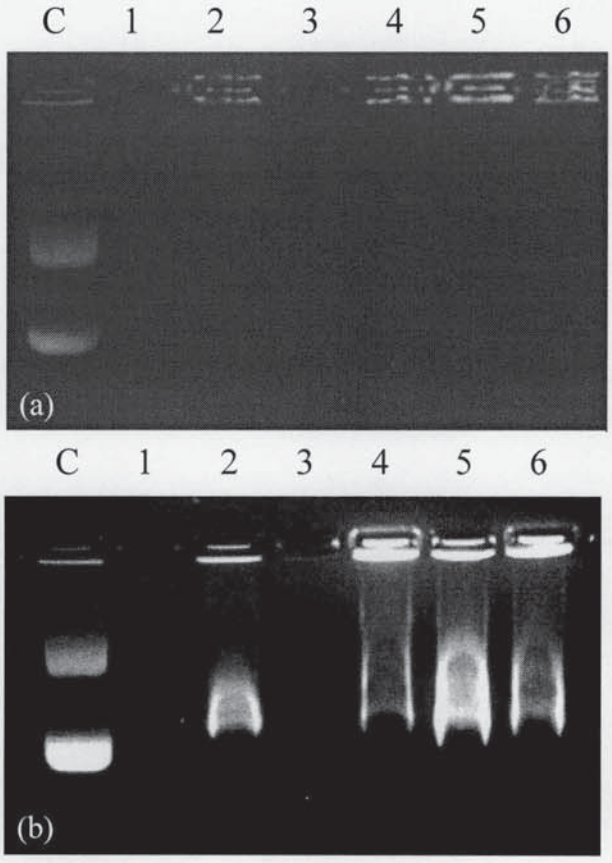


Fig. 2.3 Gel electrophoresis of DNA loaded surfactant vesicles composed of MP:DOPE:Chol:DC-Chol; 16:8:4:4 μmoles, prepared using various methods. Lane C, naked DNA; lane 1, "empty" SUV; lane 2, SUV complexed with DNA; lane 3, "empty" DRV; lane 4, DRV(DNA); lane 5, DRV-DNA_{inc}; lane 6, DRV-DNA_{reh}. (a) In the absence of SDS showing no migration of DNA. (b) Migration of surface-complexed DNA in the presence of 1% SDS, seen in the order of DRV(DNA) < DRV-DNA_{reh} < SUV+DNA < DRV-DNA_{inc}.

Fig. 2.3a shows that, on gel electrophoresis of all four DNA loaded preparations composing MP:DOPE:Chol:DC-Chol; 16:8:4:4 μmoles, in the absence of anionic SDS molecules, DNA remained at the site of application, bound to the cationic charges of the

preparations. In contrast, gel electrophoresis in the presence of SDS (Fig. 2.3b), displaced DNA is seen to migrate towards the cathode with more DNA being displaced from the SUV-DNA and DRV-DNA complexes (Fig. 2.3b, lanes 2 and 5) than with DRV with entrapped DNA (Fig. 2.3b, lane 4). Such observations are similar to those previously demonstrated with cationic liposomes formed using the cationic lipid DOTAP (Perrie and Gregoriadis, 2000) and also with cationic lipid DC-Chol (Perrie *et al.*, 2003). It has been suggested that surfactant vesicle complexes which form after incubation of the appropriate amount of plasmid DNA with SUV, form structures in which DNA is localised within bilayers, bound electrostatically to the cationic charges (Felgner *et al.*, 1987; Zhu *et al.*, 1993). However, the observations that DC-Chol based SUV-DNA and DRV-DNA complexes lose a significant amount of their DNA content on electrophoresis in the presence of SDS indicates that plasmid DNA loaded onto these vesicle preparations is accessible for displacement in the presence of competitive anions therefore may be easily dislodged and thereafter degraded *in vivo* before it can be effectively delivered. Alternatively the displacement- resistant nature of DNA associated with DRV(DNA) (Fig. 2.3b, lane4) suggests that such DRV(DNA) preparations are likely to incorporate most of their DNA within closed bilayers, probably bound to a large extent to the inner cationic charges.

The dehydration-rehydration strategy involves mixing the material to be entrapped, together with the SUV before freeze-drying. In this way, all of the lipid can be brought into contact with all of the solute in the anhydrous state (Kirby and Gregoriadis, 1984). Concentration of the vesicles combined with the reduction of hydrophobic forces during the dehydration-rehydration cycle will result in loss of vesicle stability thereby promoting fusion of these destabilised SUV into larger multilamellar vesicles

entrapping solute as they form (Deamer and Barchfield, 1982). To establish if effective entrapment of DNA within DRV requires the plasmid DNA to be present during both dehydration and rehydration, vesicles were also formed where the plasmid DNA was not added until the rehydration stage (after dehydration) (DRV-DNA_{reh}). Results in Fig. 2.3b show more DNA is displaced from vesicles formed by the rehydration of SUV with a plasmid DNA solution (DRV-DNA_{reh}, lane 6) compared to SUV dehydrated and rehydrated in the presence of DNA (DRV(DNA), lane 4). Moreover, Fig. 2.3b shows a trend of increased DNA displacement in the order of DRV-DNA_{inc}>DRV-DNA_{reh}>DRV(DNA). As similar amounts of DNA were present in all three preparations (~95%; Table 2.2) this suggests that the rehydration of freeze-dried SUV with DNA (DRV-DNA_{reh}) can promote more DNA entrapment within the multilamellar system than is the case when DNA is surface complexed to preformed DRV (DRV-DNA_{inc}), however the presence of DNA during both the dehydration and rehydration procedures appears to promote the highest DNA entrapment with the least DNA being displaced from DRV(DNA) by the competitive SDS anion molecules (Fig. 2.3b, lane 4) and hence this method was chosen for further studies.

2.3.4 Effect of vesicle bilayer composition: a comparative study between MP and PC based systems

To assess the effect of bilayer composition on surfactant vesicle characteristics, a series of formulations were prepared using the cationic lipid DC-Chol. As demonstrated in Table 2.3, MP based formulations containing DOPE were successfully formulated but only when additional cholesterol was present within the bilayer composition. The preference to include DOPE within the MP formulation was supported by previous *in vitro* (Felgner *et al.*, 1994) investigations, which demonstrate the presence of DOPE within vesicle bilayers enhances liposome mediated gene expression. Similarly, *in vivo*

the advantage of the presence of DOPE in both liposomal (Perrie *et al.*, 2002) and MP based (Obrenovic *et al.*, 1998) vesicles which mediated DNA immunisation via the intramuscular route has also been demonstrated. Since the original report (Felgner *et al.*, 1987) in which cationic liposomes composed of the cationic lipid DOTMA (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride) and the neutral lipid DOPE were shown to be effective transfection agents, many authors (e.g. Legendre and Szoka, 1993) have reported improved transfection efficiency when DOPE (often referred to as a 'helper lipid') is incorporated in cationic lipid transfection systems compared to formulations omitting DOPE or replacing it with other lipids. Phosphotidylethanolamines such as DOPE have a relatively small head group and two bulky fatty acyl chains, which give the molecule an inverted cone shape (Israelachvili *et al.*, 1977). Due to this molecular cone shape, DOPE or membranes enriched with DOPE have a strong tendency to form an inverted hexagonal phase, and promote membrane destabilisation (Cullis *et al.*, 1986). This ability of DOPE to promote membrane destabilisation has been suggested to facilitate gene transfection (Szoka *et al.*, 1996) and liposome-mediated DNA immunisation (Perrie *et al.*, 2001) by promoting the disruption of the endosomal membrane after endocytosis/phagocytosis of liposomal-DNA systems and ensuring escape of the plasmid DNA into the cytoplasm.

To compare the effect of cationic lipid on these formulations, a second series of preparations were made replacing DC-Chol with DOTAP which possess dioleoyl hydrocarbon chains similar to DOPE. Results in Table 2.4 demonstrate that, whilst all PC based liposome formulations produced submicron sized vesicles, attempts to prepare formulations containing a combination of MP, DOPE and DOTAP were also

unsuccessful similar to their DC-Chol counter parts (Table 2.3), resulting in the formation of a precipitate. The inability of this combination to form a stable bilayer

Vesicle composition	Vesicle size (nm)	Zeta potential (mV)	Entrapment (% of total)
PC:DOPE:DC-Chol (16:8:4 μmol)	856 ± 89	41.4 ± 2.8	93.3 ± 3.3
MP:DOPE:DC-Chol (16:8:4 μmol)	PPT	ND	ND
PC:DOPE:Chol:DC-Chol (16:8:4:4 μmol)	703 ± 40	40.4 ± 1.6	95.8 ± 4.1
MP:DOPE:Chol:DC-Chol (16:8:4:4 μmol)	1755 ± 345	40.3 ± 2.8	90.0 ± 7.0
PC:DOPE:Chol:DC-Chol (16:4:8:4 μmol)	799 ± 43	44.9 ± 1.3	97.0 ± 2.6
MP:DOPE:Chol:DC-Chol (16:4:8:4 μmol)	1624 ± 350	42.9 ± 1.2	93.6 ± 3.1

Table 2.3 DC-Chol based DRV: the effect of bilayer composition on vesicle size, zeta potential and DNA entrapment. Plasmid DNA (pRc/CMV-HBS; 100 μg) was incorporated into cationic DRV to produce DRV(DNA) of various lipid compositions and lipid molar ratios as shown. In addition each formulation contained 4 μmol of the cationic surfactant DC-Chol. The vesicle size and zeta potential of the DRV were measured in double-distilled water and 0.001 M PBS respectively at 25°C. Incorporation values were based on PicoGreen analysis. PPT: precipitate; ND: not determined; Results represent means ± S.D., n=3.

structure appears to be attributed to the combined presence of MP and DOPE since substitution of either PC for MP, or DOPE for cholesterol both resulted in the formation of stable DRV (DNA) approximately 2 μm in size or below (Table 2.4). Attempts to formulate a MP/DOTAP system containing DOPE by adding cholesterol to the system (MP:DOPE:Chol:DOTAP; 16:8:4:4 molar ratio; Table 2.4) were also unsuccessful.

Whilst there were no notable formulation problems with any of the PC-based liposome compositions tested, recent studies (Perrie *et al.*, 2003) have demonstrated that the combination of DOPE and DOTAP can result in a small decrease in DRV(DNA) stability. Investigating the DNA retention capability of various DRV(DNA) in the

presence of competitive anions, it was noted that DRV(DNA) compositions containing high dioleoyl chain concentration (resulting from the combination of DOPE and

Vesicle composition	Vesicle size (nm)	Zeta potential (mV)	Entrapment (% of total)
PC:DOPE:DOTAP (16:8:4 μmol)	775 ± 68	42.1 ± 1.3	94.0 ± 2.8
MP:DOPE:DOTAP (16:8:4 μmol)	PPT	ND	ND
PC:Chol:DOTAP (16:8:4 μmol)	932 ± 123	53.9 ± 1.5	90.0 ± 3.0
MP:Chol:DOTAP (16:8:4 μmol)	2021 ± 399	45.6 ± 2.1	85.5 ± 9.7
PC:DOPE:Chol:DOTAP (16:8:4:4 μmol)	721.8 ± 56	43.0 ± 2.0	94.8 ± 4.4
MP:DOPE:Chol:DOTAP (16:8:4:4 μmol)	PPT	ND	ND

Table 2.4 DOTAP based cationic DRV: the effect of bilayer composition on vesicle size, zeta potential and DNA entrapment. Plasmid DNA (pRc/CMV-HBS; 100 μg) was incorporated into cationic DRV to produce DRV(DNA) of various lipid compositions and lipid molar ratios as shown. In addition each formulation contained 4 μmol of the cationic surfactant DOTAP. The vesicle size and zeta potential of the DRV was measured in double-distilled water or 0.001 M PBS respectively at 25°C. Incorporation values were based on PicoGreen analysis. PPT: precipitate; ND: not determined; Results represent means ± S.D., n=3.

DOTAP) were more susceptible to DNA loss under such conditions compared with similar formulations where DOPE was substituted for Chol, or where DC-Chol replaced DOTAP (Perrie *et al.*, 2003). This again may be contributed to the reduced stability of DOPE containing membranes already discussed. As previously reported with other plasmids and DRV liposome formulations (Gregoriadis *et al.*, 1996b; Perrie and Gregoriadis, 2000; Perrie *et al.*, 2001, 2002, 2003), plasmid (pRc/CMV-HBS) entrapment (% of total used) assayed based on PicoGreen analysis (Fenske *et al.*, 2002) in all PC and MP based systems successfully prepared was considerable (85-97%; Tables 2.3 and 2.4) and can be attributed in part to the electrostatic interaction between the anionic DNA and the cationic vesicles as demonstrated by their measured cationic

zeta potential (40-53 mV; Tables 2.3 and 2.4). The entrapment values measured for DNA incorporation into the PC and MP formulations using this intercalating dye strongly correlate with entrapment values based on ^{35}S-radioassay previously reported (Gregoriadis *et al.*, 1996b; Arunothayanun *et al.*, 1998; Perrie and Gregoriadis, 2000; Perrie *et al.*, 2001). Such entrapment values, based on the assay of ^{35}S radioactivity have been shown (Perrie and Gregoriadis, 2000) to be reproducible and to predominantly reflect actual DNA entrapment within the bilayers as opposed to complexation of DNA with the vesicle surface.

Notable from both Tables 2.3 and 2.4 is the almost two-fold increased vesicle size of MP based system compared with their PC counterparts. This increased vesicle size may be, to a degree, the result of increased vesicle aggregation in the presence of electrolytes (non-complexing salts such as NaCl) in the buffered solutions used in the vesicle suspensions increasing the ionic strength of the medium which in turn causes the collapse of the electrical double layer thus resulting in vesicle aggregation and increase in size (Lawrence *et al.*, 1996). Indeed, in both MP based SUV (Fig. 2.4a) and DRV(DNA) (Fig. 2.4b) z-average diameter increased significantly ($P<0.05$) when suspended in phosphate buffered saline and 0.9% saline compared to when suspended in distilled water. In contrast, the equivalent SUV and DRV(DNA) liposome formulations demonstrated no significant change in z-average diameter when suspended in each of the three media. However even in the absence of electrolytes, the MP based DRV(DNA) formed were significantly larger in size compared to their equivalent liposomal DRV(DNA) (1160 nm compared with 769 nm, respectively; Fig. 2.4b) suggesting again this increase in vesicle size can be attributed to the inclusion of the non-ionic surfactant MP in the DRV(DNA) formulation.

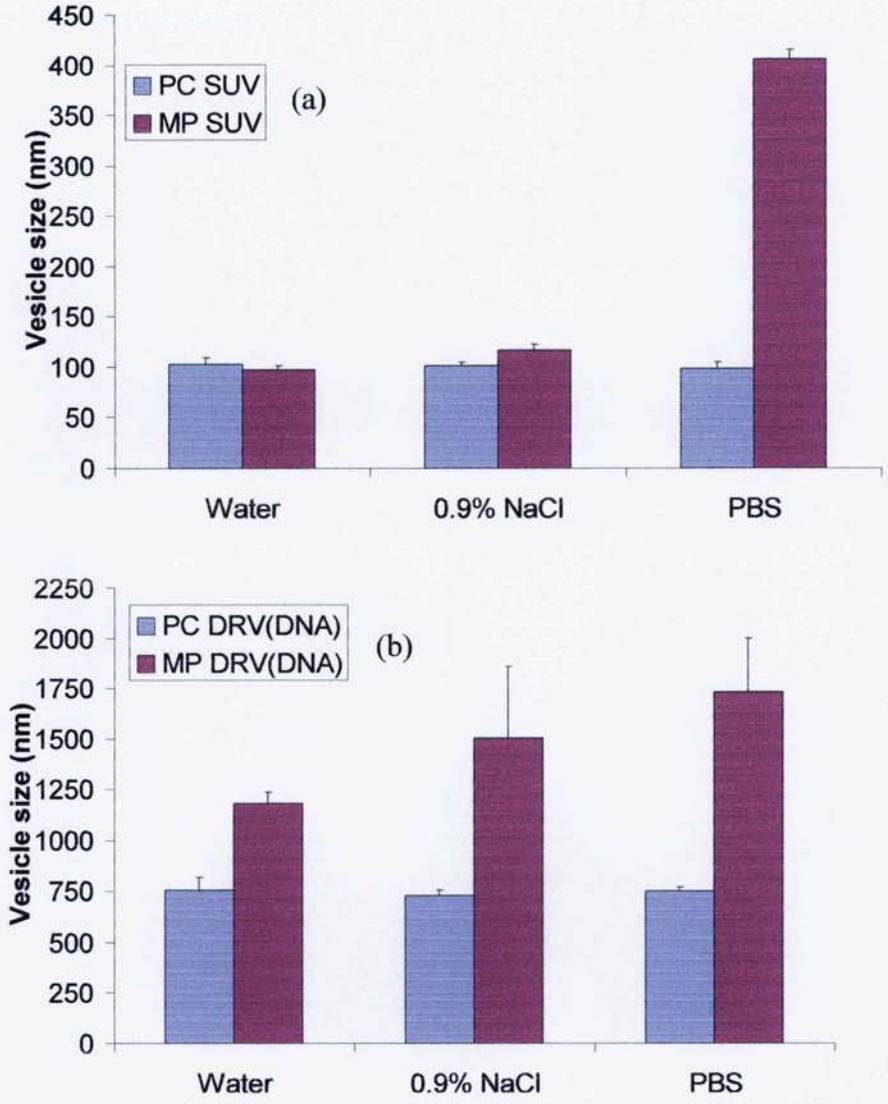


Fig. 2.4 z-average diameter of surfactant vesicles composed of PC or MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles) prepared as SUV (a) and DRV(DNA) (b) in double distilled water, 0.9% saline and phosphate buffered saline (pH 7.4). Vesicle z-average diameter was determined using Brookhaven *ZetaPlus* at 25°C. Results represent means ± S.D., n=3-5.

2.3.5 Gel electrophoresis studies

To investigate the DNA spatial location within the various cationic DRV, preparations were subjected to gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) at a concentration (0.05%) below the critical micelle concentration of the surfactant (Florence and Attwood, 1998). It has previously been shown (Perrie and Gregoriadis, 2000) that at this concentration the anionic SDS although unable to solubilise the DRV

surfactants, is able to electrostatically compete with the DNA bound to the cationic surface charges and release it into the medium. Released DNA would then be expected to migrate to the cathode, with DNA unavailable (presumably entrapped) to SDS displacement remaining at the site of application.

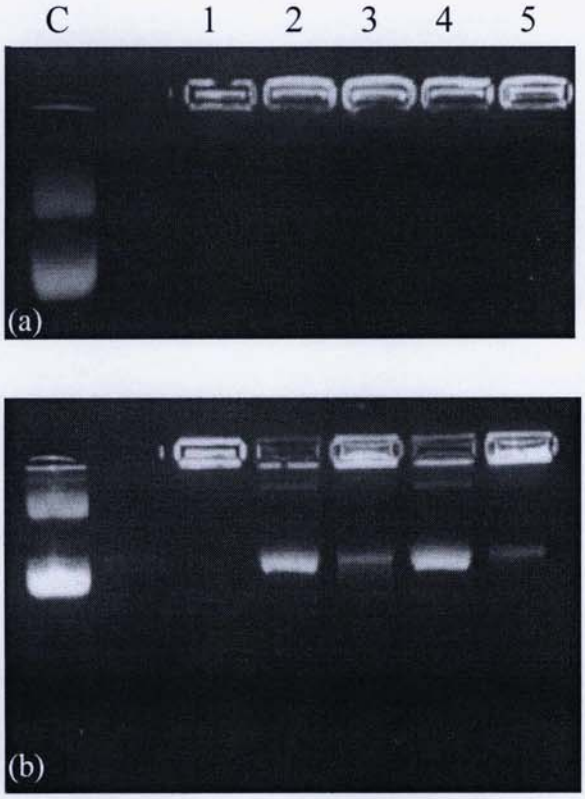


Fig. 2.5 Gel electrophoresis of DRV(DNA) preparations composed of – lane C: naked DNA; lane 1: MP:DOPE:Chol:DC-Chol, 16:8:4:4 μmoles; lane 2: PC:DOPE:DOTAP, 16:8:4 μmoles; lane 3: PC:DOPE:DC-Chol, 16:8:4 μmoles; lane 4: PC:DOPE:Chol:DOTAP, 16:8:4:4 μmoles; lane 5: PC:DOPE:Chol:DC-Chol, 16:8:4:4 μmoles. (a) in the absence of anionic SDS; (b) as in (a) but in the presence of anionic SDS.

Fig. 2.5a shows that, on gel electrophoresis of all five DRV preparations in the absence of anionic molecules, DNA remains at the site of application, bound to the cationic charges of the preparations. Similarly, following electrophoresis in the presence of SDS (Fig. 2.5b) no measurable DNA is displaced from the MP/DC-Chol preparation (Fig.2.5b, lane 1). However, from Fig. 2.5b it appears that despite the similar cationic nature and zeta potential of the formulations tested (Tables 2.3 and 2.4) the bilayer composition of the DRV(DNA) can influence the amount of DNA displaced from DRV(DNA) in the presence of competitive anions. DRV(DNA) formulations

containing DOTAP (Fig. 2.5b, lanes 2 and 4) displaced more DNA loss under the described conditions compared with their DC-Chol containing DRV(DNA) counterparts (Fig. 2.5b, lanes 3 and 5, respectively). Therefore, from this data it becomes apparent that by substituting DOTAP for DC-Chol or replacing MP with PC within the vesicle formulation results in an increased displacement of DNA from the DRV(DNA) formulations. Since it is proposed that DNA is predominantly entrapped within the bilayers of DRV, presumably bound to the inner cationic charges with only a minor portion of DNA interacting with surface cationic charge of the vesicles, it is apparent any change in the surface zeta potential (an indirect measure of surface charge) of the DRV(DNA) would influence the ability of the DRV(DNA) to retain this surface bound DNA. However, from Tables 2.3 and 2.4 it is clear that there is no apparent difference between the analogous DOTAP and DC-Chol formulations suggesting that other factors may be involved. It is unclear, at present why the high dioleoyl chain concentration (resulting from the combination of DOPE and DOTAP as in Fig. 2.5b, lanes 2 and 4) of the DRV(DNA) results in an increased displacement of DNA from the DRV(DNA) formulations however this may be contributed to the reduced stability of DOPE containing membranes (Farhood *et al.*, 1995) which can promote membrane mixing and fusion (Stamatataos *et al.*, 1988; Wrobel and Collins, 1995). Further, increasing the quantity of DOPE in cationic lipid-complexes appears (Wong *et al.*, 1996) to increase dissociation of DNA from cationic liposomes. De Gier *et al.*, (1968) demonstrated that the introduction of double bonds into the alkyl chains causes a marked enhancement in the permeability of the vesicle. Therefore it may be that the combination of DOPE and DOTAP within the DRV(DNA) results in bilayer systems which are less resistant to destabilisation in the presence of competitive anions compared with vesicles containing a combination of DOPE and DC-Chol. Further, replacing PC with non-ionic surfactant

MP (Fig. 2.5b, lane 1), results in a formulation, which appears resistant to the induced destabilisation and resultant DNA loss. Conformation of the enhanced stability of MP based DRV(DNA) can be seen with a similar MP and PC analogous vesicle pair which contain an increased cholesterol content (Fig. 2.6). Again, on gel electrophoresis of both formulations in the absence of anionic molecules, DNA remains at the site of application, bound to the cationic charges of the preparations with no measurable DNA displacement under the described conditions (Fig. 2.6, lanes 1 and 2, respectively). However, in the presence of SDS, again DNA displacement is only detected from the PC based liposome preparation (Fig. 2.6, lanes 3 and 4) and not the MP based preparation (Fig. 2.6, lanes 5 and 6).

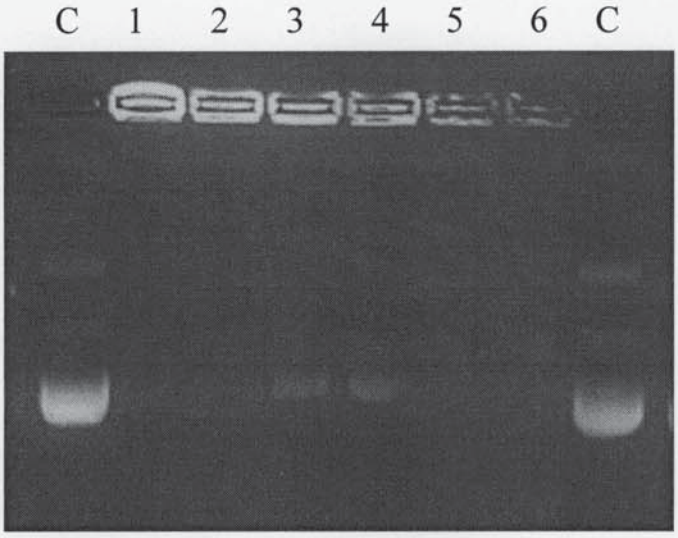


Fig. 2.6 Gel electrophoresis of DRV(DNA) preparations composed of – lane C: naked DNA; lane 1: PC:DOPE:Chol:DC-Chol, 16:4:8:4 μmoles; lane 2: MP:DOPE:Chol:DC-Chol, 16:4:8:4 μmoles; lanes 3 and 4: as in lane 1 in the presence of SDS; lanes 5 and 6: as in lane 2 in the presence of SDS.

2.3.6 Reduction of DRV vesicle size and bilayer stabilisation: effect of disaccharides

Data presented in previous sections demonstrates that MP based surfactant vesicle system (MP:DOPE:Chol:DC-Chol; 16:8:4:4 μmoles) prepared using dehydration-rehydration method, incorporating plasmid DNA have shown to produce large vesicle sizes, almost twice that of their PC based counter parts (Tables 2.3 and 2.4). It was suggested (Gregoriadis *et al.*, 1999) that transfection of APCs with encapsulated DNA

could be enhanced by optimising the vesicle size besides the surface charge and surfactant/lipid composition. Large vesicles over a micron are readily taken up by the reticulo endotheilial system (RES) by which they are normally intercepted (Gregoriadis, 1995) however, this may not always be beneficial. Prolonged systemic half-life is usually achieved with small vesicle sizes (submicron), a characteristic that is required for vesicle access to and uptake by regional lymph nodes (Velinova *et al.*, 1996) after local injection.

One of the profound arguments for large vesicle sizes of DRV is that these preparations are subjected to membrane destabilisation during freeze-drying process and thereby leading to vesicle fusion and/or aggregation and this result in loss of structural integrity (Crowe *et al.*, 1986). Indeed, this process is exploited in the DRV method where freeze drying of SUV in the presence of plasmid DNA enables plasmid DNA to come into intimate contact with the surfactant/lipid membranes as the bilayers destabilize during drying, and upon rehydration the solute is entrapped when vesicle reformation occurs. Sugars such as sucrose and trehalose have been used previously as cryoprotectants and shown to prevent fusion of surfactant vesicle preparations during freeze-drying (Crowe *et al.*, 1989; Van Bommel and Crommelin, 1984; Madden *et al.*, 1985; Crowe *et al.*, 1998). Cryoprotectants are non-eutectic in nature, i.e., they do not crystallize, and typically undergo a transition upon freezing from a viscous gel to an amorphous frozen matrix with less molecular mobility at a temperature referred to as the glass transition temperature (T_g). Critical to efficient freeze drying is that the sample temperature remains below T_g to avoid shrinkage or collapse. It is well known that, while a hydrophobic force drives the formation of membrane structures when surfactants or lipids are dispersed in aqueous solution, a bound hydration layer surrounding the head

group of surfactants plays an important role in the interaction of a membrane with solutes at the interface on the membrane (Gruner, 1992). Water participates in the hydrogen bond network through the hydration layer (Prats *et al.*, 1987). It was suggested (Crowe *et al.*, 1986) that sugars when used as cryoprotectants form a pseudohydration layer on the vesicle surfaces replacing water molecules and thereby prevent fusion during dehydration process. The protective effect of carbohydrates during the freeze-drying of surfactant vesicles is based on a narrow balance between the interaction of the sugar and the surfactant/lipid head groups and the glass-forming properties of the carbohydrate (Crowe *et al.*, 1997). An efficient cryoprotectant should have a sufficiently high T_g and also provide good interaction with the lipid bilayer. The T_g of monosaccharides such as glucose is generally too low (-45°C) to stabilise vesicles in the dried state, whereas oligo- and polysaccharides are able to form a stable glass, but, due to their large size, are unable to interact with lipid head groups. Disaccharides are small enough to be able to interact with vesicles and have a sufficiently high T_g to form a glassy-state (for example, sucrose and trehalose has a T_g of -32°C and -29.5°C respectively).

Surfactant vesicles were prepared by modified dehydration-rehydration procedure (Zadi and Gregoriadis, 2000) described in section 2.2.2.4.4. The preformed SUV composing MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles), were mixed with 100 μg of plasmid DNA (pRc/CMV-HBS) and freeze-dried at –40°C (below T_g of sucrose) for 72 hours in the presence of variable concentrations of sucrose such that the sugar to surfactant/lipid mass ratio was between 1 and 30. Different sugar to surfactant/lipid mass ratio was employed to examine their effect on physico-chemical properties of the DRV such as, vesicle size, zeta potential and DNA incorporation. Results (Fig. 2.7) showed that there

was no significant difference in vesicle sizes when prepared in the absence of sucrose and also when the sucrose was present at a mass ratio of 1 and 3, with sizes being approximately 1.5 microns (1511 ± 228, 1724 ± 195 and 1594 ± 377 nm respectively;

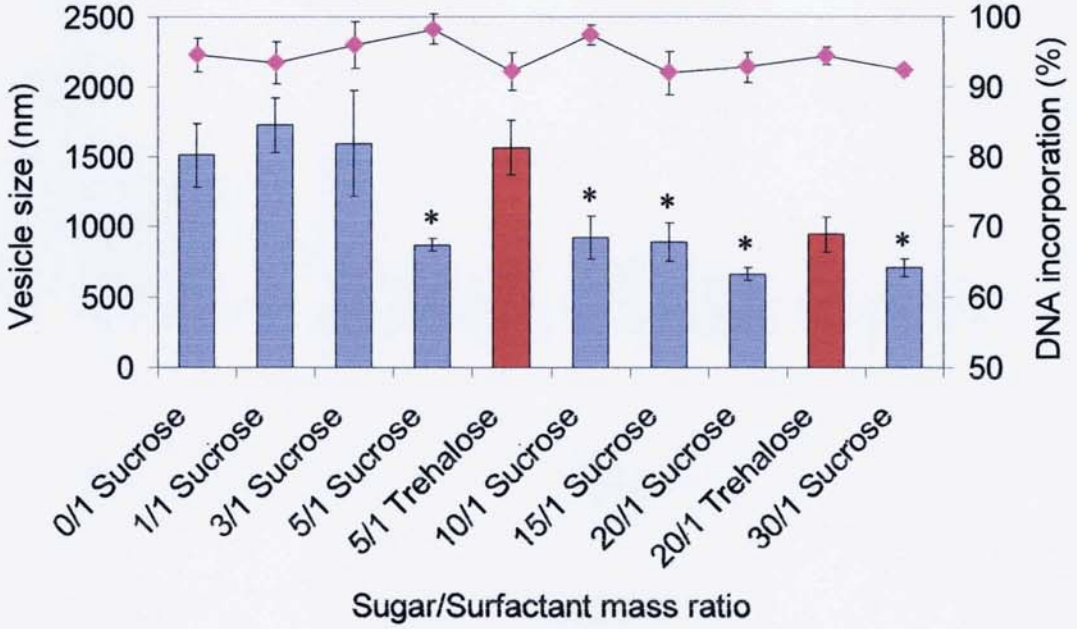


Fig. 2.7 Vesicle size (bars) and DNA incorporation (♦) in DRV(DNA) composed of MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles) in the presence or absence of various concentrations of sucrose or trehalose as cryoprotectants. Results represent means ± SD., n = 3-5. * represent a significant (P<0.05) reduction in vesicle size compared to the preparation in the absence of sucrose.

Fig. 2.7) presumably due to a pronounced fusion and/or aggregation of bilayers during dehydration, that lead to formation of large vesicles upon subsequent rehydration. Increasing the mass ratio (to 5, 10, 15, 20 and 30) resulted in a significant reduction (P<0.05) in vesicle size producing submicron sized vesicles (Fig. 2.7) suggesting the protection offered by the presence of excess sugar against destabilisation and vesicle fusion by replacing hydrated water molecules on the surfactant head groups with sugar molecules (Crowe and Crowe, 1993). Further decrease in vesicle size was not noticed between the mass ratios of 20 and 30 (Fig. 2.7).

Trehalose was employed separately as a second cryoprotectant in the preparations at a mass ratio of 5 and 20 for comparison with that of its sucrose counter parts. Interestingly, the vesicle size at a mass ratio of 5 was significantly higher (1564 ± 198 nm; Fig. 2.7) than its sucrose counter part (871 ± 41 nm; Fig. 2.7) ($P<0.05$). Trehalose is considered to be more hydrophobic in nature than sucrose with a K_{av} value of 0.68. K_{av} is the partition coefficient of trehalose for polystylene gel in water. The benzene ring of polystylene interacts with hydrophobic plane of trehalose molecule formed by the CH- and CH_2 groups of trehalose, K_{av} values increase with the number of glucose residues in trehalose (Janado and Yano, 1985). Therefore, one explanation is that because of its high hydrophobic affinity, it is likely to penetrate the polar head groups and enter into the hydrophobic regions of the surfactant molecules in the bilayer (Fig. 2.8) during dehydration step when either total or partial amount of bound water is displaced and it would remain in the bilayers even after the rehydration of the freeze-dried powder has been performed (Viera *et al.*, 1993) thus increasing the overall vesicle size of the system which corroborates the phenomena that trehalose tends to promote expansion of bilayer lattice (Crowe *et al.*, 1984; Goodrich *et al.*, 1991; Crowe *et al.*, 1988) upon rehydration of freeze-dried sample.

No significant effect on zeta potential was noticed in all the preparations upon varying the sugar/surfactant mass ratio whose values remained fairly constant (between 38 and 44 mV; data not shown) suggesting no electrostatic interaction between the sugars and the vesicle surface as would be expected. Entrapment values measured on the basis of PicoGreen analysis were observed to be high (~95 to 98%; Fig. 2.7) and consistent irrespective of presence or absence of the sugars in all preparations.

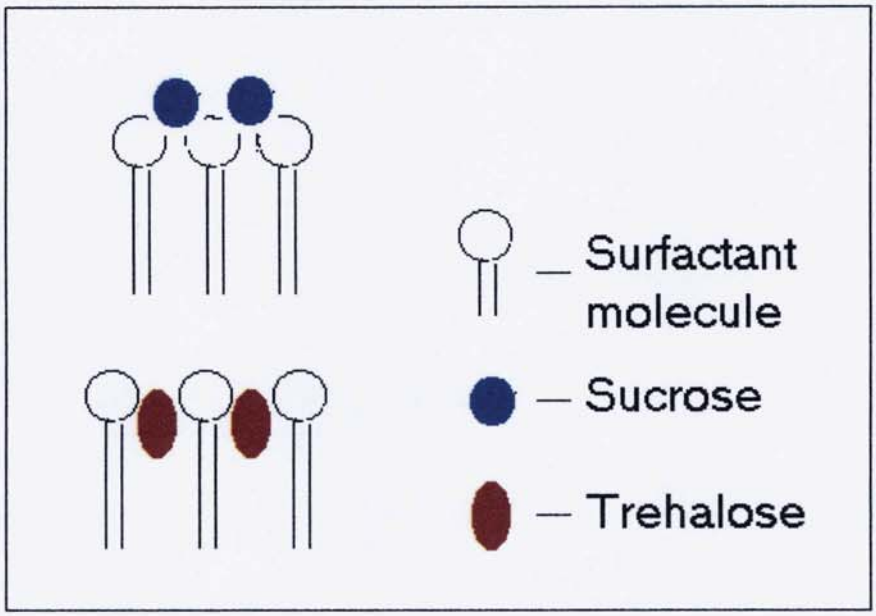


Fig. 2.8 Schematic diagram showing interaction of sugar molecules with hydrophilic head groups of surfactant molecules. Hydrogen bonding, van der Waals' forces and also hydrophobic interaction occur during freeze-drying process and causes trehalose molecules to penetrate deep into the vesicle bilayer due to its relatively high hydrophobic nature unlike sucrose and remain within the bilayer even after rehydration of the freeze-dried powder thus increasing bilayer lattice of trehalose incorporated vesicles and thereby the vesicle size.

Gel electrophoresis of DRV(DNA) composed of MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles) prepared in the presence of various concentrations of sucrose was conducted to determine the influence of sucrose on surface bound DNA in these samples. As anticipated, no release of surface bound DNA was observed in the presence of 40% sucrose (Fig. 2.9a) due to the absence of anionic molecules to compete with the DNA bound to the vesicle surface. When a standard concentration, 0.05% SDS was applied on to the preparations in order to displace the un-encapsulated, surface bound DNA through anionic competition, no release was noticed (Fig. 2.9b) which again suggests the strong surface affinity of the anionic DNA to the positively charged vesicle surface and that the concentration of SDS was not sufficient for displacing the DNA. Further more when the SDS concentration was increased to 1%, surface bound DNA was

eventually displaced from the vesicle surfaces (Fig. 2.9c) due to the anionic competition induced and the release was observed to decrease with the increase in the sucrose to surfactant mass ratio presumably due to either high internalisation of the plasmid DNA in the DRV or due to the carbohydrate having a stabilising effect on the surfactant bilayer.

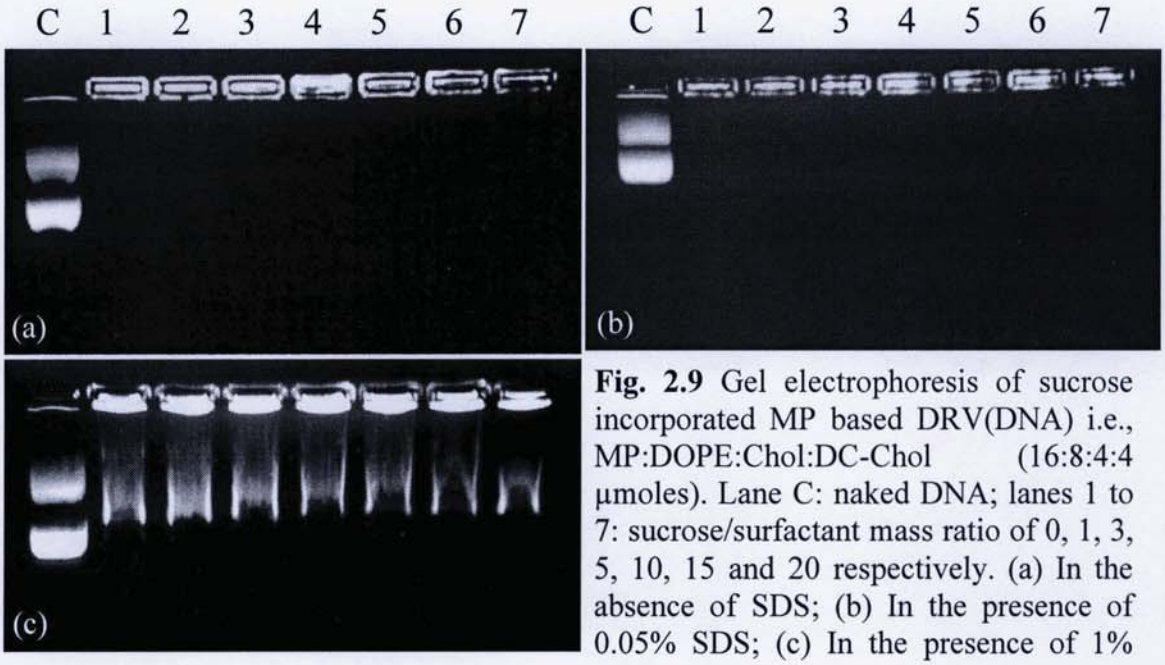


Fig. 2.9 Gel electrophoresis of sucrose incorporated MP based DRV(DNA) i.e., MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles). Lane C: naked DNA; lanes 1 to 7: sucrose/surfactant mass ratio of 0, 1, 3, 5, 10, 15 and 20 respectively. (a) In the absence of SDS; (b) In the presence of 0.05% SDS; (c) In the presence of 1% SDS.

2.4 CONCLUSIONS

Despite some differences in physico-chemical characteristics, all DRV(DNA) formulations showed high entrapment values irrespective of the surfactants employed suggesting that the electrostatic interactions between the cationic lipid present in the formulation and anionic DNA was a prime factor for such high values. Comparison of zeta potential and vesicle size of MP and PC based DRV suggests that despite the similar cationic nature, the z-average diameter of MP based DRV(DNA) has measured approximately twice that of their PC counter parts. Moreover, in gel electrophoresis studies MP based DRV(DNA) displayed an increased stability and increased DNA retention in the presence of competitive anions when compared with similarly

formulated PC based analogues. Presence of cryoprotectants such as sucrose showed a significant reduction in the vesicle size of MP based system maintaining high entrapment. The hydrophobic and hydrophilic portions of MP are ester-linked and may be degraded by esterases *in vivo* to glycerol and a fatty acid (palmitic). Such biodegradability is an important property for any carrier system and without it carrier material *in vivo* may accumulate intracellularly in the lysosomal compartment of phagocytic cells (Hunter *et al.*, 1988).

Results from the present studies also show that cationic DRV with entrapped DNA have a positive zeta potential and thus satisfy the criteria for an efficient synthetic gene-transfer vector as outlined by Behr (1993) which include the need for a synthetic vector to not only condense DNA but also mask its anionic nature. A positive zeta potential for surfactant vesicle-DNA complexes has also been reported to directly relate to transfection efficiency *in vitro* (Takeuchi *et al.*, 1996) with higher zeta potentials been shown to have higher transfection efficiency. Moreover, though the cationic charge of vesicles is known to be masked by the binding of serum proteins (Black & Gregoriadis, 1976), transfection *in vivo* is greatly influenced by the judicial choice of cationic lipids (Gregoriadis *et al.*, 1996a; Legendre and Szoka, 1993; Nabel *et al.*, 1993; Solodin *et al.*, 1995; Thierry *et al.*, 1995; Zhu *et al.*, 1993) and it may be that cationic lipids, even although masked by protein may be important in facilitating the transfer of DNA from endosomes (after the phogocytosis of liposomes) into the cytoplasm (Legendre and Szoka, 1993). Presumably, the ratio of cationic lipid to anionic DNA will also affect the zeta potential of the formulation and this and presence of other surfactants in the formulation and their effect on the transfection in mammalian cells (COS 7) is discussed in chapter 3.

Chapter Three

Surfactant Vesicle-DNA Complexes in Gene Delivery: Characterisation and In Vitro Studies

3.1 INTRODUCTION

3.1.1 Cationic surfactant vesicle –DNA complexes

Despite extensive investigations into cationic liposomes as carrier systems for plasmid DNA, the relationship between liposome structure and transfection efficiency is yet to be fully understood (Escriou *et al.*, 1998; Zuidam & Barenholz, 1998; Birchall *et al.*, 1999; Almofti *et al.*, 2003). Efficient delivery of DNA by liposomes is known to be dependent on numerous factors including: type of cationic lipid used, cationic lipid to DNA ratio, cell type and the type of neutral (helper) lipid used (Zabner, 1997). All these factors contribute, not only to the formation of liposome-DNA complexes, but also to liposome-DNA complex/cell interactions. Through the spontaneous electrostatic interactions of cationic lipids with DNA, a diverse array of liposome structures can be formed for complexes prepared at different lipid to DNA ratios, and even for those prepared at the same ratio. Therefore, a very heterogeneous population of vesicle-DNA complexes are produced with only a fraction of them are responsible for transfection (Faneca *et al*, 2002; Zhang and Anchordoquy, 2004), making it difficult to distinguish between the 'active' and 'non-active' structures. Identifying the most effective structures will provide a very important advancement into understanding and improving gene transfer.

Each lipid component used to formulate liposomes is said to determine and influence liposome-DNA complex structure and function when applied *in vitro* and *in vivo*, indicating that the liposomes primary function can be optimised by utilising the most appropriate lipids for the delivery of plasmid DNA (Gregoriadis, 1995). In addition to a cationic lipid, the inclusion of a helper lipid is required, not only to stabilise the cationic liposome, as pure cationic lipids repel each other, but also to induce high levels of

transfection, mediating cellular interactions and entry into the cytoplasm (Farhood *et al.*, 1995). However, efficiency varies depending on the type of helper lipid incorporated within the liposome bilayer. Helper lipids are said to influence the way the lipid self-assembles and additionally affect the electrostatics between the cationic liposomes and DNA within the complex (Hirsh-Lerner *et al.*, 2005). The morphology of the liposome-DNA complexes can vary depending on the helper lipid employed and as previous studies have revealed there is an existence of two different phases: a multilamellar structure (L_α^C) with the DNA confined between two lipid bilayers and columnar inverted hexagonal (H_{II}^C) liquid-crystalline structure where the lipid surrounds the DNA and arranges itself into a hexagonal lattice (Congiu *et al.*, 2004). Complexes containing dioleoyl phosphatidylethanolamine (DOPE), which forms H_{II}^C structures, in equal combination with a cationic lipid exhibit higher levels of transfection *in vitro* than those containing any other helper lipid, such as cholesterol (Chol), which on the other hand is considered to be better *in vivo* (Templeton *et al.*, 1997; Sternberg *et al.*, 1998; Hirsch-Lerner *et al.*, 2005).

3.1.2 Aims and objectives

In initial studies, the effect of various cationic lipids in the presence of DOPE on transfection efficiency was examined. Cholesterol as a helper lipid has not yet been investigated in detail *in vitro*, therefore the effect of cholesterol on transfection efficiency of liposome-DNA complexes was studied in comparison to the common helper lipid, DOPE in order to get an insight on how certain liposome-DNA complexes are better transfection agents than others and provide further information on influential parameters such as size, surface charge, cationic lipid/DNA charge ratio and morphology of complexes in transfection of plasmid DNA. Further, effect of inclusion of non-ionic surfactants varying in alkyl chain length and in alkyl chain number on the

overall transfection efficiency was studied. Transmission electron microscopy (TEM) and environmental scanning electron microscopy (ESEM) were used to examine the morphology of the complexes.

3.2 MATERIALS AND METHODS

3.2.1 Materials

3.2.1.1 Chemicals

Dulbecco's modified eagle medium (D-MEM), OptiMEM® I (reduced serum medium), foetal bovine serum (FBS), L-glutamine/Penicillin-Streptomycin, Trypsin-EDTA was purchased from GIBCO™ - Invitrogen™ life technologies, Carlsbad, CA. Lipofectin® reagent was purchased from Invitrogen™ life technologies, Carlsbad, CA. Trypan blue, triton X-100, phosphate buffer saline tablets (PBS), ethidium bromide and agarose, sucrose, sodium dodecyl sulphate were purchased from Sigma-Aldrich, Poole, Dorset, UK. Luciferase assay system and CellTiter 96® AQ_{ueous} One solution cell proliferation assay system were purchased from Promega, Madison, WI, USA. COS 7 (African green monkey kidney fibroblast) cells were obtained from European collection of animal cell cultures (ECACC), Salisbury, UK. 75 cm^2 tissue culture treated flasks, sterile pipettes, 12-well and 96-well culture plates were purchased from Corning Costar® corporation, NY, USA. All other reagents and solvents were of analytical grade.

3.2.1.2 Surfactants and Lipids

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dipalmitoyl-3-trimethylammonium propane (DPTAP), dimethyldioctadecylammonium bromide (DDA) were purchased from Avanti® Lipids, Alabaster, AL, USA. 1-monopalmitoyl glycerol (C16:0) (MP), 1-

monostearyl glycerol (C18:0) (MS), 1-monolauryl glycerol (C12:0) (ML), 1,2-dipalmitoyl glycerol (C16:0) (DP), 1,2,3-tripalmitoyl glycerol (C16:0) (TP), cholesterol (Chol), cholesteryl 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) were purchased from Sigma-Aldrich, Poole, Dorset, UK. Egg phosphatidylcholine (PC) was purchased from Lipid Products, Nutfield, Surrey, UK.

3.2.1.3 Plasmid DNA

Plasmid DNA (pRc/CMV-luc, 6.7 kb), also known as gWiz luciferase, containing the human cytomegalovirus (CMV) promoter and encoded with the firefly luciferase reporter gene was purchased from Aldevron, Fargo, USA.

3.2.2 Methods

3.2.2.1 Liposome preparation

MLV and SUV incorporating appropriate amounts of neutral and cationic lipids were prepared as outlined in sections 2.2.2.4.1 and 2.2.2.4.2 respectively and the resultant SUV suspension was filtered through a 0.22 μm pore size sterile filter.

3.2.2.2 Preparation of cationic liposome/DNA complexes

Cationic liposome-DNA complexes were prepared by incubating 20 μl of SUV solution containing appropriate concentration of cationic lipid (such as 0.39, 0.59, 0.78, 1.17, 1.56, 2.34 μmole/ml) in 400 μl serum-free and antibiotic-free medium (opti-MEM) for 40 minutes at room temperature. 400 μl of opti-MEM containing 4 μg plasmid DNA was added to the SUV solution and gently mixed followed by incubation for a further 15 min at room temperature to allow for the formation of SUV-DNA complexes. The

resultant SUV-DNA complexes were then diluted to a final volume of 4 ml with opti-MEM giving a final DNA concentration of 1 μg/ml.

3.2.2.3 Determination of size and surface charge

The z-average diameter and zeta potential of empty vesicles and SUV-DNA complexes were determined as outlined in sections 2.2.2.4.5 and 2.2.2.4.6 respectively in a 1/100 solution of optiMEM (diluted in double distilled water).

3.2.2.4 Agarose-gel electrophoresis studies

SUV-DNA complexes were subjected to agarose gel electrophoresis to determine the retention of plasmid DNA in the complexes under various conditions. In brief, 10 μl of various SUV-DNA complexes (containing 0.01 μg DNA) were mixed with either gel-loading buffer (consisting of 0.05% SDS) or 40% sucrose (w/v) and the gel was run in the conditions described in section 2.2.2.3.

3.2.2.5 Cell culture

COS 7 cells were cultured in 75 cm^2 culture flasks in Dulbecco's modified eagle medium (D-MEM) with 4 mM L-glutamine and supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (FBS) and also containing penicillin (100 IU/ml) and streptomycin (100 μg/ml) and maintained at 37°C in a humid atmosphere containing 5% CO_2. The cells were passaged every 3-4 days.

3.2.2.6 In vitro transfection

Transfection experiments were carried out with slight modification of a method described previously (Felgner *et al.*, 1987). COS 7 cells were seeded at a density of 1 ×

10^5 cells/well in supplemented D-MEM medium in a 12-well plate and incubated at 37°C in a humid, 5% CO_2 atmosphere for 24 hours. 20 µl of defined mole ratios of sterile filtered liposome samples were diluted up to 400 µl with serum-free optiMEM in microfuge tubes and allowed to stand for 40 min at room temperature. 400 µl of luciferase plasmid DNA, diluted to a concentration of 10 µg/ml in optiMEM was added to the samples and was incubated at room temperature for another 15 min to form complexes which was further diluted in 3.2 ml optiMEM (total volume 4 ml) to a final plasmid concentration of 1 µg/ml. Meanwhile, the sub-confluent cells (i.e., about 85% confluent) on the 12 well culture plates were washed with 1ml of optiMEM and 1 ml of surfactant vesicle-DNA complexes (0.0078 µmole of total lipid containing 1µg plasmid DNA) was added to the cells each well and the plates were incubated for 5 hours at 37°C in humid, 5% CO_2 atmosphere to allow uptake of the DNA. After incubation, the vesicle-DNA complexes were removed and the cells were surface rinsed thoroughly and fed with 2 ml of supplemented growth medium (D-MEM). The cells were returned for a further 48 hours incubation to allow intracellular gene expression to proceed.

3.2.2.7 Luciferase assay

Transfection efficiency of each formulation was determined by measuring the luciferase activity in each well and results were expressed as a percentage of the internal control (i.e. the luciferase activity obtained with Lipofectin as the transfection agent). Luciferase assay reagent was thawed at room temperature from -70°C. Culture plates were removed from the incubator and the transfected cells were washed twice with PBS and lysed with 80 µl of 1 x lysis buffer in each well. The cells were scraped gently with a cell scraper and 60 µl of lysate from each well was transferred into microfuge tubes. The tubes were vortexed briefly and centrifuged at 12000 ×g for 15 seconds at room

temperature to liberate the cell extract supernatant from the sedimented cell debris. 10 µl each of supernatants was pipetted into 96 well plates and read on luminometer (Spectra Max Gemini XPS from Molecular Devices, USA) for luminescence (luciferase activity) before and after addition of luciferase reagent (100 µl/well).

3.2.2.8 Cell proliferation assay

The CellTiter 96® AQ_{ueous} one solution reagent (MTS, chemically known as 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) (a novel tetrazolium compound from Promega) was thawed from -20°C in water bath at 37°C and 20 µl of this reagent was pipetted into each well of the 96 well culture plates consisting of COS 7 cells (1×10^5 cells/well) in 100 µl culture medium which were incubated for 48 hours at 37°C in 5% CO_2, humid atmosphere. The surfactant vesicle-DNA complexes were incubated with cells as per the transfection protocol (5 hours) before replacing with the 100 µl of culture medium. The plates in the presence of MTS were returned for incubation for another 3 hours. The absorbance of the water-soluble formazan produced by the cellular reduction of MTS is measured at 490 nm using a 96 well plate reader (Bio-rad, Japan). Culture medium without cells was used as a blank. The relative cell viability (%) compared to control wells containing cell culture medium was calculated by $[abs]_{sample} / [abs]_{control} \times 100$.

3.2.2.9 Crystal violet assay

The culture plates incubated with COS 7 cells (1×10^5 cells/well) were removed from the incubator and the medium was replaced with 500 µl of crystal violet solution (0.4% w/v in 30% methanol) and was allowed to stand for 20 min at room temperature. The stain was sucked off and 1 ml of distilled water was added to each well delivered down

from the side of the well. The plates were gently swirled for 1 min and the liquid was sucked off again. The washing procedure was repeated for two more times and allowed to air dry before the image of the plates was captured. The dye was later extracted in 500 µl of 1% w/v sodium dodecyl sulphate. Absorbance at 570 nm was measured to determine the variation in cell growth.

3.2.2.10 Transmission electron microscopy (TEM)

Morphological analysis of the SUV-DNA complexes was carried out by TEM using a JEOL 1200 EX TEM fitted with a LaB6 filament, with an operating voltage from 40 to 120 kV. 10 µl of freshly prepared sample was placed on a polymer filmed copper grid and allowed to stand for 2 minutes. The excess sample was removed using filter paper and replaced with same volume of freshly filtered 2% w/v aqueous uranyl acetate (UA). The UA solution was removed after 2 minutes and the grids were washed in distilled water and allowed to air dry, forming a thin film which was viewed and imaged at 70 kV.

3.2.2.11 Environmental scanning electron microscopy (ESEM)

Morphological analysis was also analysed using Philips XL-30 field emission gun environmental scanning electron microscope. Briefly, 50 µl of the formulation was loaded on ESEM sample holder and examined under saturated water vapour conditions. Gradual reduction of pressure in the sample chamber resulted in controlled dehydration of the sample environment enabling to view the preparations. A working temperature of 5°C was maintained during the experiments.

3.3 RESULTS AND DISCUSSION

3.3.1 Cell growth in culture plates

Prior to performing cell culture experiments, an initial study was conducted to ensure the uniformity of COS 7 cell growth in 12 well and 96 well culture plates. Crystal violet assay was performed as described in section 3.2.2.9 by culturing the cells in supplemented growth medium (D-MEM). Typical absorbance values of 0.4 % w/v crystal violet reagent measured at 570 nm on a 96 well plate reader after extracting it with 1 % w/v of sodium dodecyl sulphate are observed to be uniform (Fig. 3.1a; overlapped on respective wells of the culture plate for clarity). The difference between the highest (1.384) and the lowest (1.303) values was approximately 5%, which is considered to be with in the limits of variation, implying that a uniform cell growth was achieved in each well. Similar results were also noted in 96 well culture plates (Fig. 3.1b, values not shown).

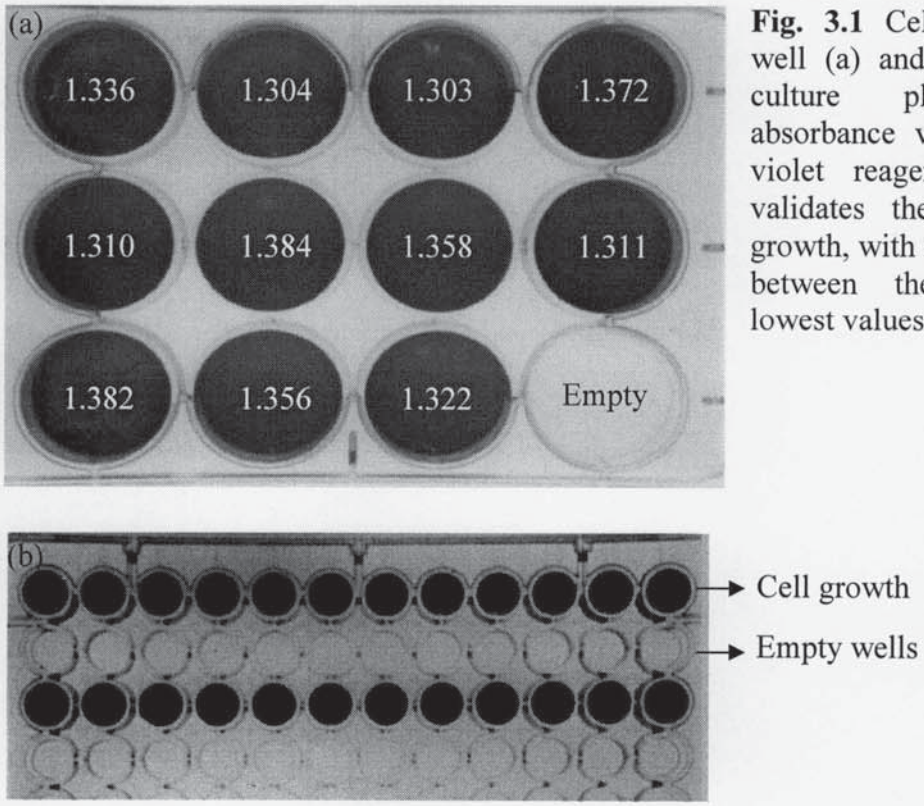


Fig. 3.1 Cell growth in 12 well (a) and in 96 well (b) culture plates. Typical absorbance values of crystal violet reagent at 570 nm validates the uniformity of growth, with a variation of 5% between the highest and lowest values.

3.3.2 Effect of cationic lipid in DNA transfection

The interaction and complex formation between plasmid DNA and cationic liposomes is dependent on the type of cationic lipid present within the liposomal formulation. Felgner *et al.*, (1987) first synthesised the cationic lipid DOTMA. This cationic lipid, in combination with the helper lipid DOPE, is commercially available as Lipofectin®, and has been extensively used to transfect a wide variety of cells. However there are also a range of other cationic lipids available including cationic derivatives of cholesterol, DOTAP and its analogues. In an initial study, the transfection efficiency of formulations consisting of five different cationic lipids (DDA, DOTAP, DOTMA, DPTAP and DC-Chol) in combination with DOPE (at a 1:1 molar ratio) was assessed. All cationic lipids tested differ in their chemical structure and therefore their ability to transfect cells is likely to vary as a result of differences in their interactions with the helper lipid, DNA, and potentially due to the structural features of the resultant lipid-DNA complexes.

As shown in Fig. 3.2, the transfection efficiency of liposome-DNA complexes were in the order of DC-Chol > DPTAP > DDA ≈ DOTMA ≈ DOTAP, with efficiency of DC-Chol being significantly ($P<0.05$) greater than all other lipids tested and around 6 times higher than those complexes composed of DOTAP, DOTMA or DDA. One of the reasons to which this may be attributed is, within their structures, DOTAP, DOTMA and DDA all contain quaternary amines that tend to inhibit protein kinase C (PKC) required for endocytosis of complexes by the cell, which inversely correlates with transfection efficacy (Farhood *et al.*, 1992). On the other hand, DC-Chol has no PKC inhibitory activity suggesting an endosomal pathway for the entry of the complexes into the cells (Zabner, 1997). The toxicity profile of these cationic lipids was also assessed

via cell proliferation and as shown in Fig. 3.2, of the 5 lipids tested only DDA showed signs of toxicity with cell proliferation values being significantly lower ($P<0.05$) than the remaining lipids. The toxicity of DDA has been previously reported for macrophage cell lines (Filion and Phillips, 1997) and the measured toxicity was suggested to be further enhanced synergistically when combined with DOPE. The low transfection efficiency of DDA (Fig 3.2) may also be attributed to such lipid assemblies not being observed to structurally enclose the DNA over a range of lipid:DNA charge ratios i.e., 0.3:1 to 3:1 (+/-) (Birchall *et al.*, 1999) with the DNA remained uncomplexed or loosely associated with the lipid matrix. This lipid is likely to be lost from the complex prior to interaction with the cell membrane thus making this system less transfection efficient (Birchall *et al.*, 1999). However, interestingly, DDA has been effectively used as an immuno-stimulatory agent in numerous vaccination studies (Andersen, 1994; Wedlock *et al.*, 2002), a factor which may be directly related to the toxicity profile already mentioned.

The variation in transfection efficiency between the different cationic lipids could also be attributed to the manner in which the complexes form. By employing the dextran step gradient method, Eastman *et al.*, (1997) found that though the complexes are formed in the presence of different cationic lipids, all form by charge neutralisation, however, the definitive characteristics of the resultant complex varies between the different cationic lipids, by variation in complex size and charge densities. The extent to which the cationic liposomes interact and bind with the plasmid DNA is also likely to have a large effect on the way the DNA dissociates from the complex. Studies (Zuidam and Barenhloz, 1997) have shown that DC-Chol vesicles are only moderately charged

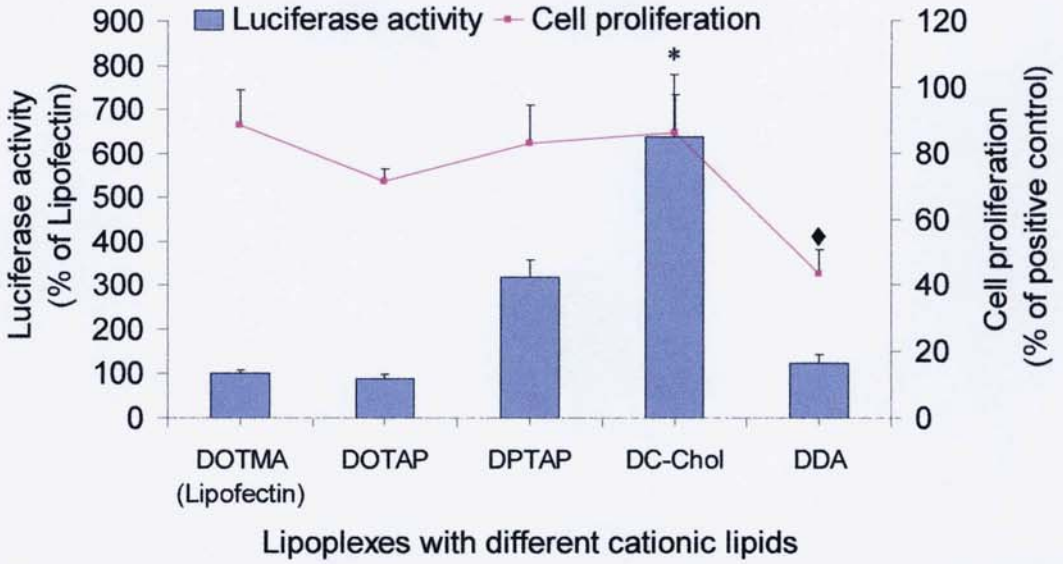


Fig. 3.2 Screening of different cationic lipids in liposome-DNA complexes based on their transfection efficiency and cytotoxicity in COS 7 cells. Liposomes composed of DOPE:cationic lipid (0.78:0.78 μmole/ml) were complexed with luciferase plasmid DNA and were subjected to transfection. Luciferase activity is plotted as the percentage of lipofectin. * signifies that the cationic lipid, DC-Chol generates significantly ($P<0.05$) higher levels of activity than DOTMA, DOTAP, DPTAP and DDA. Results denote mean ± SD, $n = 3$. Cell proliferation (an indication of cytotoxicity), represented as percentage of positive control (cells in the presence of growth medium) showed a significant toxicity (♦) ($P<0.05$) for the cationic lipid, DDA compared to the rest tested. All measurements were obtained from at least 8 different wells and an average ± SD is reported for each sample.

i.e., the tertiary amine of DC-Chol in DC-Chol/DOPE (1:1 mole ratio) liposomes is only 50% charged at pH 7.4, in contrast to the 100 % of quarternary amines, DOTAP or DOTMA. The different protonations of these cationic lipids might result in a different distance between the polar head group and the phosphate group of the plasmid DNA which as a consequence may make it easier for the DNA to dissociate from the DC-Chol based complex without requirement of high energy while on the other hand, vesicles incorporating cationic lipids such as DOTAP or DOTMA being completely charged would require more energy/electrostatic interaction for DNA release to occur. This may provide an additional explanation of the lower transfection efficiency values

of complexes containing cationic lipids such as DOTAP or DOTMA (Fig. 3.2) as the dissociation of DNA from the lipoplex has been suggested as an important rate-controlling step in the transfection process (Zabner *et al.*, 1995).

3.3.3 Comparison of efficacy of helper lipids - DOPE and cholesterol in DNA transfection

3.3.3.1 Effect of lipid to DNA charge ratio

DNA carries two negative charges per a base pair, whereas each DC-Chol molecule has one positive charge on its head group, the complex would be stoichiometrically neutral only when the numbers of DNA bases and DC-Chol molecules are equal. At low cationic lipid:DNA charge ratio (0.5:1), DOPE:DC-Chol liposomes exhibited minimal transfection levels (10% of lipofectin control; Fig. 3.3), whereas in comparison, Chol:DC-Chol liposome-DNA complexes formulated at similar charge ratio gave significantly ($P<0.05$) higher transfection (210%; Fig. 3.3). When the charge ratio was increased to 0.75, transfection activity increased to 130% and 250% for the DOPE and Chol lipoplexes respectively, again showing the enhanced ability of the chol based systems to promote enhanced transfection. At these lower charge ratios, Chol as a helper lipid performed significantly ($P<0.05$) better than DOPE. At a 1:1 charge ratio both Chol and DOPE based systems showed a similar activity (~450%; Fig 3.3) and this level found to be maximal for chol-based lipoplexes with no further significant changes in transfection levels measured with increased charge ratios up to 3:1. In contrast, optimum transfection for DOPE based system was not achieved until a charge ratio of 1.5:1 was reached, however transfection efficiency was significantly higher ($P<0.05$) than those measured for chol-based lipoplexes (1070% vs 450% respectively; Fig. 3.3). Increasing the lipid:DNA ratio further to 2:1 resulted in a reduction of activity for

DOPE complexes down to 860% (Fig. 3.3), which was however, still significantly higher than levels measured with chol-based lipoplexes at the same charge ratio.

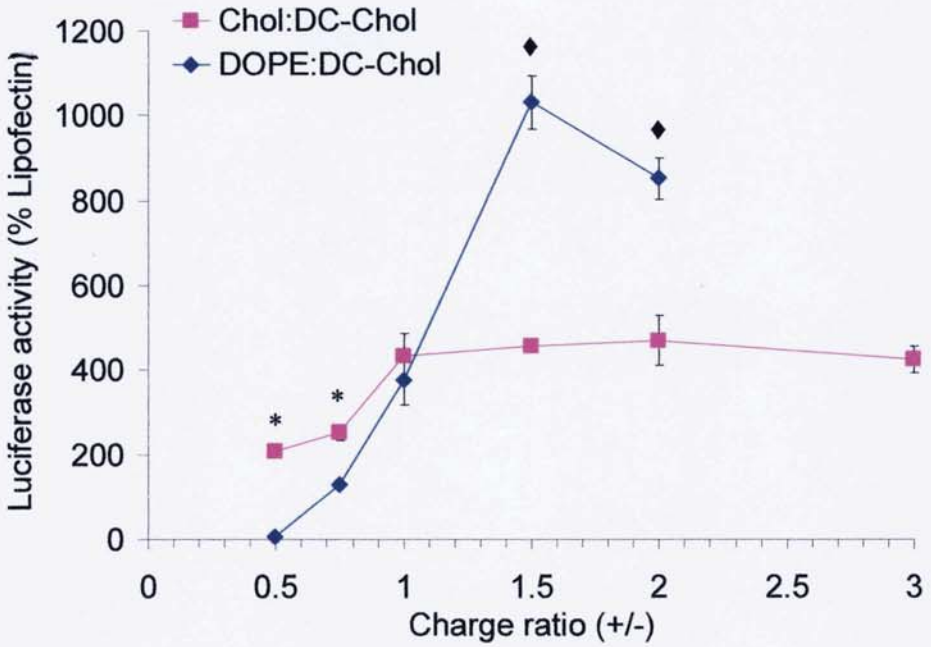


Fig. 3.3 Effect of cationic lipid:DNA charge ratio on transfection efficiency of liposome-DNA complexes. Liposomes composed of either DOPE:DC-Chol or Chol:DC-Chol (at equimolar ratios) formulated with increasing amounts of total lipid which were mixed with a fixed amount of gWiz luciferase DNA (1μg/ml) to give various lipid:DNA charge ratios were added to COS 7 cells. * signifies that Chol:DC-Chol complexes at lower charge ratios transfect cells significantly (P<0.05) better than DOPE:DC-Chol complexes. ♦ signifies that at a charge ratio of 1.5 and also 2, DOPE:DC-Chol complexes transfect cells significantly (P<0.05) better than Chol:DC-Chol complexes. All measurements were obtained for at least 3 independently synthesized batches and an average activity ± SD is reported for each sample.

3.3.3.2 Cell viability

In terms of the cytotoxicity of the DOPE and Chol based SUV-DNA complexes, the viability of COS 7 cells remained almost unaltered when the lipid:DNA charge ratio was increased up to 1 (Fig. 3.4). A further increase in lipid:DNA charge ratios apparently reduced the viability of the cells in correspondence with the increased charge ratios at 1.5 and 2, with a significant reduction in viability, indicating that the higher the cationic lipid content, the toxic they became to the cells which could be an explanation

as to why the transfection efficiency decreased in DOPE:DC-Chol complexes and plateau in case of Chol:DC-Chol complexes at a charge ratio of 2 (Fig. 3.3) as would be expected, since previous reports (Guo and Lee, 2000; Hyvonen *et al.*, 2000) suggested that cationic lipids in high concentrations are toxic to the cells.

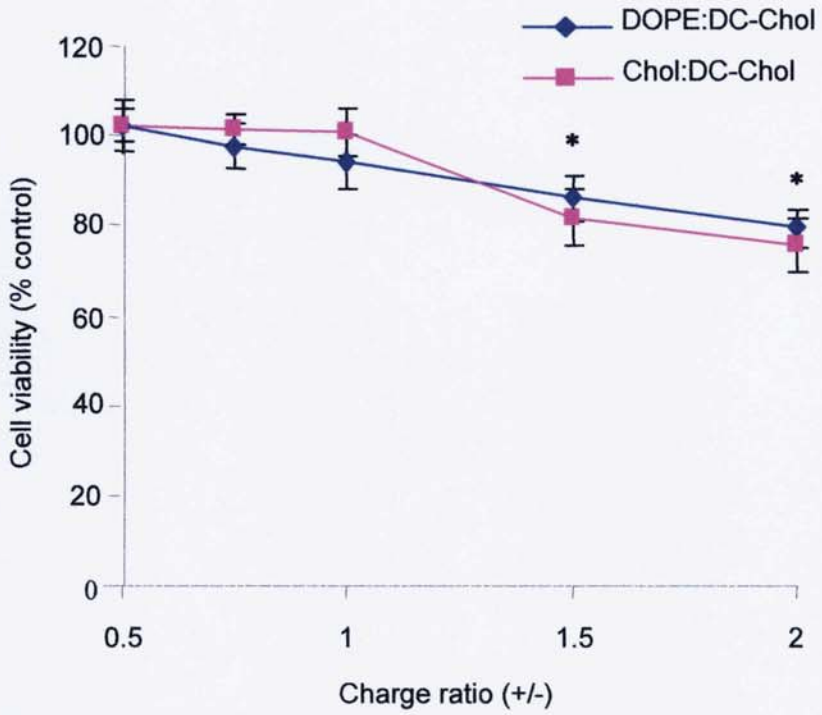


Fig. 3.4 Cytotoxicity of DOPE:DC-Chol and Chol:DC-Chol based liposome-DNA complexes. The effect of variation in lipid-DNA charge ratio was measured in terms of cell viability with respect positive control (cells in growth medium). * represents that liposome-DNA complexes at a charge ratio of 1.5 and 2 significantly ($P<0.05$) reduce cell viability. All measurements were obtained from at least 8 different wells and an average ± SD is reported for each sample.

3.3.3.3 Size, zeta potential and gel electrophoresis studies

Both the z-average diameter and zeta potential of DOPE-DC-Chol:DNA and Chol-DC-Chol:DNA complexes were dependent on the lipid:DNA charge ratio. At the lower charge ratios (i.e. 0.5 and 0.75) the zeta potential for both DOPE and Chol formulations were highly negative as anticipated, due to the presence of excess amount of non-condensed DNA compared to lipid, with no significant differences found between the

two formulations (Fig. 3.5 and Fig. 3.6). However, when the lipid:DNA charge ratio was increased further, the measured zeta potential of the two formulations also significantly increased ($P<0.05$) with zeta potentials approaching neutrality around charge ratios of 1.5 and 2 as the negative charges of DNA are progressively neutralised. This can be attributed to the electrostatic interactions that occur during the complexation process, when cationic liposomes interact and bind onto the anionic surface of the plasmid DNA, structures previously described as being like 'beads on a string' develop (Gershon *et al.*, 1993) consequently, with the enhanced amount of cationic charges present, the anionic charges of the DNA will be neutralised resulting in a neutral or positive zeta potential (Fig. 3.5 and Fig. 3.6). As confirmed by gel electrophoresis (Fig. 3.7a and b), the amount of non-condensed and free DNA decreased as the lipid:DNA charge ratio was increased, demonstrating that the complexation efficiency is dependent on the lipid:DNA charge ratio. Complexes exhibiting a negative zeta potential at the lower charge ratios contains a large amount of free DNA, however, as the zeta potential reaches neutrality most of the DNA on the gel is retained in the site of application, indicating that the DNA is complexed and bound to the cationic charges of the liposomes (Perrie and Gregoriadis, 2000).

To further confirm the presence of free DNA and to ensure that DNA displacement is not a consequence of the anionic components present within the loading buffer (i.e. bromophenol blue) that electrostatically compete with the DNA, gel electrophoresis was also performed in the absence of anionic components (i.e., in 40% sucrose) where the level of DNA displacement decreased with increase in charge ratio (Fig. 3.7c and d) which showed similar levels of DNA displacement both in the presence and absence of bromophenol blue (Fig. 3.7a vs. c and Fig. 3.7b vs. d for DOPE and Chol systems respectively).

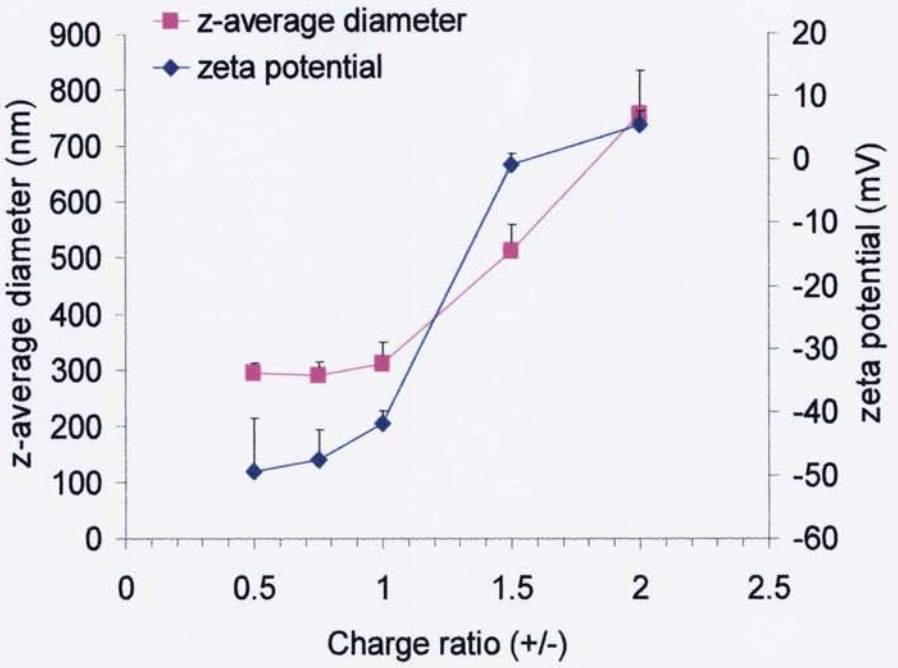


Fig. 3.5 Effect of cationic lipid:DNA charge ratio on z-average diameter and zeta potential of DOPE/DC-Chol:DNA complexes. An initial size of about 300 nm up to a charge ratio of 1, has increased to about 800 nm between the charge ratios of 1.5 and 2, while a similar trend in the rise of zeta potential was noticed with the complexes becoming neutral or slightly positive at a charge ratio of 2. All measurements were obtained for at least 3 independently synthesized batches, the z-average diameter and zeta potential ± SD is reported for each sample.

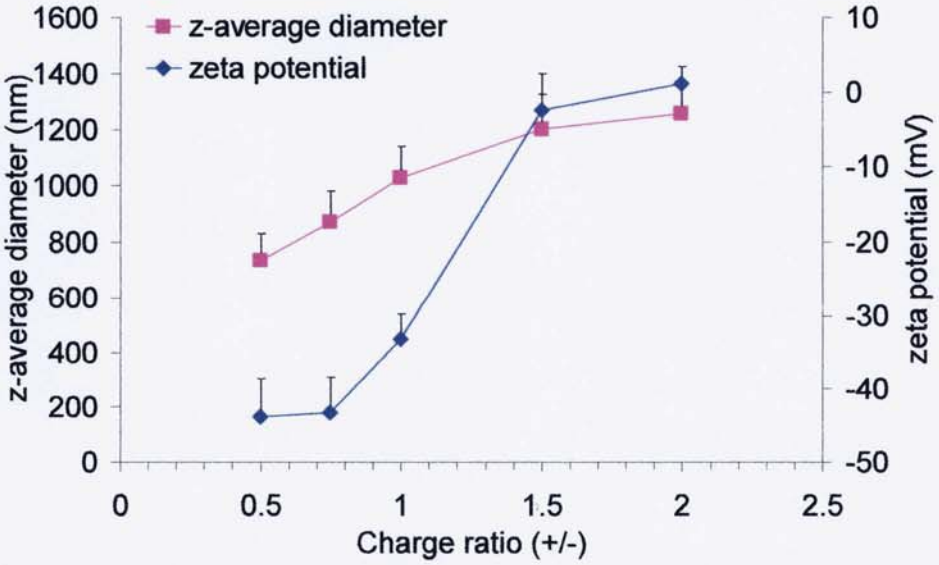


Fig. 3.6 Effect of cationic lipid:DNA charge ratio on z-average diameter and zeta potential of Chol/DC-Chol:DNA complexes. A linear trend in the size from an initial size of ~750 nm to ~1250 nm was noticed as the charge ratio was increased while zeta potential has shown a rise more rapidly at a charge ratio of 1.5 with complexes reaching neutrality. All measurements were obtained for at least 3 independently synthesized batches, the z-average diameter and zeta potential ± SD is reported for each sample.

When the sizes of the SUV-DNA complexes are considered, there is a much more interesting relationship with transfection efficiency. As previously reported (Sternberg *et al.*, 1994; Birchall *et al.*, 1999) upon addition of DNA, small cationic vesicles undergo dramatic changes in configuration, whereby the oppositely charged DNA causes the vesicles to aggregate and fuse, eventually forming larger structures (e.g. DOPE-containing liposomes with empty SUV sizes of 75 nm produced complexes with sizes of 550 nm upon addition of DNA at a charge ratio of 1.5; Fig 3.5). Fig. 3.5 also reveals that the z-average diameter of DOPE containing complexes only increases slightly (with no significant difference measured) until a charge ratio of 1 is reached and subsequently the size increases as the lipid:DNA ratio was increased further, with a proportional rise in surface charge of the complexes to neutrality. These size changes have been ascribed to the fusion between individual vesicles induced by the anionic nature of the plasmid DNA (Almofti *et al.*, 2003). As more cationic lipid is added, fused liposomes envelope the entire length of DNA causing complete neutralisation of the DNA's negative charges. Fig. 3.5 clearly show that as the cationic lipid content increases, the pattern of increased complex size correlated with the decrease in the negative zeta potential and as zeta potential become less negative and eventually neutral, the liposome-DNA complexes reach a stage where a balance exists between the net positive charge from cationic lipid and the net negative charge from the DNA, termed as isoelectric point. At this point, the van der Waals' attractive forces are able to overcome the weak electrostatic repulsions between vesicles (Safinya *et al.*, 1998; Pedroso de Lima *et al.*, 2001) which cause them to interact and form into larger structures surrounding the plasmid DNA.

The trend in the correlation between complex size and surface charge of Chol based complexes is less pronounced than DOPE based counter parts, as even at the lower

ratios, the liposome-DNA complexes are significantly ($P<0.05$) larger in size (Fig. 3.5 and Fig. 3.6) and exhibit a linear trend of increase in size as lipid:DNA charge ratio is increased. For Chol containing complexes, an interesting observation is the correlation between the size (Fig. 3.6) and transfection efficiency (Fig. 3.3) as these results run in parallel. Indeed, at the low charge ratios (i.e. 0.5 and 0.75), where Chol-DC-Chol:DNA complexes were larger in size (between 750nm and 900nm) revealed greater levels of transfection than their DOPE counter parts (~300nm) at those ratios. Additionally, DOPE-DC-Chol:DNA complexes exhibited enhanced activity as complex size increased. Optimum transfection of DOPE containing complexes was achieved when complex size reached sizes around 550 nm (Fig. 3.3 and Fig. 3.5). In this instance vesicle size appears to play a predominant role in transfection efficiency and is an important parameter to consider when formulating a successful liposomal gene delivery system. In agreement with the previous report (Felgner *et al.*, 1995), liposome-DNA complexes with diameters ranging between 500-1000 nm produced elevated levels of activity when compared to smaller complexes (300-350 nm) (for example, in case of DOPE; Fig. 3.5). Complexes which are relatively large are more likely to be heavier, which may favour their sedimentation over the cell surface, as a consequence of gravity, promoting liposome-DNA complex cell interactions, increasing endosomal entry into the cytoplasm and eventual delivery of plasmid DNA.

Faneca *et al.*, (2002) also concluded that large complexes contain an abundance of DNA copies, thus delivering an ample amount of DNA to the cell and as previously reported, in order for complexes to transfect cells efficiently they need to incorporate and carry a sufficient amount of DNA to the cell surface. Nevertheless, in addition to this, upon reaching the cell the complex should possess certain properties in order to mediate entry of the DNA into the cytoplasm. It is at this stage in which the helper lipid

may play an influential role in mediating successful transfection. By incorporation of DOPE into the bilayer of the cationic lipid delivery system transfection activity is enhanced, producing the highest level of activity at a charge ratio of 1.5, which is in agreement with previous studies, although with a different cationic lipid (Almofti *et al.*, 2003). DOPE is the common helper lipid used for *in vitro* transfection due to its high non-bilayer forming activity and ability to transform into the inverted H_{II} hexagonal phase after endocytosis in acidic conditions facilitated by its cone-shaped molecular structure (Israelachvili, 1991). Liposome-DNA complexes enriched with DOPE promote membrane fusion thus facilitating the destabilisation of the endosomal membrane and escape of plasmid DNA into the cytosol (Farhood *et al.*, 1995). These fusogenic properties of DOPE are absent in Chol based complexes and although activity is relatively high at lower charge ratios, it is significantly ($P<0.05$) lower than DOPE-DC-Chol:DNA complexes at what is considered as an optimum charge ratio at 1.5 (Fig. 3.3). This highlights the benefit of the destabilising effect of DOPE-containing complexes *in vitro* and emphasizes its ability to facilitate entry of plasmid DNA into the cytosol.

At the higher charge ratio of 2, transfection activity either reduces or plateaus for DOPE and Chol respectively (Fig. 3.3). At this ratio, the zeta potential for both helper lipids became neutral (Fig. 3.5 and Fig. 3.6), demonstrating that the plasmid DNA has been completely condensed. Therefore, reduction in activity may be due to the high intensity of DNA condensation where the DNA is tightly packed and bound to the cationic charges of the liposomes in the complex, rendering it difficult to dissociate and consequently preventing the unloading from the complex and thus reducing the efficiency in the delivery as suggested previously (Faneca *et al*, 2002).

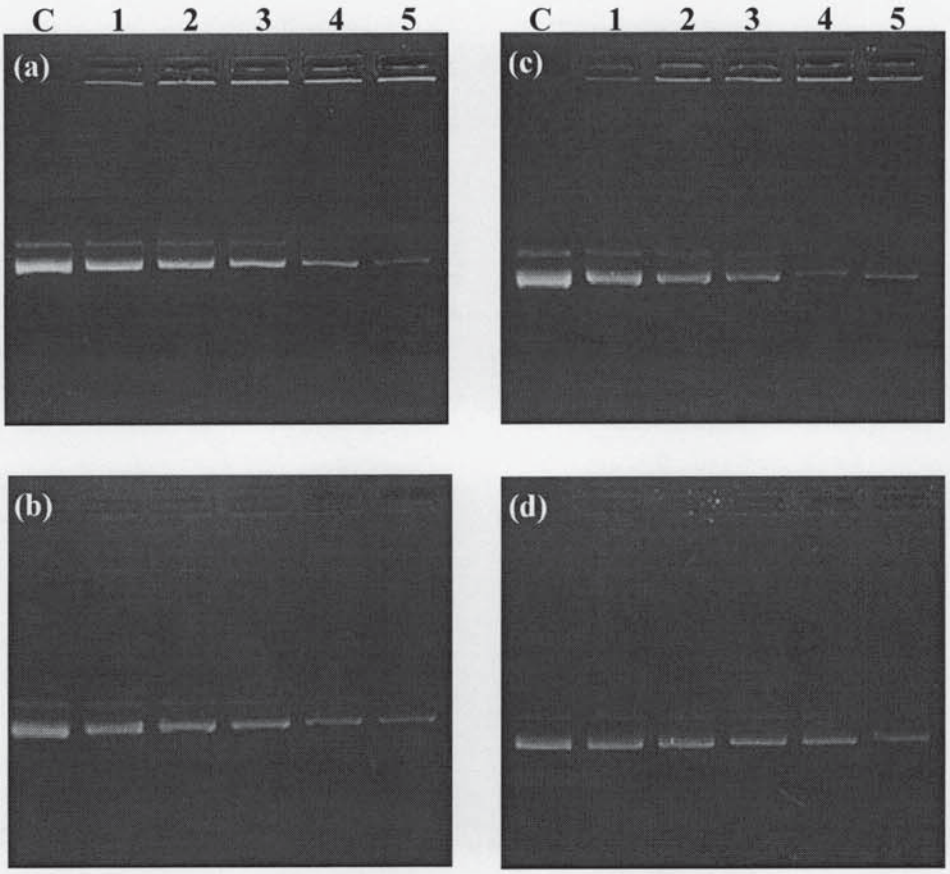


Fig. 3.7 Gel electrophoresis of liposome-DNA complexes composed of DOPE:DC-Chol (a) and Chol:DC-Chol (b) preparations at lipid:DNA charge ratios of 0.5 (lane 1); 0.75 (lane 2); 1.0 (lane 3); 1.5 (lane 4); 2.0 (lane 5) and naked DNA (lane C). (c) as in (a) but in the absence of anionic competition (in 40% sucrose). (d) as in (b) but in the absence of anionic competition (in 40% sucrose).

3.3.3.4 Effect of vesicle size reduction on transfection efficiency within DOPE-DC-Chol:DNA complexes

The size and surface charge of lipid-DNA complexes such as DOPE/DC-Chol-DNA complexes will not only influence their transfection efficiency as seen in previous section (3.3.3.3) but also their physical stability, *in vivo* distribution and cellular interaction (Mahato *et al.*, 1995; Lasic and Templeton 1996; Tomlinson and Rolland 1996). To gain further understanding about the impact of the preparation procedure on the stability of the complexes and in turn on their transfection efficiency, DOPE:DC-Chol vesicles of different size range and lamellarity were prepared by sonicating MLV

for increased time intervals (30, 90, and 210 seconds). These vesicles were than mixed with DNA at a charge ratio of 1. The initial vesicle sizes of empty MLV were about 600 nm (Fig. 3.8) which when sonicated for 30 seconds resulted in rapid reduction in vesicle sizes (below 200 nm; Fig. 3.8) and further reduction was seen by increasing the duration of sonication though the reduction was nominal (100 nm; Fig. 3.8). Interestingly, no significant variation was observed in the surface charges of the vesicles in all the sonicated samples, where the zeta potential was maintained at about 40 mV (Fig. 3.8). Addition of DNA to these vesicle preparations resulted in significant increases in sizes ($P<0.05$; Fig. 3.8) compared to their empty vesicles, a clear indication of electrostatic interactive forces between the opposite charges of cationic lipid and DNA were seen with a strong negative zeta potential (about -40 mV; Fig. 3.8) implying the presence of excess free, non-condensed DNA in the suspension that resisted the impact of vesicle size changes through sonication showing no significant variation on the zeta potential of the complexes (Fig. 3.8).

Transfection using DOPE:DC-Chol multilamellar vesicles (~ 1000 nm in size; Fig. 3.8) at a charge ratio of 1 was highest (Fig. 3.9). Interestingly, of the 4 vesicle size ranges, MLV produced significantly ($P<0.05$) higher transfection levels than the other 3. However there was no clear trend between vesicle size and transfection with the smallest of the sonicated vesicles (sonicated for 210 sec; ~ 100 nm prior to complex formation (Fig. 3.8)) showing the second highest levels of transfection (Fig. 3.9). No significant difference was found between those preparations that were sonicated for 30 and 90 seconds. The transfection efficiencies that were observed to be higher when plasmid DNA was mixed with MLV than with SUV (Fig. 3.9) were in agreement with previous reports (Liu *et al.*, 1997; Zelphati *et al.*, 1998). Though the issue of efficacy of

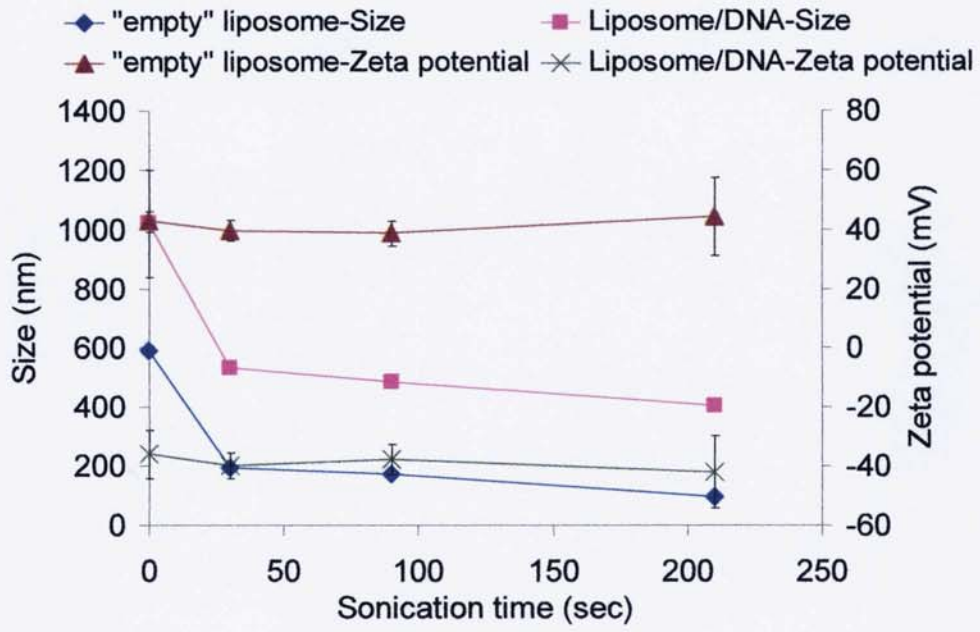


Fig. 3.8 Effect of sonication time on vesicle size and zeta potential. Empty DOPE-DC-Chol liposomes were sonicated for different time intervals and the variation in size and zeta potential was studied. No significant variation in the zeta potential was observed in the samples with and without sonication and also the sonication time whose values retained at about 40 mV while the zeta potential of corresponding DOPE-DC-Chol/DNA complexes at a charge ratio of 1 was observed to be between -30 and -40 mV. All measurements were obtained for at least 3 independently synthesized batches, the z-average diameter and zeta potential ± SD is reported for each sample.

MLV versus SUV as a starting agent for lipoplex formation is reported to be contradictary (Bennet *et al.*, 1996), since some of the data, for example, data by Templeton *et al* (1997) show superiority of unilamellar vesicles over MLV in transfection efficiency, though this may not be compared directly with the previous data, including the present work in which the lipoplexes were formed spontaneously and used as such unlike the modified form of unilamellar vesicles of Templeton *et al* (1997) where the vesicles were subjected to ultrasonic irradiation followed by low pressure extrusion encapsulating the DNA. Nevertheless, both forms of vesicles have their own up and down sides, for example, multilamellar vesicles are much less homogeneous with a high polydispersity but are much easier to prepare while SUV

have the advantage of being much more homogeneous with respect to size distribution than MLV and therefore they are easier to sterilise and characterise. In addition, larger mole fractions of their lipids are exposed to immediate interaction with the DNA.

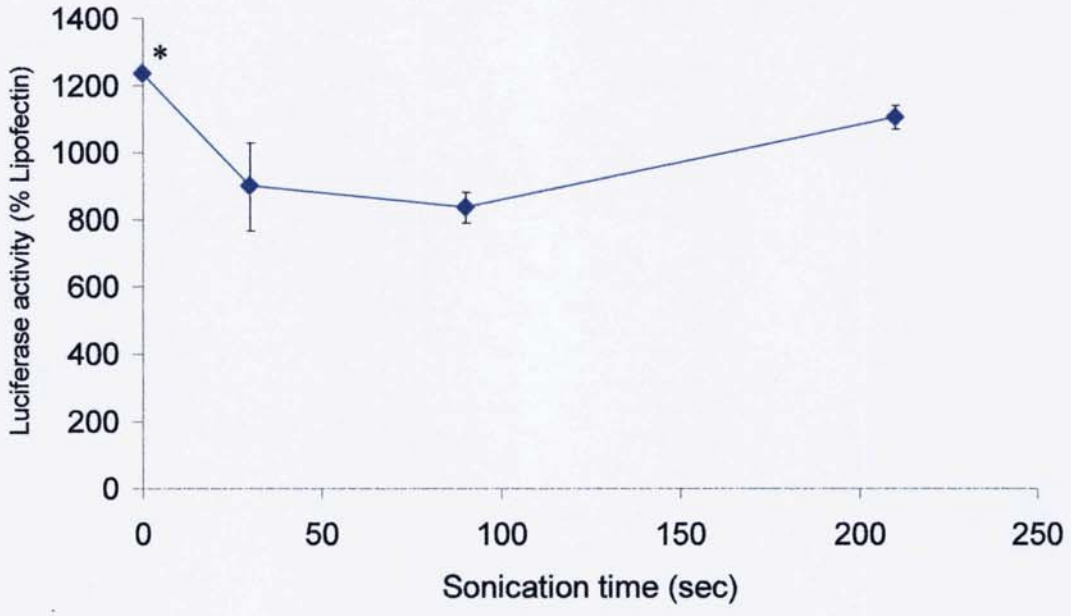


Fig. 3.9 Effect of vesicle size on transfection efficiency of liposome-DNA complexes. Multilamellar vesicles composed of DOPE:DC-Chol (0.78:0.78 μmoles) were sonicated for different time intervals to reduce the vesicle size before mixing with plasmid DNA (1μg/ml). The complexes were added to COS 7 cells and allowed to undergo transfection. * represent that the complexes of vesicles without sonication transfected the cells significantly ($P<0.05$) better than their sonicated counter parts. All measurements were obtained for at least 3 independently synthesized batches and an average activity ± SD is reported for each sample.

3.3.3.5 Morphological analysis of DOPE vs cholesterol liposome-DNA complexes by TEM and ESEM

DOPE containing complexes that produced the highest transfection activity are enriched with heterogeneous particles, as seen in TEM micrographs (Fig. 3.10a and b), demonstrating the presence of both aggregated liposomes (Fig. 3.10a) and fused liposomes (Fig. 3.10b). Similarly, Zabner (1997) found that transfection levels were highest when the liposome-DNA complex preparations contained heterogeneous structures compared to small homogenous particles. Although, this does make it

difficult to identify between the 'active' and 'non-active' structures, this heterogeneity could alternatively indicate that a variety of complex structures are required for successful transfection, therefore there is still a need to further investigate the various morphological structures.

At a low lipid:DNA charge ratio (0.5), the DOPE based cationic liposomes begin to aggregate around and along the DNA strand, showing initial signs of fusion between individual liposomes (Fig. 3.10c), with the DNA behaving as a fusogenic agent and neutralising the repulsive forces acting between the liposomes (Li *et al.*, 1996). However, at this ratio the preparation shows relatively small homogeneous particles due to the low amount of lipid coated with DNA, preventing aggregation (Eastman *et al.*, 1997) and a large amount of free DNA, which may explain the lower levels of transfection efficiency at this charge ratio.

TEM images of DOPE/DC-Chol:DNA complexes revealed two morphological structures resembling 'meat ball' and 'spaghetti-like', as reported previously (Sternberg *et al.*, 1994) using freeze-fracture electron microscopy. At a high cationic lipid:DNA charge ratio (1.5), cationic liposome aggregation occurs possibly surrounding the anionic DNA, forming a 'meat ball' structure (Fig. 3.10a), which is further substantiated by zeta potential results showing relatively higher values (Fig. 3.5) than the complexes that were formed at lower charge ratio (0.5) (Fig. 3.10c).

Another morphology of DOPE/DC-Chol:DNA complexes revealed 'spaghetti-like' structures (Fig. 3.10b) formed by the adherence of cationic liposomes along the DNA strand. As the cationic lipid:DNA ratio increases, as previously demonstrated by

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

On the other hand, the low transfection efficiency of Chol-DC-Chol:DNA complexes at a similar charge ratio (1.5) as that of its DOPE counter part, could possibly be explained by the non-fusogenic properties of the helper lipid, cholesterol, as seen in the Fig. 3.10d, e and f. Chol complexes do not exhibit the fusogenic properties as DOPE, instead the inclusion of Chol rigidifies the lipid bilayer and increases the stability of liposomal vesicles (Gregoriadis, 1993). Upon addition of DNA to these complexes, it appears that the liposomes aggregate around the DNA but do not show any signs of liposome-liposome fusion and therefore these complexes are unlikely to structurally enclose the DNA (Fig. 3.10d and e). Considering this, complexes containing Chol as the helper lipid may bind along and surround the DNA strand eventually forming large aggregates. Chol-DC-Chol:DNA complexes also revealed several morphologies resembling the structures for DOPE-DC-Chol:DNA complexes (i.e. 'meatball' and 'spaghetti-like' structures) (Fig. 3.10a vs. d and 3.10b vs. e respectively) with sizes matching those measured by photon correlation spectroscopy (Fig. 3.6).

1. DOPE/DC-Chol:DNA complexes **2. Chol/DC-Chol:DNA complexes**

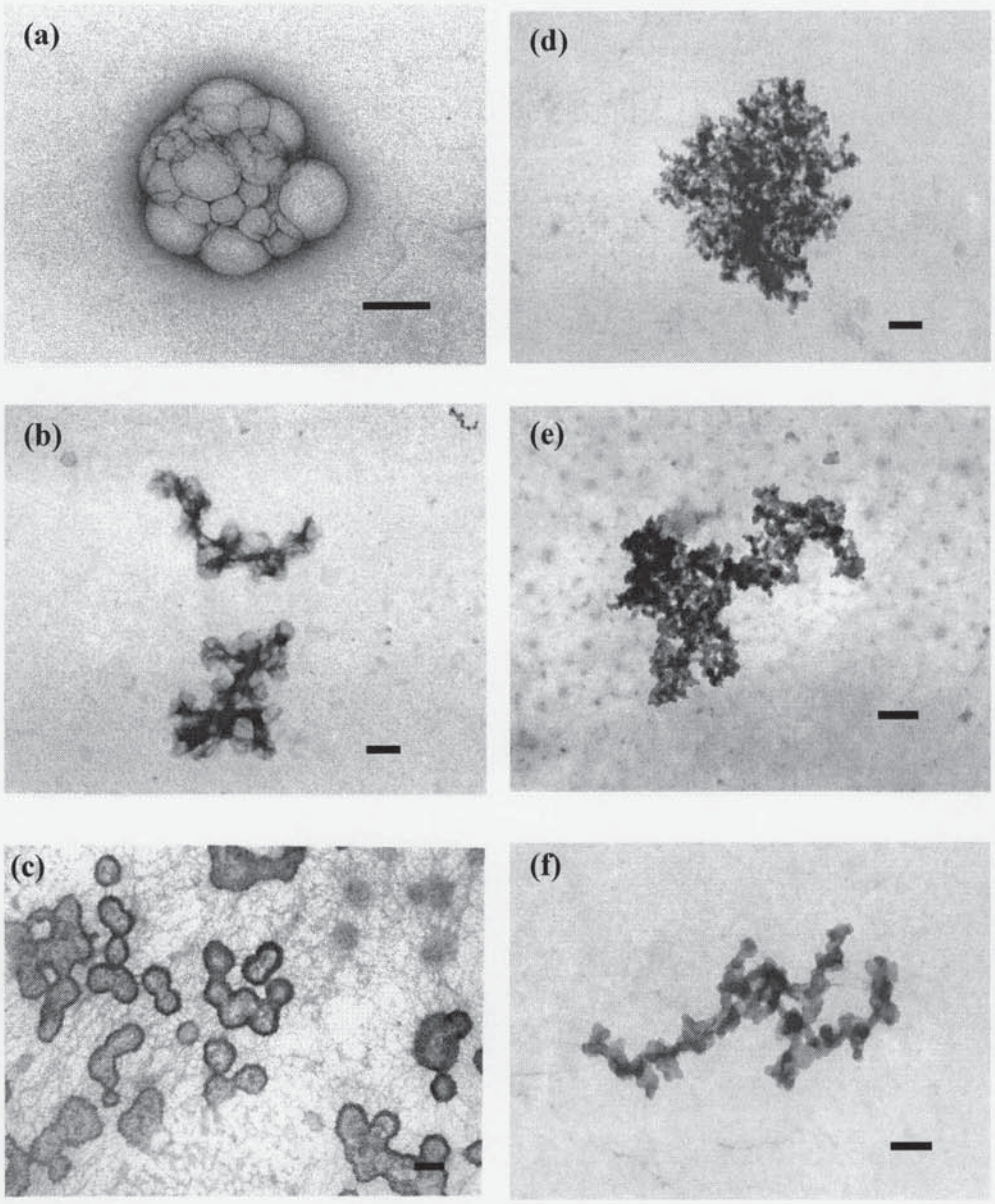


Fig. 3.10 Transmission electron microscopy (TEM) of liposome-DNA complexes composed of DOPE:DC-Chol at a charge ratio of 1.5 (a, bar = 100 nm; b, bar = 500 nm) and 0.5 (c, bar = 100 nm); Chol:DC-Chol at a charge ratio of 1.5 (d, bar = 200 nm; e, bar = 200 nm; f, bar = 100 nm) [Done in conjunction with S. McNeil].

In DOPE:DC-Chol complexes, where there is an indication of fusion of liposomes leading to formation of a 'lipid coat' around the DNA strand (Fig. 3.10c), possibly with inverted hexagonal lipid orientation around the DNA also is revealed in the ESEM micrograph (Fig. 3.11), where the liposomes fuse together and form a rod-like structure, similar observations were reported by Gershon *et al.*, using the Kleinschmidt method of

rotary shadowing electron microscopy visualising the structures formed when DNA was complexed with cationic liposomes comprising DOTMA and DOPE (Gershon *et al.*, 1993). At low lipid:DNA charge ratios (0.5), cationic liposomes are adsorbed at the surface of DNA molecules forming aggregates that progressively surround large segments of DNA. Continuous addition of cationic liposomes to a critical concentration and density (at a charge ratio of 1.5) results in DNA-induced membrane fusion and liposome-mediated DNA collapse and condensation. It was also suggested (Gershon *et al.*, 1993) that these processes result in coating of DNA with lipid bilayer along the entire length of the plasmid which is, we believe, for the first time demonstrated here using ESEM technique where similar observations were evident (Fig. 3.11). A general model for the interaction of cationic liposomes with DNA was proposed (Felgner *et al.*, 1995) according to which the cationic liposomes approach opposite sides of the anionic DNA strand and fuse with each other. The final rearrangement of the liposome-DNA complex involves coating of the DNA strand with a single lipid bilayer.

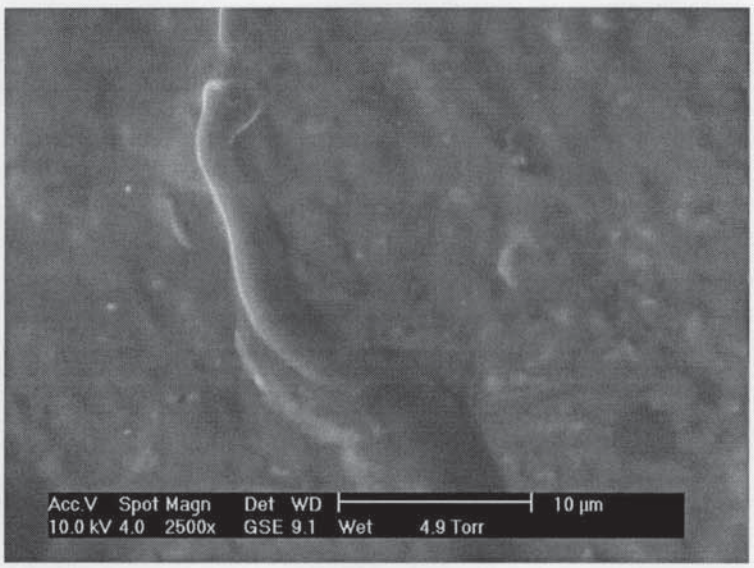


Fig. 3.11 Environmental scanning electron microscopy (ESEM) of DOPE-DC-Chol:DNA complexes at a charge ratio of 1.5. SUV forming a 'lipid coat' around the DNA strand, possibly with an inverted hexagonal lipid orientation around the DNA is seen. Liposome fusion is induced by the anionic nature of DNA, which in turn causes the DNA to collapse and become entrapped in 'lipid coat' forming rod-like structures. Bar = 10 µm. [Done in conjunction with S. McNeil].

3.3.4 Incorporating non-ionic surfactants in DOPE-DC-Chol formulation

3.3.4.1 Optimisation of composition

The thermodynamic driving force for the spontaneous formation of the complexes is the lowering in total free energy of the complexes when compared with those of the surfactant vesicles and the DNA individually. The main forces which contribute to the total free energy of the complexes include the electrostatic forces, elastic (bending and stretching) forces, and for vesicles made of more than one component, also contributions from mixing or demixing of surfactants/lipids (Zuidam *et al.*, 1999). In general, in order to obtain complexes spontaneously, the lowering of electrostatic free energy due to neutralization of the positively charged membranes by the negatively charged nucleic acids must be larger than the elastic energy involved in the surfactant/lipid molecule adapting to the complex geometry (May and Ben-Shaul, 1997; Harries *et al.*, 1998). Complexing DOPE-DC-Chol (1.17:1.17 μmol) with DNA at a charge ratio of 1.5 was seen to result in highest transfection (Fig. 3.3) and hence this composition was chosen for further modification by supplementing with different concentrations of non-ionic surfactant, 1-monopalmitoyl glycerol (MP) in the presence of cholesterol (0.39 μmoles) (to give a final composition of 0.39, 0.59, 0.78 or 1.17:0.39:1.17:1.17 μmoles of MP:Chol:DOPE:DC-Chol respectively) in order to study the combined effect of co-lipids/surfactants in the complexes.

In Fig. 3.12, it was observed that when the MP concentration was increased in the complexes, a reduced trend in the transfection efficiency was observed, although the reason for this is unclear at present, it could be potentially due to the presence of excess amount of lipid/surfactant in the SUV-DNA complexes which could entrap plasmid DNA in the bulk of the aggregate structures, making it difficult for DNA to dissociate

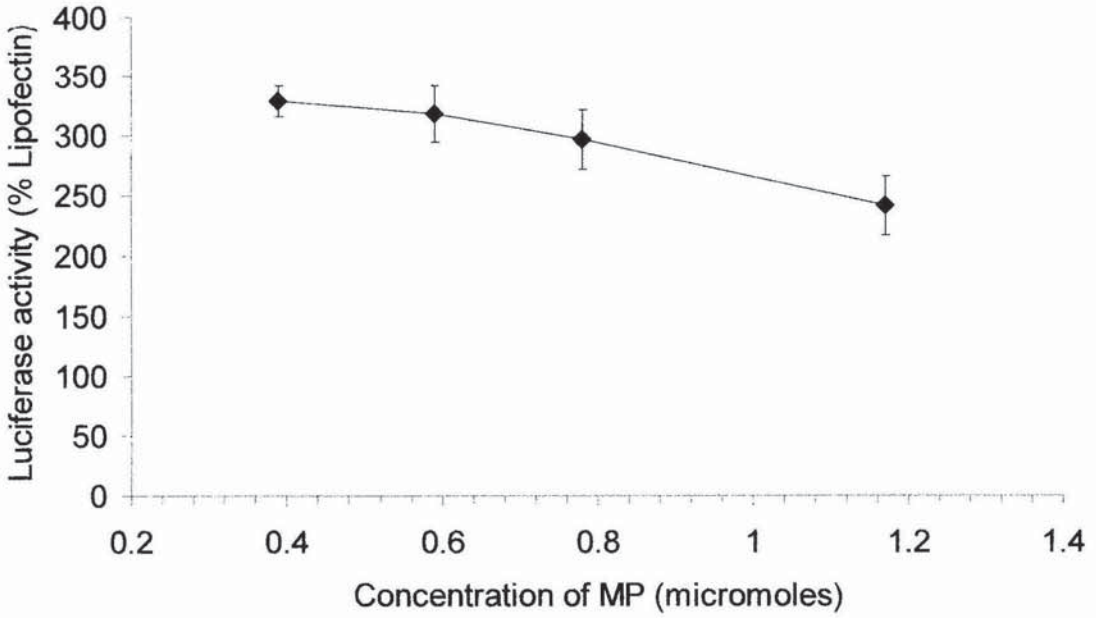


Fig. 3.12 Transfection efficiency of MP based surfactant vesicle-DNA complexes. Varying amounts MP in the presence of Chol (0.39 μmoles) were incorporated into DOPE-DC-Chol (1.17:1.17 μmoles) formulation and complexed with DNA at a charge ratio of 1.5. Increase in the MP concentration showed a reduced trend in the luciferase activity. All measurements were obtained for at least 3 independently synthesized batches and an average activity ± SD is reported for each sample.

from the complex and access the cell membrane in order to undergo transfection or could also be due to a variety of other reasons such as increased toxicity or decreased binding of DNA, as the availability of cationic lipid at the proximities of DNA might be reduced or increase in vesicle size due to the incorporation of excess surfactant.

3.3.4.2 Variation of alkyl chain length and DOPE ratio

It is now well understood that the neutral helper lipids incorporated in the complexes determines the morphologies and structures of the composite condensates (complexes) and affects the DNA transfection efficiency sometimes dramatically (Congiu *et al.*, 2004). In addition to facilitating endosomal disruption due to acidification of endosomal lumen and thereby activating the DOPE's fusogenic properties, DOPE was also suggested to play a role in facilitating the disassembling or dissociation of the lipid-

DNA complex after their internalisation and help in escape of DNA from endocytic vesicles (Harvie *et al.*, 1998) This was based on the assumption that the amine group of DOPE can compete with cationic lipid for DNA phosphate groups upon internalisation of the complex, thus leading to weakening of the binding forces between cationic lipids and DNA (Harvie *et al.*, 1998).

Fig 3.13 shows a significant reduction ($P<0.05$) in the transfection efficiency in the DOPE:DC-Chol formulation was observed when the DOPE concentration was reduced by half (i.e., to 0.59 μmoles). It was reported (Farhood *et al.*, 1995) that the enhanced helper activity of the DOPE was seen only when an optimum amount (1:1 mole ratio) of DOPE was employed. This was compared with another helper lipid dioleoylphosphatidylcholine (DOPC) which do not possess the ability to destabilise endosome membranes has failed to show helper activity. Such data is consistent with the notion that cationic lipid:DNA complexes enter the cell through an endosomal pathway and that the neutral lipid DOPE's function is to destabilise the endosome membrane (Farhood *et al.*, 1995) and is in conjunction with the electron microscopy results (Zhou and Huang, 1994) where the cells transfected with DOPE:DC-Chol showed an endosome destabilizing effect of DOPE unlike DOPC which failed to do the same.

The benefits of using DOPE were attributed to the fact that DOPE, possesses a lower head group to tail ratio and thus introduces better matching in charge density between lipid and DNA surfaces (Zuidam and Barenholz, 1997), easier counterion release from the lipid surface by the DNA (Zuidam and Barenholz, 1998) and lower hydration of the lipid surface (Zuidam *et al.*, 1999). It has been proposed that the destabilisation of the

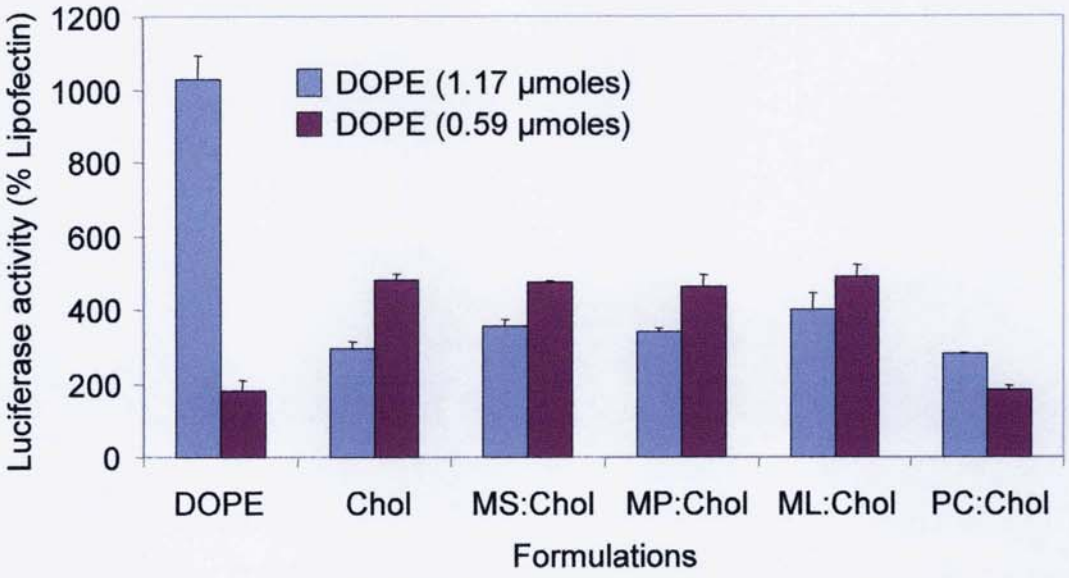


Fig. 3.13 Effect of incorporation of non-ionic surfactants and altering the DOPE concentration on transfection efficiency. DOPE:DC-Chol (1.17:1.17 µmoles) composition [DOPE *on graph*] was modified by incorporating Chol (0.39 µmoles)[Chol *on graph*] and other single alkyl chain non-ionic surfactants with varying chain lengths i.e., MS, MP, ML and also PC (0.39 µmoles each) making the final composition as surfactant:Chol:DOPE:DC-Chol, 0.39:0.39:1.17:1.17 µmoles respectively. All measurements were obtained for at least 3 independently synthesized batches and an average activity ± SD is reported for each sample.

endosomal membrane by the internalised complexes induces flip-flop of anionic lipids from the cytoplasmic leaflet to the luminal leaflet. The formation of a charge-neutral ion pair is thought to result in the displacement of the DNA from its complex with the cationic lipid leading to the release of DNA into the cytoplasm (Xu and Szoka, 1996). As a consequence of hexagonal lattice, the alkyl chains are oriented toward the aqueous phase, thereby promoting attractive hydrophobic and van der Waals' forces over the repulsive electrostatic and hydration forces (Regelin *et al.*, 2000). When DOPE concentrations were reduced by 50% these forces could presumably be diminished resulting in a 5 fold reduction in transfection efficiency of DOPE:DC-Chol (1.17:1.17 µmoles vs. 0.59:1.17 µmoles; Fig. 3.13).

Formulations supplemented with the addition of cholesterol (Chol:DOPE:DC-Chol), 1-monostearoyl glycerol (MS) (C18:0) (MS:Chol:DOPE:DC-Chol) or MP (MP:Chol:DOPE:DC-Chol) showed a significant increase ($P<0.05$) in the transfection efficiency (Fig. 3.13) at low DOPE concentration compared to high DOPE concentration while no significant variation ($P>0.05$) was noted in the formulation consisting of 1-monolauryl glycerol (ML) (C12:0) (ML:Chol:DOPE:DC-Chol). A low transfection efficiency observed for PC based system (Fig. 3.13) correlates with the previous reports (Duncan *et al.*, 1997; Tsan *et al.*, 1997) suggesting that PC mediates destabilisation of the lipid-DNA complexes prior to transfection. PC is a major component of pulmonary surfactant and has been shown to inhibit cationic lipid-mediated gene transfer to epithelial cells *in vitro* by mixing with the cationic lipid and disrupting the lipid-DNA complex (Duncan *et al.*, 1997). One of the reasons for the overall reduction in the transfection efficiency when the non-ionic surfactants were added to DOPE:DC-Chol vesicles along with cholesterol could be that the glycerol head group which is relatively more hydrated by possessing two free hydroxyl groups in conjunction with more orderly packed saturated alkyl chains could presumably discourage the lamellar-to-inverted hexagonal reorganisation of DOPE in the bilayers.

Using agarose gel electrophoresis studies (Fig. 3.14) the amount of accessible non-condensed DNA in all the complexes prepared (incorporating Chol and other non-ionic surfactants in DOPE:DC-Chol formulation) was shown to be similar (Fig. 3.14, lane 2 to lane 7) indicating that incorporation of different surfactants in the vesicle composition did not affect the spatial location of DNA and its stability. The importance of maintaining the stability and integrity of DNA during formulation and delivery process was discussed previously (Middaugh *et al.*, 1998) suggesting that introduction of a single break in the DNA phosphodiester backbone leads to conversion of

supercoiled to open circular and ultimately linear forms of DNA with further breaks. Although, the conversion of DNA from a supercoiled to an open circular form usually has a minor effect on gene transfer efficiency, subsequent conversion to linear forms can substantially reduce gene expression (Adami *et al.*, 1998). However, gel electrophoresis micrograph (Fig. 3.14) showed that the displaced non-condensed DNA was intact and supercoiled as it is observed to be migrated equally to a similar extent as that of the naked supercoiled DNA (Fig. 3.14, lane 1).

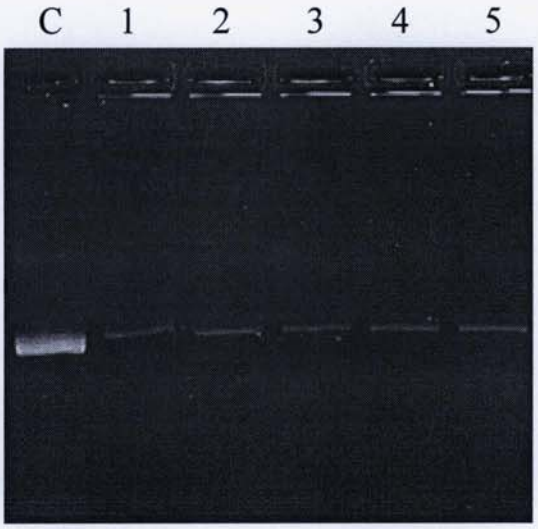


Fig. 3.14 Gel electrophoresis of surfactant vesicle-DNA complexes composed of Chol:DOPE:DC-Chol, lane 1; MS:Chol:DOPE:DC-Chol, lane 2; MP:Chol:DOPE:DC-Chol, lane 3; ML:Chol:DOPE:DC-Chol, lane 4; PC:Chol:DOPE:DC-Chol, lane 5; and lane C, naked supercoiled DNA.

3.3.4.3 Vesicle size and zeta potential

The mean sizes of the complexes prepared with the addition of cholesterol and non-ionic surfactants were found to be independent of the composition (Fig. 3.15) with no significant difference between the preparations possessing a polydispersity index (PI) between 0.2 and 0.5 suggesting a heterogeneous size distribution (results not shown). The large complex sizes seen could be a result of aggregation rather than intrinsically larger complexes as noted by the zeta potential values where the surface charges of the complexes were in the range of near neutral (+/– 10 mV; Fig 3.15). The similarity in the zeta potential values irrespective of the preparations composed of different surfactants was indeed anticipated, since the cationic lipid to DNA charge ratio in all the

preparations was kept constant. One explanation for the observed aggregation behaviour may involve the intrinsic thermodynamic instability of the system. Thus, the large surface area of the complexes may lead to an energetically favourable reduction in surface free energy through aggregation phenomena.

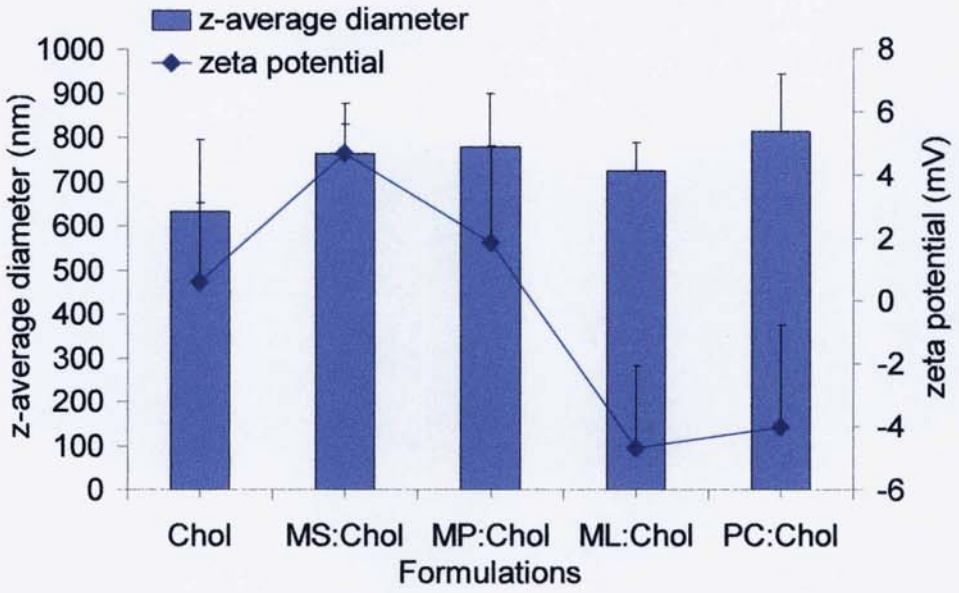


Fig. 3.15 Effect of incorporation of Chol and other non-ionic surfactants into DOPE:DC-Chol vesicles on z-average diameter and zeta potential. The complex sizes were observed to be in the range of 600 and 900 nm with no significant difference between the systems. Zeta potential of the complexes was observed to be between ± 4 mV indicating a near neutral charge for all the systems. All measurements were obtained for at least 3 independently synthesized batches, the z-average diameter and zeta potential ± SD is reported for each sample.

3.3.4.4 Morphological analysis of MP vs. PC based liposome-DNA complexes by TEM

The electrostatic interaction of cationic vesicles with the negatively charged phosphate backbone of plasmid DNA allows the two to self-assemble into vesicle-DNA complexes. When forming a complex with DNA, a profound structural rearrangement of the liposome occurs. Gershon *et al.*, (1993) suggested an interaction in which the cationic liposomes bind initially to DNA molecules to form clusters of aggregated vesicles along the nucleic acid strand. At a critical lipid:DNA charge ratio (around 1:1 +/-), any further increase in the lipid causes a synergistic DNA-induced liposome fusion

and lipid-induced DNA collapse. The result is the formation of a condensed DNA structure completely entrapped within a fused lipid bilayer. Fig. 3.16 shows TEM micrographs of complexes that have been freshly prepared from MP based (MP:Chol:DOPE:DC-Chol, 0.39:0.39:0.59:1.17 μmoles) and PC based (PC:Chol:DOPE:DC-Chol, 0.39:0.39:0.59:1.17 μmoles) vesicles at a lipid:DNA charge ratio of 1.5.

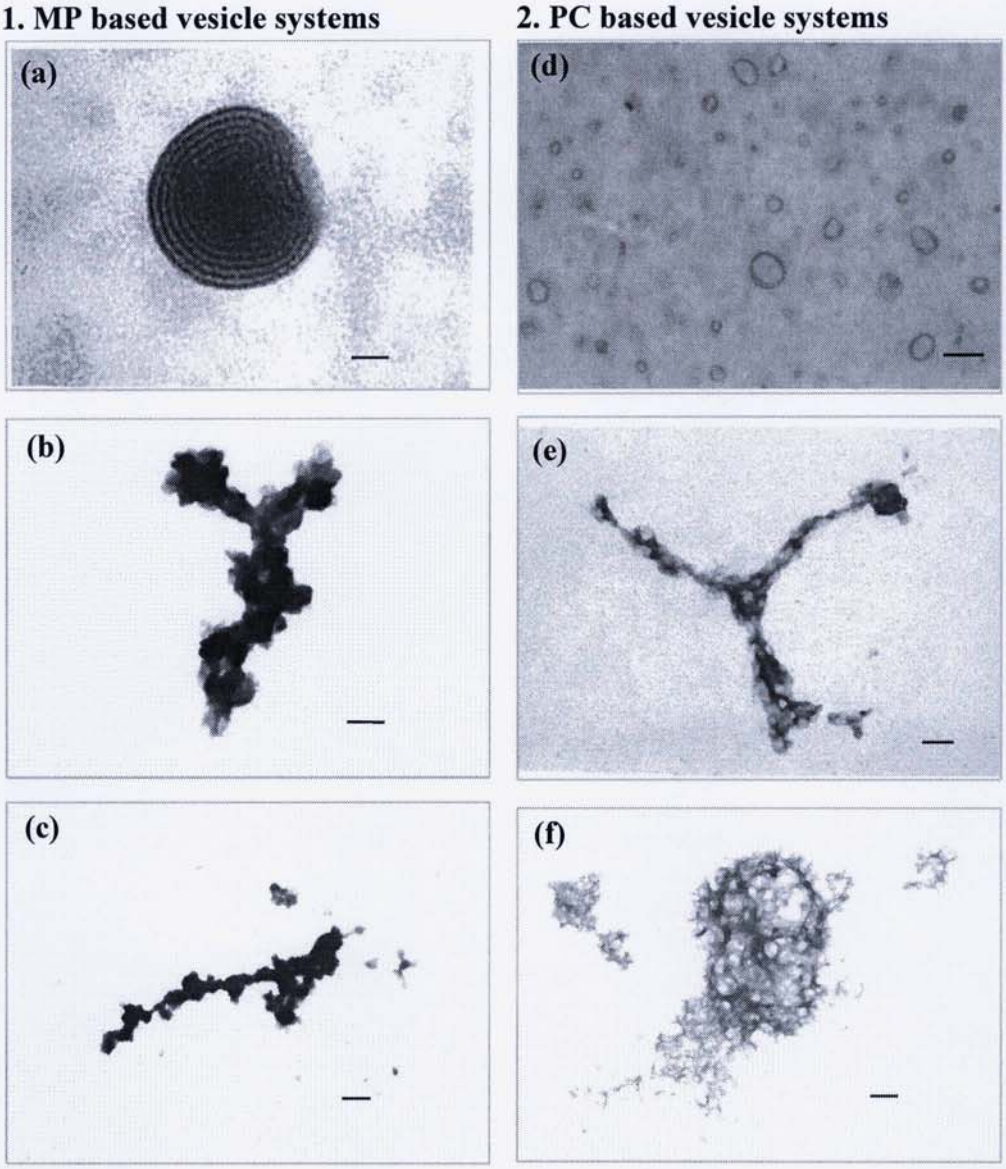


Fig. 3.16 Transmission electron microscopy (TEM) of MP and PC based surfactant vesicle-DNA complexes. MP:Chol:DOPE:DC-Chol (0.39:0.39:0.59:1.17 μmoles), (a) 'empty' vesicle, bar = 20 nm, (b) SUV-DNA, bar = 300 nm, (c) SUV-DNA, bar = 500 nm and PC:Chol:DOPE:DC-Chol (0.39:0.39:0.59:1.17 μmoles), (d) 'empty' SUV, bar = 300 nm, (e) SUV-DNA, bar = 500 nm, (f) SUV-DNA, bar = 1 μm.

Interestingly, sonication of empty MP based MLV though were reduced in vesicle size to about 80 nm still retained their multilamellar nature (Fig. 3.16a) while the PC based vesicles formed unilameller vesicles (Fig. 3.16d). In Fig. 3.16b, a number of vesicles appear to have approached the plasmid DNA and attached to its surface to form a new aggregated structure. The micrograph shows DNA and surface adsorbed cationic liposomes forming a structure which appears like two DNA segments that are spirally coiled. The adsorbed vesicles lie in close proximity with each other with the DNA acting as a fusogenic agent, neutralising the repulsive electrostatic forces on the cationic lipid and drawing them together to form semi-fused vesicles (Li *et al.*, 1996). Fig. 3.16c shows the same formulation at a 1.5 charge ratio but in which the vesicles appear to be adsorbed to a single segment of DNA resembling a 'spaghetti-like' appearance (Sternberg *et al.*, 1994) also referred to as 'bead on string' arrangements (Gershon *et al.*, 1993). The size of the structures seen in Fig. 3.16b,c are representative of the large aggregates measured by PCS (Fig. 3.15). In case of PC based systems, 'spaghetti-like' structures (Fig. 3.16e) were revealed where the ends of DNA segments were observed to be fused by the lipid vesicles apart from being adsorbed all along the DNA strands which is in contrast to spiral orientation of DNA strands as observed in MP based system (Fig. 3.16b). To further demonstrate the heterogeneity within the same PC based formulation, Fig. 3.16f depicts yet another structure where the unilamellar vesicles have attached to the surface of DNA to form an aggregated liposome complex resembling a 'meat-ball' structure (Sternberg *et al.*, 1994). Fig. 3.16b,c,e,f demonstrate that a high degree of polymorphism of cationic vesicle-DNA complexes exists with in similar formulations, and it clearly raises the question of which of the various forms of complex from within a single formulation contribute in efficient transfection. Methods designed to produce homogeneous complexes and to identify the complex that is most

effective at delivering the gene could provide an important advancement in improving gene transfer (Zabner *et al.*, 1995). Though, on the contrary, preparations of cationic lipid-DNA that are enriched in the small homogeneous particles were shown to be less transfection efficient (Zabner, 1997) than the heterogeneous cationic lipid-DNA complexes such as those represented in Fig. 3.16b, c, e, and f.

3.3.4.5 Effect of varying alkyl chain number and lipid mole ratio

To further study the effect of co-lipids/surfactants in the vesicle-DNA complexes, non-ionic surfactants varying in alkyl chain number, such as 1-monopalmitoyl glycerol (MP) (16:0, single alkyl chain), 1,2-dipalmitoyl glycerol (DP) (16:0, double alkyl chain) and 1,2,3-tripalmitoyl glycerol (TP) (16:0, triple alkyl chain) all possessing a glycerol head group (chemical structures included in appendices) were incorporated in different combinations in the presence or absence of DOPE and Cholesterol. The cationic lipid:DNA charge ratio was maintained constant at 1.5. One of the important reasons for the lipoplexes exhibiting a large degree of polymorphism apart from the charge ratio and the composition of the medium, is the specific surfactant/lipid composition of vesicles (Felgner *et al.*, 1996; Lasic, 1997). Indeed, the lamellar to hexagonal phase of DOPE as discussed in previous sections is presumed to be affected by the packing parameter, electrostatic interactions, hydration repulsion and van der Waals' attraction when incorporated with additional lipids/surfactants.

In Fig. 3.17, a high transfection efficiency was observed when MP (0.39 μmoles) was included in the DOPE:DC-Chol vesicle preparation, a similar trend that was observed in DOPE:DC-Chol preparation on its own (Fig. 3.3) while replacement of MP with DP or TP resulted in 3 fold reduction the transfection efficiency (Fig. 3.17). One reason that

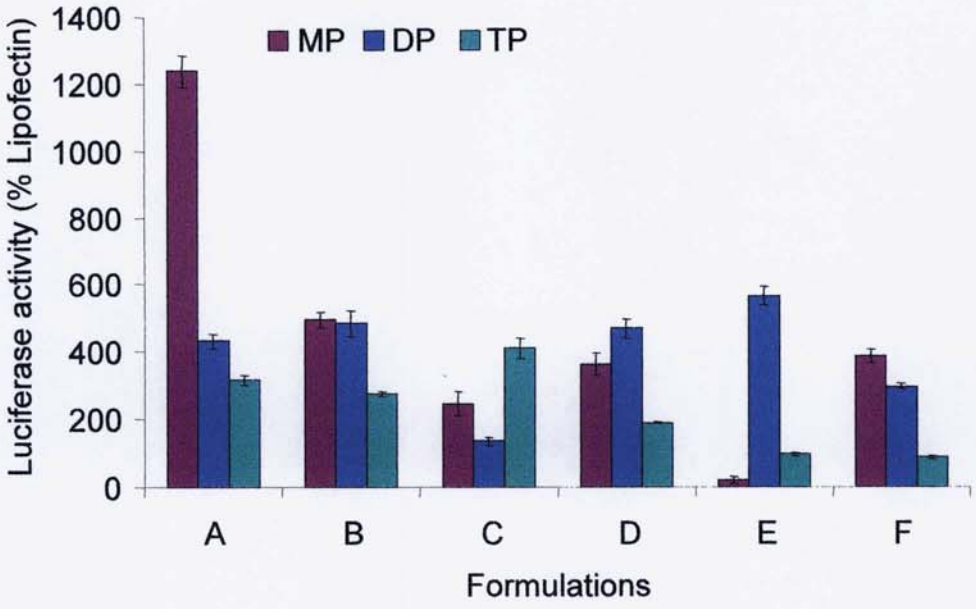


Fig. 3.17 Comparison of transfection efficiencies between different surfactant (Sf) combinations. A- Sf:DOPE:DC-Chol, 0.39:1.17:1.17 μmoles; B- Sf:DOPE:DC-Chol, 0.39:0.59:1.17 μmoles; C- Sf:Chol:DOPE:DC-Chol, 0.39:0.39:1.17:1.17 μmoles; D- Sf:Chol:DOPE:DC-Chol, 0.39:0.39:0.59:1.17 μmoles; E- Sf:Chol:DC-Chol, 0.39:0.39:1.17 μmoles; F- Sf:DOPE:DC-Chol, 0.39:0.39:1.17 μmoles. All measurements were obtained for at least 3 independently synthesized batches and an average activity ± SD is reported for each sample.

could explain this trend is, since MP possesses molecular features (single alkyl chain) that would be able to accommodate the cone-shaped DOPE and presumably display dynamics for perturbation of the target membranes as well as plasmid release which could bear an important consequence for transfection, might be difficult for DP and TP counter parts possessing double and triple alkyl chains to facilitate the flip-flop mechanism of DOPE. However, transfection efficiency was significantly ($P<0.05$) reduced even in case of MP based preparation when the DOPE concentration was reduced by half (0.59 μmoles) similar to the trend observed in Fig. 3.13 while there was no significant ($P<0.05$) alteration in the activity of DP and TP based systems with the change in DOPE concentration (Fig. 3.17). Addition of cholesterol (0.39 μmoles) to these preparations when the DOPE concentration was 1.17 μmoles further significantly

($P<0.05$) reduced the activity of MP and DP based systems. Interestingly, a significant ($P<0.05$) improvement the efficiency of TP based system was observed which was in the order of TP>MP>DP. When the composition of this preparation was further modified by reducing the DOPE concentration once again to 0.59 μmoles, the efficiency was reversed showing in the order of DP>MP>TP. A further more interesting result here is the efficiency of transfection shown by DP based system (DP:Chol:DC-Chol, 0.39:0.39:1.17 μmoles respectively) where an approximately 600% (of control) activity was observed (Fig. 3.17) even in the complete absence of DOPE in the preparation. Several advantages are being proposed for cholesterol as a co-lipid such as, cholesterol maintains the transfection in the presence of serum by improving cell binding and uptake (Crook *et al.*, 1998) this effect has been suggested due to the decreased charge density in the complexes incorporating cholesterol while Semple *et al.*, (1996) observed that the half-life of vesicles in the systemic circulation of mice can be increased by including cholesterol in the formulation. Finally, replacement of Chol with DOPE at a similar mole ratio (0.39 μmoles) showed an activity in the order of MP>DP>TP again reinforcing the presumption of increasing in the number of alkyl chains leads to the diminished performance of DOPE in the bilayers restricting its flip-flop mechanism with the endosomal membranes which in turn compromises the transfection. The reason for a relatively high activity of DP based system when DOPE was replaced by Chol in relation to its MP and TP is not clear at present and needs further investigation.

3.4 CONCLUSIONS

The work in this study was set out to define the structure and physico-chemical properties of cationic lipid-DNA gene delivery complexes in relation to their

transfection efficiencies. High levels of transfection efficiency were achieved with cationic liposomes containing DC-Chol, proving to be at least 6 times more efficient than DOTMA, DOTAP and DDA. The inclusion of the helper lipid DOPE induced high levels of transfection, but the efficiency of transfection varied depending on the type of helper lipid incorporated within the liposome bilayer. When comparing the transfection efficiency of the helper lipids DOPE and Chol in combination with DC-Chol (1:1 molar ratio), overall the optimum level of transfection efficiency was achieved with DOPE at a charge ratio of 1.5:1. However, at the lower charge ratios Chol produced significantly higher levels of activity compared to DOPE, demonstrating that even in the absence of fusogenic properties offered by DOPE, DNA can be effectively delivered. The same experiment also highlighted that the efficiency of transfection depended not only on the type of helper lipid used but also on the effective lipid:DNA charge ratio. As anticipated, when lipid:DNA charge ratio was increased, the displacement of DNA from the complex was minimised as observed in the gel electrophoresis studies, also at high ratios, neutrally charged lipid-DNA complexes, due to the reduction in the electrostatic repulsive forces formed heterogeneous aggregates confirmed by visualising these complexes by TEM and for the first time using ESEM technique which correlate with the PCS vesicle size measurements. Inclusion of non-ionic surfactants varying in alkyl chain length and also in alkyl chain number has revealed some interesting observations depicting the variability in transfection efficiency by altering the helper lipid concentration though further characterisation of these preparations is thought to be needed to gain a better understanding of the underlying interplay between surfactant-lipid-DNA-cell component entities. An important implication of the reversibility in neutralisation and changes in DNA structure is that, most of the time DNA must be available to lipid molecules, which supports the claims of Eastman *et al.*, (1997) in that,

in lipoplexes formed spontaneously at the charge ratios in the range of 0.2 – 2.0, most DNA although condensed, is actually exposed and not encapsulated by the lipid layer. In this respect, the differences in size, stability, shape and exposure of plasmid DNA between spontaneously and non-spontaneously (using external energy) formed lipoplexes, are expected to have major implications for transfection efficiency, especially *in vivo* (Templeton *et al.*, 1997). The correlation between the transfection efficiencies *in vitro* and *in vivo* is always a challenge (Lasic, 1997) as the *in vivo* transfection is much more complicated process than the *in vitro* transfection, especially interaction of lipoplexes with serum components which modify the structure and properties of the lipoplexes (Sternberg *et al.*, 1998) and affect transfection. Thus the *in vivo* transfection, in terms of genetic vaccination, of various DNA incorporated preparations was further studied and discussed in the 4th chapter.

Chapter Four

Hepatitis-B Encoded DNA Vaccines: Characterisation and Immunisation Studies

4.1 INTRODUCTION

4.1.1 Genetic Immunisation

As already noted in chapter one, Hepatitis B poses a serious worldwide problem and lack of efficient antiviral treatment against Hepatitis B makes the development of a vaccine highly desirable. The major shortcoming of the current antigen-based vaccines is the inability to induce cellular immunity, which is essential for prophylaxis and treatment of the disease (Xiao-wen *et al.*, 2005). DNA vaccines may offer an effective alternative to these systems with studies (Lee *et al.*, 2001; Jasper *et al.*, 2000; Geissler *et al.*, 1998) already demonstrating that immunisation with eukaryotically expressed plasmids encoding the small, middle and large viral envelope proteins, core proteins, or polymerase proteins of Hepatitis B virus being effective at eliciting strong and durable humoural and cell-mediated immunity including CTL and cytokines in mice. In this respect, it has been proposed as a good strategy for the immunisation of non-responders to the current recombinant Hepatitis B antigen (HBsAg) vaccines and for application of a therapeutic vaccine (Rottinghaus *et al.*, 2003). It has been previously reported that liposome-encapsulated DNA prepared by the dehydration-rehydration method (DRV liposomes) (Kirby and Gregoriadis, 1984; Perrie and Gregoriadis, 2000) has been shown to promote enhanced humoural and cell-mediated immune responses against the encoded antigen in immunised mice (Gregoriadis *et al.*, 1997, 1999; Perrie *et al.*, 2001, 2003) compared to naked DNA or DNA complexed to identical preformed liposomes. Similar observations have been made with plasmid DNA-entrapped within non-ionic surfactant based vesicles prepared by similar method (Obrenovic *et al.*, 1998; Obrenovic and Gregoriadis, 1999; Perrie *et al.*, 2004).

In contrast to naked DNA, DNA entrapped within multilamellar vesicles such as those obtained by the dehydration-rehydration method, is protected from nuclease attack in

biological milieu. Liposomes also offer an additional advantage when used as carriers for DNA vaccines since, after administration, liposomes are likely to deliver their DNA content to antigen presenting cells (APC) infiltrating the site of injection or in the draining lymph nodes (Tuner *et al.*, 1983; Velinova *et al.*, 1996; Perrie *et al.*, 2001). In addition, vesicle-mediated DNA delivery may offer the choice of the subcutaneous route, which for naked DNA, has so far failed to elicit immune responses (Gregoriadis, 1998). Indeed, recently subcutaneous liposome-mediated DNA immunisation employing two DRV(DNA) liposome formulations revealed humoural responses engendered by the plasmid encoded nucleoprotein were substantially higher after two 10 μg doses of liposome-entrapped DNA compared to equal doses of naked DNA (Perrie *et al.*, 2003). These results suggests that, not only can DNA be effectively entrapped within liposomes using the DRV method but that such DRV liposomes containing DNA may be a useful system for subcutaneous delivery of DNA vaccines (Perrie *et al.*, 2003).

4.1.2 Aims and Objectives

The current work was intended to incorporate plasmid DNA (pRc/CMV-HBS) in different compositions of surfactant vesicles which are known to have their own inherent adjuvant effect. The DRV method was used as one of the techniques for the preparation of DNA loaded vesicles as this method possesses the advantage of yielding high entrapment values (rather than surface complexation (Perrie and Gregoriadis, 2000)) for the plasmid. The effectiveness of these preparations to induce humoural and cell mediated responses in mice after immunisation via the subcutaneous route was studied.

4.2 MATERIALS AND METHODS

4.2.1 Materials

4.2.1.1 Chemicals

2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) tablets (ABTS), bovine serum albumin (BSA), concanavalin A, heparin, hydrogen peroxide, incomplete fruend's adjuvant, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium carbonate, sodium hydroxide, sodium phosphate dibasic, tetramethylbenzidine (TMB), Trizma® base, Tween 20 were purchased from Sigma-Aldrich, Poole, Dorset, UK. Citric acid was purchased from Fisher Scientific UK Ltd., Loughborough, UK. Marvel was from Premier Int. Foods Ltd, Lincs, UK. RPMI 1640 cell culture medium, Opti-MEM® I (reduced serum medium), foetal bovine serum (FBS), L-glutamine/Penicillin-Streptomycin were purchased from Gibco-Invitrogen, Paisley, UK. Scintillation fluid, Optiphase Hisafe III was from Fisher Scientific UK Ltd., Loughborough, UK. [3H] thymidine was purchased from Amersham plc, UK. DuoSet® capture ELISA, Mouse IL-2 and IL-6 were purchased from R&D systems, MN, USA Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin IgG, IgG1, & IgG2a subclasses were obtained from Oxford Biotechnology, UK. ELISA plates (flat bottomed, high binding) were purchased from Greiner Bio-One Ltd, UK. All other reagents and solvents were of analytical grade.

4.2.1.2 Surfactants and Lipids

1-monopalmitoyl glycerol (C16:0) (MP), 1,2-dipalmitoyl glycerol (C16:0) (DP), cholesterol (Chol), cholesteryl 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) were purchased from Sigma-Aldrich, Poole, Dorset, UK. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti®

Lipids, Alabaster, AL, USA. Egg phosphatidylcholine (PC) was purchased from Lipid Products, Nutfield, Surrey, UK.

4.2.1.3 Plasmid DNA

Plasmid DNA used in this study was pRc/CMV-HBS (5.6 kb), (also referred to as pCMV-HBs(S)), expressing the sequence coding for the S (small) region of HBsAg (subtype ayw) cloned by Dr. Robert Whalen, Pasteur Institute (Davis *et al.*, 1993b), was supplied by Aldevron, Fargo, USA. The backbone of this plasmid is pUC-based encoding a bacterial origin of replication and ampicillin-resistance gene for growth and selection. It also carries a cytomegalovirus (CMV) promoter and terminator sequence (Cox *et al.*, 2002).

4.2.2 Methods

4.2.2.1 Preparation of surfactant vesicles

SUV and DRV were prepared as outlined in sections 2.2.2.4.2 and 2.2.2.4.3 respectively.

4.2.2.2 Immunisation of mice

Female BALB/c mice, 6 – 8 weeks old, were obtained from B & K Universal Ltd., Hull, England. All procedures had been approved by the Aston University's Ethical Review Committee and were conducted in accordance with the Animals (Scientific Procedure) Act 1986. Mice were immunized with 20 μg of 'free' or surfactant vesicle formulated plasmid DNA (pRc/CMV-HBS). The surfactant vesicle based vaccines were prepared by mixing or encapsulating 20 μg of pRc/CMV-HBS incorporated in, or complexed with, DRV of various formulations. All immunization formulations were administered in 10% sucrose and mice were immunised subcutaneously in groups of

five with the vaccines (0.2 ml/dose) at the back of the neck using 1 ml calibrated disposable plastic syringes on days 0, 14, 35, 74, and were boosted with 1 μg of recombinant hepatitis B antigen (HBsAg) on day 127 (Fig 4.1). An intramuscular injection of 3 μg HBsAg (ayw subtype) emulsified in 50 μl of freund's incomplete adjuvant was given in quadriceps for a positive control.

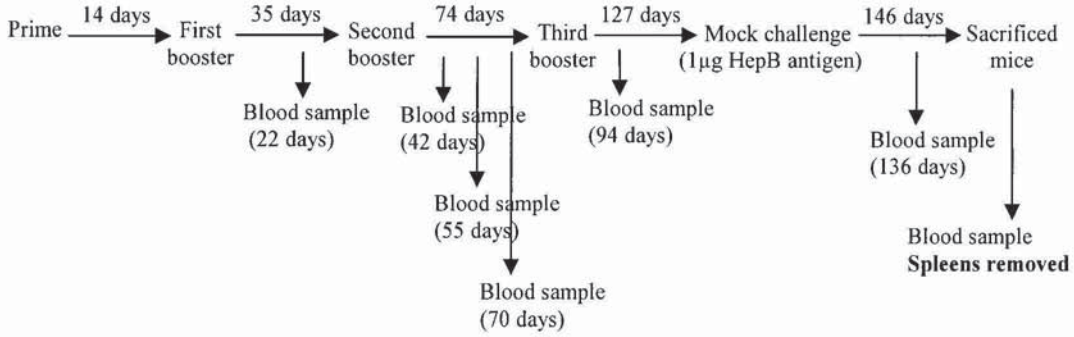


Fig 4.1 Immunisation regimen and time plan for blood sampling.

Mice were bled from the tail vein at scheduled time intervals (Fig 4.1). Blood samples were obtained by causing a small nick in the tail vein and 50 μl of blood was collected into a capillary tube that had been previously coated with 0.1% w/v heparin solution in PBS and gently blown the excess solution leaving a dried coat of anti-coagulant in the capillary tubes. The blood sample was then diluted 10-fold in 450 μl of PBS and centrifuged at 13000 rpm for 5 min to separate the sera from blood cell components. The sera (diluted 20-fold, assuming a 50% haematocrit) for each mouse was transferred into an eppendorf tube and stored at -20°C until analysed.

4.2.2.3 Antibody enzyme-linked immunosorbent assay (ELISA)

Sera samples obtained at different time intervals after immunisation were analysed for the presence of anti-HBsAg IgG1, IgG2a and IgG antibodies by the enzyme-linked

immunosorbent assay (ELISA) (Gregoriadis *et al.*, 1993). Buffers such as PBST (wash buffer), sodium carbonate buffer (Na_2CO_3) are prepared as per the recipe (see appendices) on the previous day. ELISA plates (flat bottomed, high binding; Greiner Bio-One Ltd, Glos. UK) were coated with 60 µl of HBsAg *per* well (2 µg/ml) in sodium carbonate buffer. Plates were incubated at 4°C overnight for adsorption of antigen to the plates. Unbound antigen was aspirated and the plates were washed 3 times with PBST using a plate washer. Residual washings were removed by blotting firmly onto paper towel. To avoid non-specific binding, plates were treated with 0.2 ml per well of 1% w/v Marvel (Premier Int. Foods Ltd, Lincs, UK) in PBS and incubated for 2 h at 37°C followed by washing 3 times with PBST. Serially diluted serum samples (50 µl *per* well) were then transferred to ELISA plates and incubated for 1 h at 37°C. After washing the plates as previously, 50 µl *per* well of horseradish peroxidase conjugated anti-mouse isotype specific immunoglobulin (goat anti-mouse IgG1, IgG2a or IgG; Oxford Biotechnology, Oxfordshire, UK) were added to designated wells at a working dilution of 1 in 5000 in PBS and incubated for 1 hour at 37°C. Plates were washed as above and 50 µl of substrate solution, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma, Poole, Dorset, UK) prepared in citrate buffer and incorporating 5 µl of H_2O_2/50 ml was added to each well and the reaction was allowed to proceed by incubating the plates for 30 min at 37°C. Absorbance of the colour development was measured spectrophotometrically at 405 nm on microplate reader (Bio-Rad, Herts, UK). A known positive serum and pooled naïve mice sera collected after immunisation were employed as positive and negative controls respectively.

4.2.2.4 Splenocyte cell proliferation assay

Upon termination of experiments, mice were humanely culled and their spleens aseptically removed and placed into ice-cold sterile PBS. Spleens were treated as follows: A crude suspension of spleen cells in 10ml working media (RPMI 1640 cell culture medium supplemented with 10 % (v/v) foetal bovine serum (FBS), 4 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml)) was prepared by gently grinding the spleen on a fine wire screen. After allowing the cell suspension to settle for approximately 5 minutes the liquid was transferred to sterile 20 ml 'Falcon' tubes, without disturbing the cellular debris at the bottom. The cell suspension was centrifuged (Mistral 3000*i*, UK) at 200 × *g* for 10 min. After centrifugation the supernatant was removed, the cell pellet was resuspended in 10ml fresh working media and the centrifugation procedure was repeated. Following this final centrifugation the cell pellet was resuspended in 13 ml fresh working media. These single cell suspensions were used to assess antigen specific cytokine production and antigen specific *recall* responses.

For study of antigen specific proliferative responses, aliquots of 100 µl volumes of sterile media or antigen in sterile media (at the concentrations stated) were seeded onto 96 well suspension culture plates (Greiner Bio-One Ltd. Glos. UK) and 100 µl volumes of viable splenocytes (approximately 1×10^7 cells ml^{-1}) added to each well. Covered plates were incubated in a humid, 5% CO_2 environment at 37°C. As a positive control, cells were co-cultured with concanavalin A (Sigma, Poole, Dorset, UK) at a concentration of 3µg/ml. Covered plates were incubated in a humid, 5% CO_2 environment at 37°C for 72 h. After 72 h incubation, half a microcurie of [^{3}H] thymidine (Amersham, UK) in 40 µl volumes of freshly prepared sterile working media

was added to each well, and the incubation continued for a further 24 h. The well contents were harvested onto glass filter mats (Molecular Devices Ltd., Wokingham, UK) using a cell harvester (Titertek). After drying, the discs representing each well were punched from the filter mats into 5 ml volumes of scintillation fluid (Optiphase Hisafe III, Fisher Scientific UK Ltd. Loughborough) and the incorporation of [^{3}H] thymidine into the cultured cells was measured using standard counting procedures.

4.2.2.5 Cytokine enzyme-linked immunosorbent assay (ELISA)

The supernatants of splenocyte cell suspensions prepared as outlined in section 4.2.2.4 were pooled after 24 hours incubation with 5 μg/ml recombinant HBsAg, from at least three separate wells and collected in eppendorfs and stored at -70°C until assayed for the presence of cytokines using DuoSet® capture ELISA kits (mouse IFN-γ and IL-6) purchased from R&D systems, Abingdon, UK, according to the manufacturers instructions. On the previous day of the experiment, a working concentration of capture antibody was prepared in PBS and coated 96-well microplates with 100 μl per well followed by incubation overnight at room temperature. Plates were washed with wash buffer (PBST) for 3 times. Complete removal of the liquid was ensured by inverting the plate and blotting it against clean paper towels. Plates were blocked by adding 300 μl of reagent diluent (recipe in the appendices) to each well and incubated at room temperature for 1 hour. Plates were then washed similar to that of previous washing procedure. 100 μl of samples or serially diluted standards in reagent diluent were added onto each well and incubated at room temperature. The washing procedure was repeated. Later 100 μl of detection antibody (diluted in reagent diluent) was added per well and incubated for 2 hours at room temperature and again the wash procedure was repeated. 100 μl of working dilution of streptavidin-HRP was added to each well and

incubated for 20 min at room temperature avoiding the direct light on the plate. After a final wash procedure, 100 µl of substrate solution (1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (tetramethylbenzidine; TMB) was added to each well and incubated for 20 min at room temperature avoiding direct light. 50 µl of stop solution (2N H_2SO_4) was added to each well and optical density of the sample was measured at 450 nm. A range of dilutions of purified recombinant mouse IFN-γ and IL-6 were included as standards.

4.2.2.6 Statistical analysis

Antigen-specific antibody response was measured on the basis of absorbance readings as determined by ELISA. The mean of end point serial dilutions and their standard deviation were calculated for each animal group. Significance levels at $P<0.05$ were determined using ANOVA followed by tukey test.

4.3 RESULTS AND DISCUSSION

4.3.1 Characterisation of formulations

SUV preparations composed of DOPE:DC-Chol (1.17:1.17 µmole), complexed with plasmid DNA (SUV-DNA) yielded a high transfection efficiency than its cholesterol based counter part *in vitro* (as discussed in chapter 3), addition of non-ionic surfactants, such as, 1-monopalmitoyl glycerol (MP) or 1,2-dipalmitoyl glycerol (DP) to the former, i.e., MP/DP:DOPE:DC-Chol (0.39:1.17:1.17 µmole) resulted in the sustained high transfection efficiency in case of MP based system unlike its DP based counter part. The study of such vesicle systems was further extended to immunisation experiments. In another set of formulations, MP based DRV systems (MP:DOPE:Chol:DC-Chol; 16:8:4:4 µmole) with complexed (DRV-DNA) or encapsulated (DRV(DNA))

pRc/CMV-HBS were investigated. In addition to DRV(DNA) prepared via the standard procedure (Perrie and Gregoriadis, 2000), formulations were also prepared by freeze-drying in the presence of sucrose (DRV(sucrose, DNA)) (Section 2.2.2.4.4). These formulations were compared with their similarly formulated PC based counter part i.e., PC:DOPE:Chol:DC-Chol (whose physico-chemical characteristics have been discussed in chapter 2).

4.3.1.1 The effect of suspending medium

The formulations incorporating pRc/CMV-HBS in a medium of 10% sucrose were analysed for their size and zeta potential (Table 4.1). The vesicle sizes in case of MP based DRV(DNA) were observed to be smaller when suspended in sucrose medium compared to those prepared using 0.01 M PBS (814 nm vs 1624 nm; Table 4.1 and 2.2 respectively) presumably due to the low ionic strength of sucrose medium which possess a conductance of approximately 2-10 μS as opposed to PBS with a conductance of 3500 μS. The higher ionic content of the latter could result in a shielding effect on the vesicle surface charge, resulting in the condensation of the electrical double layer (Florence and Attwood, 1998) which could result in a partial vesicle aggregation and hence could explain the increased size of the vesicles suspended in PBS vs sucrose. In such an instance a concurrent drop in zeta potential would be anticipated. However, this could not be fully investigated since analysis of the vesicle zeta potentials is conducted in 1/10 PBS buffer (0.001 M) since the high conductivity of PBS makes analysis of zeta potential unreliable in the current equipment.

DRV-DNA complexes were of a micron in size and zeta potential was observed to decrease compared to DRV(DNA). As already discussed this may be an outcome of the surface complexing of the negatively charged DNA to the surface of the positively charged DRV resulted in lowering of zeta potential and hence a consequent increase in their size. In contrast DRV(DNA) has a significant amount of plasmid DNA entrapped within the vesicles rather than surface bound (Perrie and Gregoriadis, 2000).

4.3.1.2 The effect of sucrose during freeze-drying

Freeze-drying of MP based systems in the presence of sucrose (DRV(Sucrose+DNA) incorporated at a sucrose/surfactant mass ratio of 5 showed a significant reduction ($P<0.05$; Table 4.1) in vesicle size compared to DRV(DNA), for example, from 813 ± 94 nm to 533 ± 92 nm indicating a stabilizing effect of sucrose on the vesicle bilayers during the dehydration and/or rehydratrion of the DRV as discussed in section 2.3.6 while no significant variation in the zeta potential was observed when compared to the preparation devoid of sucrose (35 ± 6 vs 41 ± 4 respectively) indicating that the presence of sucrose in the vesicle bilayers do not interfere with the electrostatic forces induced by the positively charged head group on the vesicle surface.

4.3.1.3 The effect of bilayer composition

Sizes of PC based system were significantly ($P<0.05$) smaller than its MP based counter part (587 ± 55 nm vs 814 ± 94 nm respectively, Table 4.1) because of a low surface free energy induced due to the relatively high hydrophobicity of double alkyl chained PC molecule compared to the single alkyl chained non-ionic surfactant, MP molecule adopted in the niosome formulations (Yoshioka *et al.*, 1994; Uchegbu, 1994). Nevertheless, the surface charge in both these preparations did not vary significantly

since the concentration of the cationic lipid incorporated into the system was kept constant therefore suggesting the packing parameters of PC and MP molecule in the vesicle bilayers could possibly be similar not altering the orientation of the cationic lipid in the bilayer.

DOPE:DC-Chol based SUV-DNA complexes both in the presence and absence of MP or DP molecules were found to be in the size range of about 250 nm (Table 4.1), while no significant difference was noted in the measured surface charge in these systems (Table 4.1), though a small decrease in the values was seen presumably due to the surface complexing of the negatively charged DNA.

Formulation	Vesicle type	Size (nm)	Zeta potential (mV)
MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles)	DRV(DNA)	813.5 ± 93.8	40.8 ± 3.7
MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles)	DRV-DNA	1030.2 ± 129	34.4 ± 3.3
MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles)	DRV(Sucrose + DNA)	532.7 ± 91.9	35.2 ± 5.6
PC:DOPE:Chol:DC-Chol (16:8:4:4 μmoles)	DRV(DNA)	586.7 ± 55.1	44.1 ± 2.0
DOPE:DC-Chol (1.17:1.17 μmoles)	SUV-DNA	258.8 ± 10.5	32.9 ± 3.9
MP:DOPE:DC-Chol (0.39:1.17:1.17 μmoles)	SUV-DNA	225.9 ± 8.3	31.0 ± 4.2
DP:DOPE:DC-Chol (0.39:1.17:1.17 μmoles)	SUV-DNA	230.8 ± 27.7	33.9 ± 5.4

Table 4.1 Characterisation of various surfactant vesicle preparations incorporating plasmid DNA (pRc/CMV-HBS). Vesicle size and zeta potential measured in 1/10 dilution of 10% w/v sucrose medium were obtained for at least 3 independently synthesized batches and an average value ± SD is reported for each sample.

4.3.2 Optimisation of ELISA method

Before proceeding to assay the experimental serum samples for antibody production, the ELISA method was optimised. In order to have a better discrimination between the experimental serum samples and the controls, a series of parameters were tested using serially diluted antibody-containing and antibody-free, positive and negative controls

respectively. To this end, the effect of number of plate washes in a step, the concentration of conjugated anti-mouse immunoglobulins, and the concentration of substrate solution (ABTS) all were tested using two blocking agents, i.e., 1% w/v bovine serum albumin (BSA) or Marvel (MV) and the absorbance values obtained at 405 nm were plotted.

4.3.2.1 Optimisation of substrate concentration and wash procedure for a 1 in 1000 conjugate antibody dilution

In Fig. 4.2a, with a 3 wash step procedure and with a substrate concentration of 3 ABTS tablets/50 ml citrate buffer, negative control of 1%w/v Marvel (MV) had an absorbance of ~0.2, equivalent to a normal background reading, throughout the serial dilution compared to 1%w/v BSA, which showed an undesirable increase in the absorbance with increase in the serial dilution. Positive controls with both the blocking agents gave an initial high absorbance of ~1.6, possibly due to the presence of high conjugate antibody concentration (1 in 1000 in PBS) facilitating a greater amount of non-specific as well as specific binding and thus yielding a high absorbance value, however, the positive control of 1%w/v Marvel reached the background reading (~0.2) at a serial dilution of 204800 much lower than its BSA counter part (Fig. 4.2a). The reason for a high absorbance value of BSA is unclear although it is presumed that the presence of trace amounts of serum antibodies in BSA might be contributing unlike the Marvel which is a skimmed milk powder containing casein and whey protein. Decreasing the substrate concentration to 1 ABTS tablet/100 ml citrate buffer (manufacturer's recommended concentration) lead to a decrease in the absorbance values of positive controls to 0.4 (Fig. 4.2b) with minimum discrimination against their negative counter parts presumably due to the low substrate concentration which would require a longer incubation time to react with the conjugate antibodies compared to a higher substrate

concentration which helps in faster reaction, but the incubation time was kept constant in all the cases to avoid simultaneous multiple variables. When the number of washes was increased to 5 in order to eliminate the unbound and loosely bound antibodies more efficiently, with a substrate concentration of 3 ABTS tablets/50 ml, the background reading of BSA was once again observed to be higher than that of MV (Fig. 4.2c) a similar trend was seen even with the positive controls, indicating that the relatively higher background of BSA was also influencing the positive control to give a higher absorbance compared to MV. Decreasing the substrate concentration to 1 ABTS tablet/100 ml lead to a lower absorbance value (Fig. 4.2d) for positive controls once again, as the duration of incubation was not sufficient for a complete reaction to occur.

4.3.2.2 Optimisation of substrate concentration and wash procedure for a 1 in 5000 conjugate antibody dilution

In the next set, the conjugate antibody was diluted to 1 in 5000 with a 3 wash step and 3 ABTS tablets/50 ml. In this case, both the positive controls had an initial absorbance of ~0.9 (Fig. 4.3a), with the BSA values for both the positive and negative controls slightly higher than that of MV. When the substrate concentration was decreased to 1 ABTS tablet/100 ml, the discrimination between the respective positive and negative controls reduced to a minimum (Fig. 4.3b). When the wash step was increased to 5 washes with a substrate concentration of 3 ABTS tablets/50 ml, the discrimination between the positive and negative controls was notably better till the end of the plate with the highest initial values for positive controls being ~1 (Fig. 4.3c) although the background for the BSA was seen to increase. On the other hand, decreasing the substrate concentration to 1 ABTS tablet/100 ml, lead to poor discrimination between the positive and negative controls at increased serum dilutions (Fig. 4.3d).

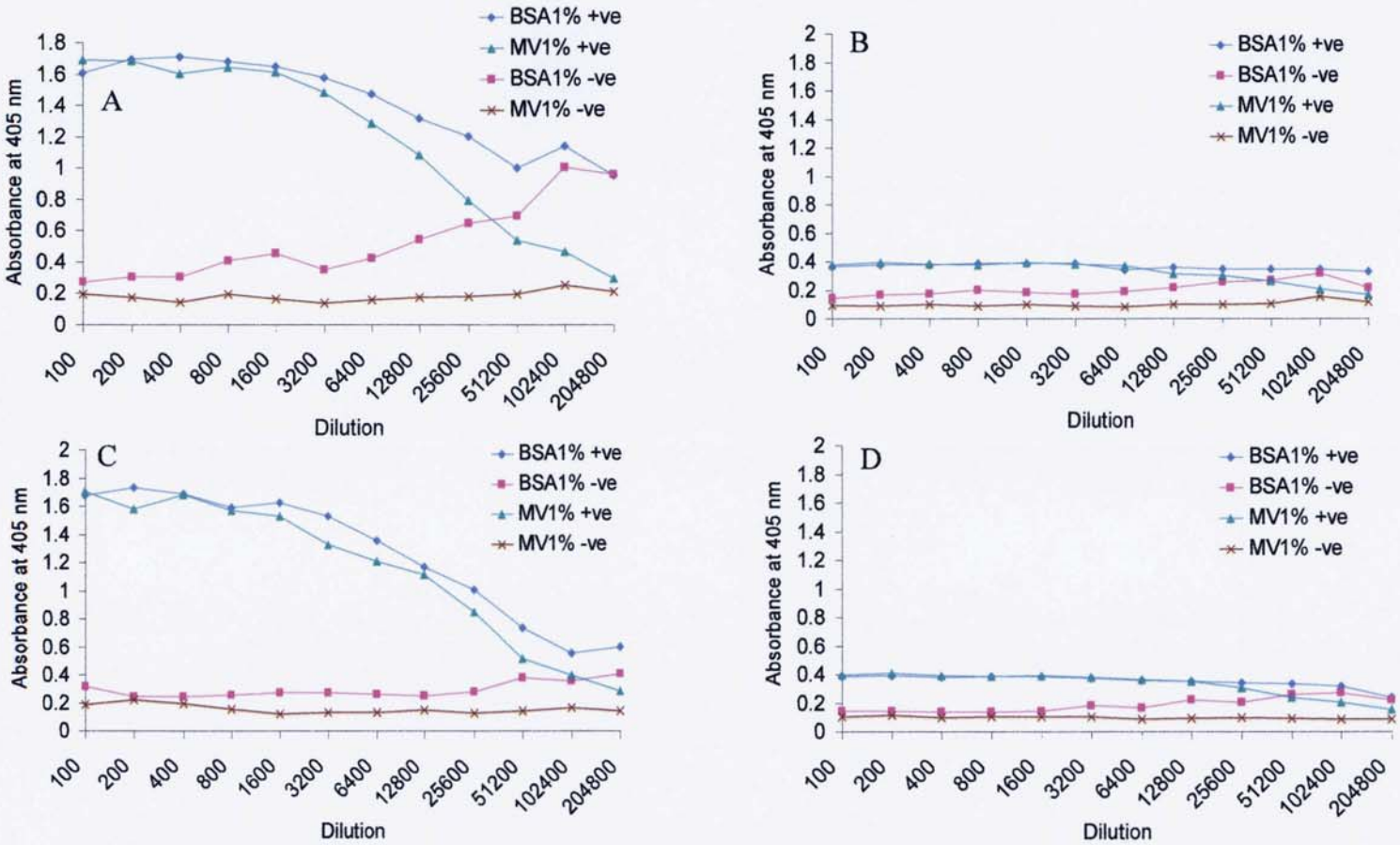


Fig. 4.2 Optimisation of antibody ELISA protocol for a 1 in 1000 conjugate antibody dilution. A series of parameters in the ELISA protocol were tested by measuring the absorbance of serially diluted antibody-containing and antibody-free, positive and negative controls respectively, using two different blocking agents, i.e., bovine serum albumin (BSA) and Marvel (MV) (1% w/v) in order to obtain a better discrimination between the experimental groups and the controls. Conjugate antibody was diluted 1 in 1000 PBS with 3 washes per step and 3 ABTS tablets dissolved in 50 ml (A) or 1 ABTS tablet dissolved in 100 ml citrate buffer (B). 5 washes per step and 3 ABTS tablets dissolved in 50 ml (C) and 1 ABTS tablet dissolved in 100 ml citrate buffer (D). Results represent average of duplicate readings on each plate.

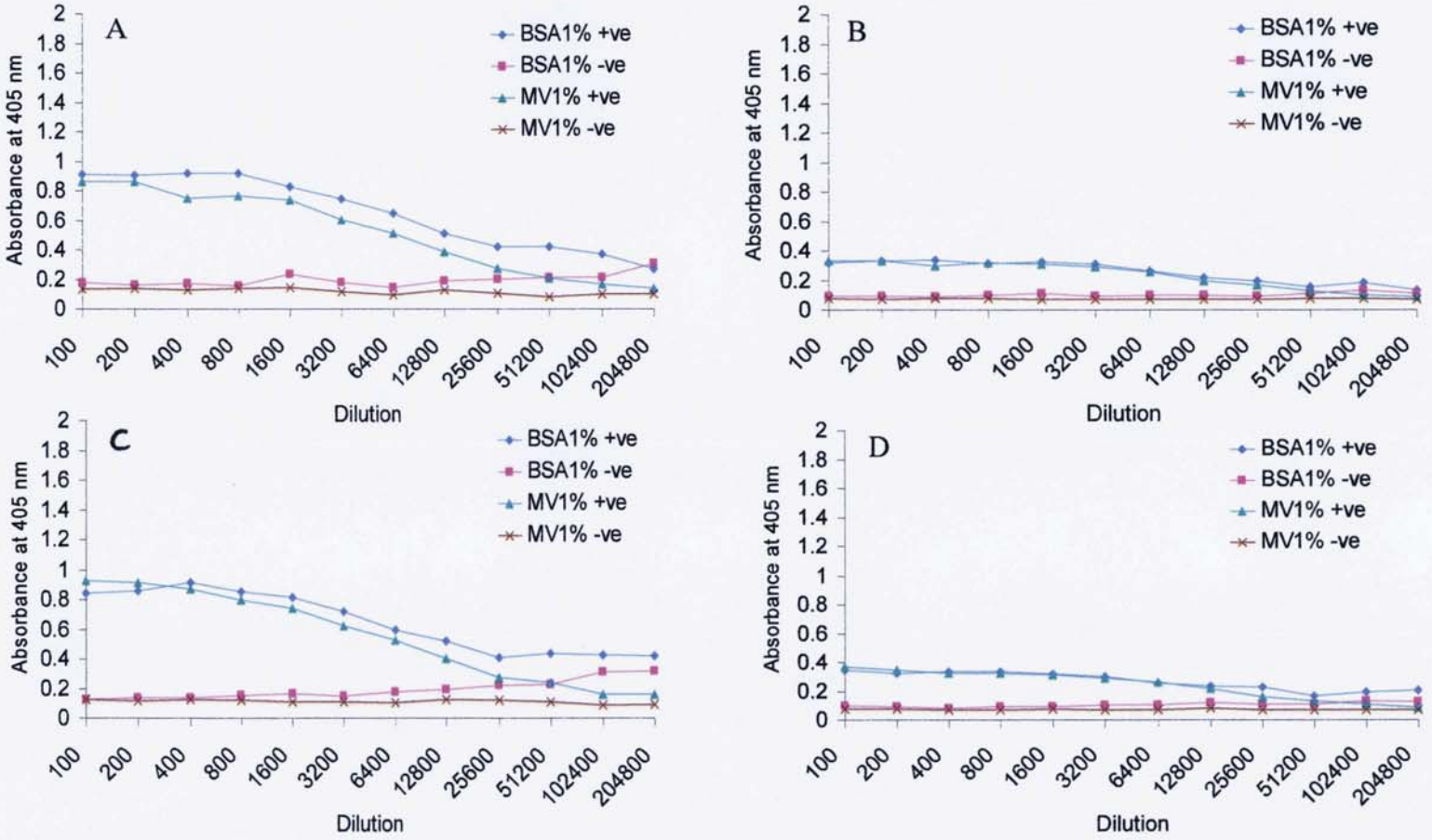


Fig. 4.3 Optimisation of antibody ELISA protocol for a 1 in 5000 conjugate antibody dilution. A series of parameters in the ELISA protocol were tested by measuring the absorbance of serially diluted antibody-containing and antibody-free, positive and negative controls respectively, using two different blocking agents, i.e., bovine serum albumin (BSA) and Marvel (MV) (1% w/v) in order to obtain a better discrimination between the experimental groups and the controls. Conjugate antibody was diluted 1 in 5000 PBS with 3 washes per step and 3 ABTS tablets dissolved in 50 ml (A) or 1 ABTS tablet dissolved in 100 ml citrate buffer (B). 5 washes per step and 3 ABTS tablets dissolved in 50 ml (C) and 1 ABTS tablet dissolved in 100 ml citrate buffer (D). Results represent average of duplicate readings on each plate.

4.3.2.3 Optimisation of substrate concentration and wash procedure for a 1 in 10000 conjugate antibody dilution

Further, when the conjugate antibody dilution was increased to 1 in 10000, with a 3 wash step procedure and a substrate concentration of 3 ABTS tablets/50 ml, the positive controls gave an initial absorbance of ~0.5 (Fig. 4.4a), with Marvel reaching the 0.2 mark (background) at 51200 dilution relatively lower than its BSA counter part. When the substrate concentration was reduced to 1 ABTS tablet/100 ml, the discrimination was once again low between the controls (Fig. 4.4b) with the reduced secondary antibody concentration compounding the low absorbance observed at the chosen incubation time. When the wash step was increased to 5, and the substrate concentration was 3 ABTS tablets/50 ml, no pronounced difference was observed (Fig. 4.4c) between the absorbance of both positive and negative controls compared to those in Fig. 4.4a. Decreasing the substrate concentration to 1 ABTS tablet/100 ml showed a poor discrimination between the positive and negative controls (Fig. 4.4d). Hence, taken together, from this data, it is clearly indicative that the negative controls using 1%w/v Marvel were yielding a minimum and a uniform background absorbance in all the cases and the protocol consisting a set of parameters including 1 in 5000 dilution of the conjugate antibody, 5 washes per step where indicated and 3 ABTS/50 ml, as seen in Fig. 4.3c was chosen for the analysis of antibody production in experimental serum samples using the ELISA method.

4.3.3 Immunisation studies

4.3.3.1 Induction of antibody response

Previous work using pRc/CMV-HBS encoding the Hepatitis B surface antigen (ayw subtype entrapped in liposomes (Gregoriadis *et al.*, 1998) and pI.18sFi/NP encoding the

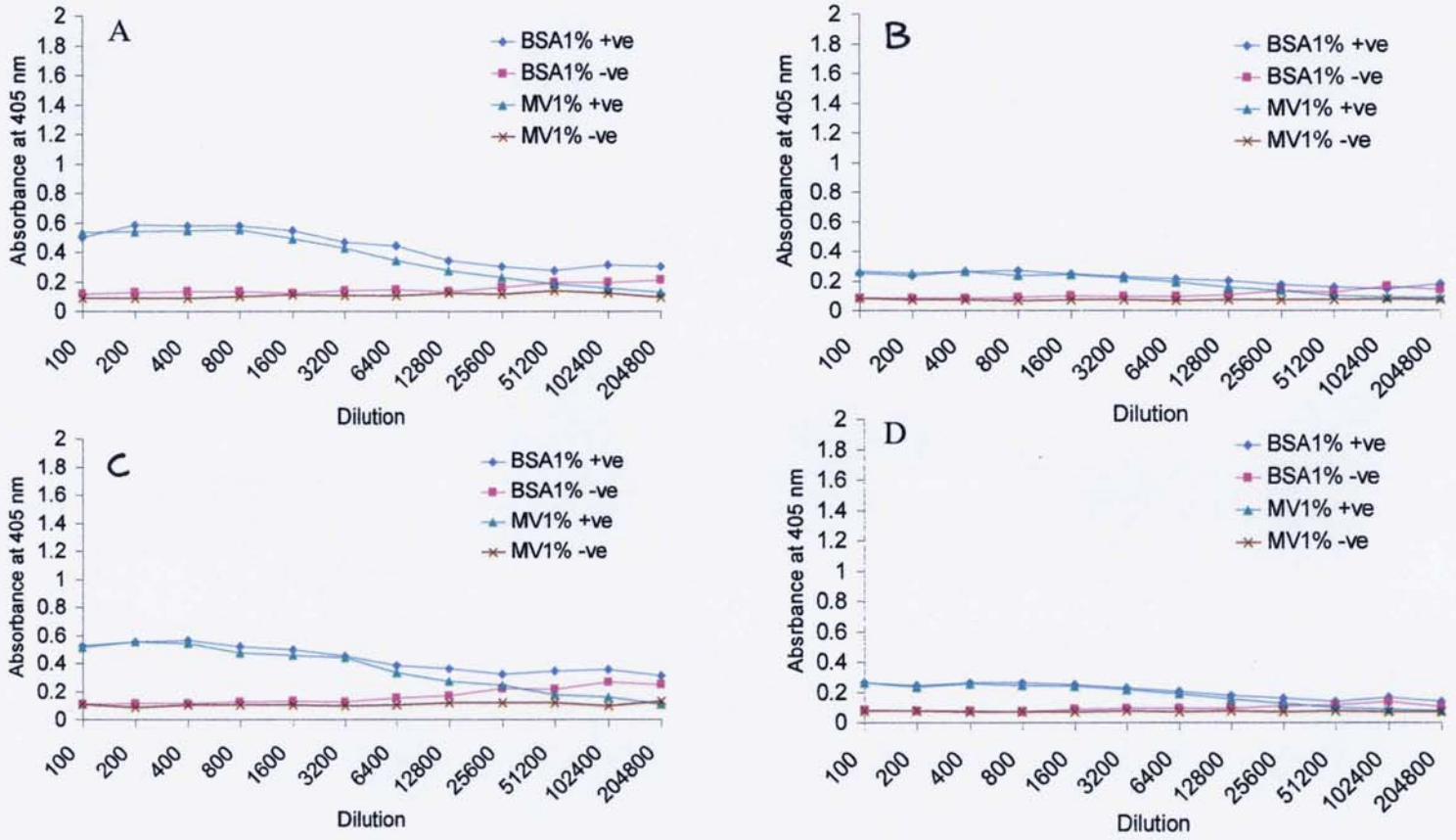


Fig. 4.4 Optimisation of antibody ELISA protocol for a 1 in 10000 conjugate antibody dilution. A series of parameters in the ELISA protocol were tested by measuring the absorbance of serially diluted antibody-containing and antibody-free, positive and negative controls respectively, using two different blocking agents, i.e., bovine serum albumin (BSA) and Marvel (MV) (1% w/v) in order to obtain a better discrimination between the experimental groups and the controls. Conjugate antibody was diluted 1 in 10000 PBS with 3 washes per step and 3 ABTS tablets dissolved in 50 ml (A) or 1 ABTS tablet dissolved in 100 ml citrate buffer (B). 5 washes per step and 3 ABTS tablets dissolved in 50 ml (C) and 1 ABTS tablet dissolved in 100 ml citrate buffer (D). Results represent average of duplicate readings on each plate.

nucleoprotein entrapped in liposomes and niosomes (Perrie *et al.*, 2003, 2004) indicated greater antibody responses against the encoded antigen compared to naked DNA when given via the subcutaneous route. This prompted the present work to further investigate the effect of surfactant vesicle composition, preparation method and characteristics on their immuno-potentiating efficiency when administered via subcutaneous route employing pRc/CMV-HBS plasmid. The plasmid dose was set at 20 μg to enable the detection of surfactant vesicle-mediated enhanced immune responses to the encoded antigen and to allow for a noticeable discrimination between the experimental groups and the naked DNA. Formulations such as DOPE:DC-Chol (1.17:1.17 μmoles) (Table 4.1; formulation 5) that were found to transfect the cells efficiently *in vitro,* as discussed in chapter 3, were tested here for immunisation. The niosome system composed of MP:DOPE:Chol:DC-Chol, 16:8:4:4 μmoles, were compared with similarly formulated vesicles where MP component of the niosome system was replaced by PC (Table 4.1; formulation 4). This niosome formulation was chosen as it showed promise in previous immunisation studies via intramuscular route (Obrenovic *et al.*, 1998; Obrenovic and Gregoriadis, 1999).

Overall this experiment was designed to investigate the effect of:

(i) liposome preparation with the comparison of (DRV(DNA) vs DRV-DNA, (DRV(sucrose+DNA) and SUV

(ii) the choice of non-ionic surfactant (MP vs DP formulations)

(iii) niosome vs liposome formulations (MP vs PC systems)

Unfortunately despite immunising the mice 4 times (days 0, 14, 35, and 74) with 20 μg of pRc/CMV-HBS, either 'naked' or formulated as described in Table 4.1 consistently low antibody responses (IgG, IgG1 and IgG2a subclasses) from all immunisation

groups was noted (Fig 4.5). Antibody responses on days 22 and 42 were similarly low (results not shown). Therefore to further study the immunological status of the mice, all groups were subjected to a mock challenge on day 127 with 1 μg of recombinant hepatitis B antigen (HBsAg) and further bled and serum samples collected on days 136 and 146 after the first injection.

A booster immunisation with recombinant hepatitis B antigen (HBsAg) (1 μg; mock challenge) was undertaken on account of previously reported strategy of combinatorial regimen involving a booster with recombinant protein following DNA immunisation that have dramatically improved the DNA-based vaccines (Meseda *et al.*, 2002; Song *et al.*, 2000; Xiao-wen *et al.*, 2005). Indeed, an increase in the IgG and IgG1 titres was apparent on day 136, though the responses from mice immunised with vesicle formulations incorporating DNA were observed to have no significant difference within the formulations or with that of free (naked) DNA at this time point, however, serum samples collected on day 146 induced a discrimination between the experimental groups and the group immunised with free DNA in both IgG and IgG1 titres (Fig. 4.5a,b,c,d) where a sharp decrease in the titres of free DNA group was noticed.

In the case of IgG2a subclass, among the experimental groups, MP based DRV(DNA) system both in the presence and absence of sucrose (Fig. 4.5e) and also the MP based SUV formulation (Fig. 4.5f) showed an increased response on day 136 and by day 146, the titre values for all formulated DNA systems along with the free DNA group showed an increased response after an initial lag time (Fig. 4.5e,f) compared to naïve group which showed no response.

The *de novo* synthesis of protein (antigen) by DNA vaccine require transfection and translation steps accounting for the time delay for the initiation of humoural response but DNA once taken up by the cells synthesizes the required antigen for longer duration and leads to production of better humoural response as compared to pure antigen (Davis, 1996). This phenomenon was however, observed only in case of IgG2a subclass (Fig. 4.5e,f). However, previous reports suggest that encapsulation of DNA within surfactant vesicles resulted in better immunological response in comparison to free DNA (Perrie *et al.*, 2004). Free DNA was reported to be susceptible to hydrolysis by deoxyribonuclease enzymes present in interstitial spaces (Gregoriadis *et al.*, 1997). The vesicles in addition to their inherent ability to be better taken up by the APCs also protect DNA from degradation by deoxyribonuclease attack. Moreover, vesicles could also act as rate-limiting membrane barrier and serve as a local depot for the sustained release of encapsulated plasmid (Schreier and Bouwstra, 1994). These might be the possible reason for a sustained IgG and IgG1 titre values in most of the experimental groups incorporating DNA in surfactant vesicles (Fig. 4.5a,b,c,d). Large standard deviations have resulted due to the presence of non-responders within the groups making it difficult assess the significance of an individual response between the groups.

Considering the vesicle characteristics, vesicle size has been shown to be inversely related to their clearance from the site of injection (Segal *et al.*, 1975), presumably because of the slower rate with which large vesicles pass through anatomical barriers (e.g., those of the lymphatic capillaries (Segal *et al.*, 1975)) though the phenomenon in the systemic circulation is reported to be vice versa (Allen *et al.*, 1995) where the larger vesicles are cleared much faster by the natural vectoring process. As discussed in section 4.3.1.3, MP based DRV systems used here were significantly ($P<0.05$) larger

than the PC counterparts and almost 4 fold larger then DOPE based SUV complexes (814 nm vs 587 nm and 814 nm vs 259 nm respectively; Table 4.1) yet, comparison of the IgG immune responses obtained revealed no significant differences between the formulations tested. However, the basis for the slight reduction (whilst not significant) of IgG1 responses after immunisation with the PC and DOPE based systems on day 146 after first injection (Fig. 4.5c,d) and also the slight delay in the enhancement of IgG2a responses in these systems between day 136 and 146 (Fig. 4.5e,f) was unclear at present.

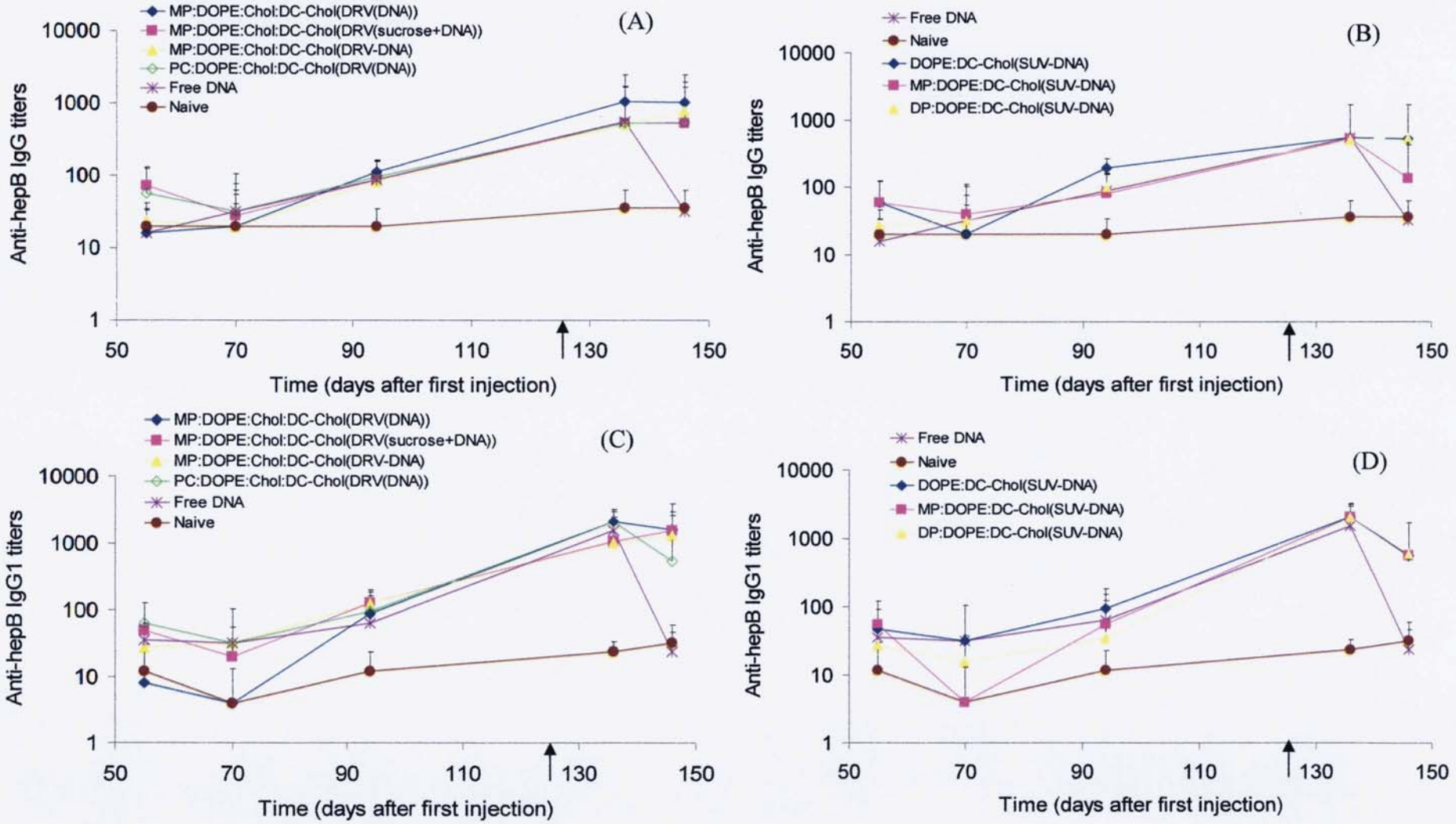
The mechanism by which cationic surfactant vesicles promote enhanced immune responses to the encoded antigen compared to naked plasmid is that cationic vesicles are endocytosed by APCs, possibly including dendritic cells, in the lymphatics where vesicles eventually end up (Velinova *et al.*, 1996). This is strongly supported by experiments in which mice were injected subcutaneously or intramuscularly with liposomes entrapping the plasmid (pCMV4.EFGP) encoding the enhanced fluorescent green protein or with the naked plasmid. Sections of the lymph nodes draining the injected site revealed much more green fluorescence when the plasmid was administered in the entrapped form (Perrie and Gregoriadis, 2001). It appears that the key ingredient of the DNA vesicular formulations in enhancing immune responses is the cationic lipid, DC-Chol. It was thought that plasmid DNA, encapsulated or complexed to preformed cationic liposomes, after being endocytosed, escapes the endocytic vacuoles via the flip-flop mechanism prior to their fusion with lysosomes (by a mechanism similar to that proposed (Szoka *et al.*, 1996, Zelphati and Szoka, 1998) for vesicle-DNA complexes as discussed in chapter 3) to enter the cytosol for eventual episomal transfection and presentation of the encoded antigen. For transfection to occur the plasmid DNA must also escape degradation within the lysosomes. This relies

largely on the combination of a cationic lipid component and the fusogenic lipid DOPE which after endocytosis, is responsible for destabilising the endosomal membrane whereupon, through lateral diffusion of anionic lipids from the cytoplasm-facing endosomal monolayer, DNA is displaced from the complex and released into the cytosol for eventual episomal transfection (Szoka *et al.*, 1996; Zelphati and Szoka, 1998). Indeed, the presence of a cationic lipid (Gregoriadis *et al.*, 1997) and DOPE (Perrie *et al.*, 2001) has been shown to be beneficial in promoting liposome- and niosome-mediated (Obrenovic *et al.*, 1998) DNA immunisation.

The low titres of the groups on day 94 (Fig. 4.5), prior to mock challenge with recombinant hepatitis B antigen do not allow us to conclude about the relative levels of subclass titres, precluding any inference about possible skewing of the immune response mediated by the delivery system as even the free DNA showed a similar trend (Fig. 4.5). However, as already mentioned, previous studies with DNA vaccination against hepatitis B surface antigen have shown effective induction of hepatitis B specific antibody as well as cytotoxic T lymphocytes (CTL) response in mice following various routes of administration including subcutaneous route.

4.3.3.2 Cell proliferation study

Primary cultures of mixed spleen cells contain antigen-presenting cells (APCs) in addition to T and B-lymphocytes. The cell proliferation assay measures the proliferation of T and B cells following their re-exposure to antigen *in vitro*. Immune cells that have expanded in response to specific immunisation *in vivo* recognise the antigen on re-exposure and clonally expand in the spleen cell culture. The magnitude of their proliferation is directly proportional to the ability of the immune system to respond to


(A)
MP:DOPE:Chol:DC-Chol(DRV(DNA))
MP:DOPE:Chol:DC-Chol(DRV(sucrose+DNA))
MP:DOPE:Chol:DC-Chol(DRV-DNA)
PC:DOPE:Chol:DC-Chol(DRV(DNA))
Free DNA
Naive
Anti-hepB IgG titers
10000
1000
100
10
1
50
70
90
110
130
150
Time (days after first injection)
(B)
Free DNA
Naive
DOPE:DC-Chol(SUV-DNA)
MP:DOPE:DC-Chol(SUV-DNA)
DP:DOPE:DC-Chol(SUV-DNA)
Anti-hepB IgG titers
Time (days after first injection)
(C)
MP:DOPE:Chol:DC-Chol(DRV(DNA))
MP:DOPE:Chol:DC-Chol(DRV(sucrose+DNA))
MP:DOPE:Chol:DC-Chol(DRV-DNA)
PC:DOPE:Chol:DC-Chol(DRV(DNA))
Free DNA
Naive
Anti-hepB IgG1 titers
Time (days after first injection)
(D)
Free DNA
Naive
DOPE:DC-Chol(SUV-DNA)
MP:DOPE:DC-Chol(SUV-DNA)
DP:DOPE:DC-Chol(SUV-DNA)
Anti-hepB IgG1 titers
Time (days after first injection)

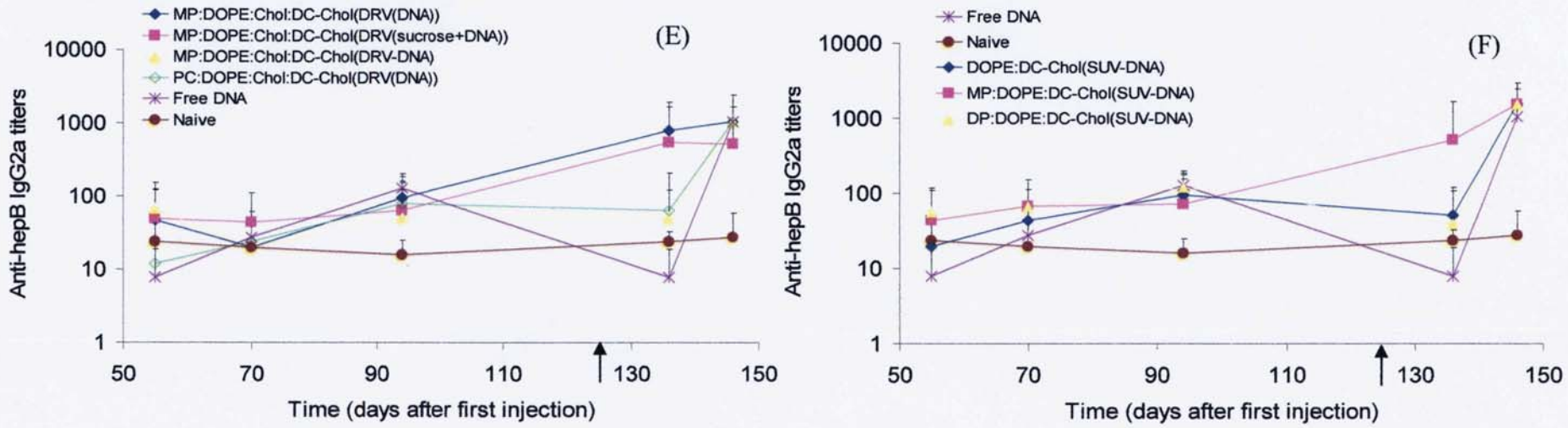


Fig. 4.5 Antibody production in response to plasmid DNA (pRc/CMV-HBS) immunisation. BALB/c mice were injected subcutaneously with free DNA or surfactant vesicle entrapped (DRV(DNA))/ complexed (DRV or SUV-DNA) described in Table 4.1 with a plasmid dose of 20 μg/200 μl on days 0, 14, 35, 74 and mock challenged with 1 μg of recombinant hepatitis B antigen on day 127 (marked with an arrow). Mice were bled on days 22, 42, 55, 70, 94, 136, 146 after the first injection, and sera tested by ELISA for IgG (A,B), IgG_1 (C,D) and IgG_{2a} (E,F). Incomplete freund's adjuvant which served as a positive control, consistent with a titre value of 2560. Results represent means ± S.D. (n = 5) of reciprocal end-point serial twofold serum dilutions.

the immunising antigen and therefore to the effectiveness of the immunisation protocol or vaccine formulation. Additionally, the concomitant measurement of antigen specific cytokine production gives insight into the type of immune response engendered and the cells responsible for proliferation. These methods are used widely throughout the literature in order to evaluate specific immune responses (Bramwell *et al.*, 2002; Westwood *et al.*, 2006). Splenocytes from the immunised mice were isolated according to the protocol outlined in section 4.2.2.4 and their proliferation following restimulation with recombinant hepatitis B antigen (HBsAg) was studied. Optimisation of the concentration of recombinant hepatitis B surface antigen was carried out based on the previous observations where it was noted that, stimulation of cells with concentrations of 20μg/ml of HBsAg showed a decrease in the observed proliferative responses (Bramwell, 2002). Although, this was reported to be in contrast to some other antigens, such as tetanus and diphtheria toxoids, which can be used at these concentrations without adverse effects (Bramwell, 2002), and are therefore more amenable to assays such as antigen specific spleen cell proliferation, giving greater discrimination between experimental groups.

Results in Fig 4.6 show that, whilst no significant differences were noted between stimulated and non-stimulated groups or between the various vaccine formulations there was a clear trend of increased splenocyte cell proliferation activity in cells harvested from mice which were immunised with SUV-DNA complexes composing DP:DOPE:DC-Chol, when the splenocytes were stimulated with HBsAg (5μg/ml) compared to the other surfactant vesicle formulations. This level of activity was comparable to those measured in splenocytes harvested from mice which received 'naked' pRc/CMV-HBS where the proliferation levels compared with the naïve group were observed to be significantly ($P<0.05$) different. Due to the presence of large intra-

group variation in the present study, as highlighted by the large standard deviations, achieving a statistical significance among the groups was proved to be difficult, however the data does suggest that the formulating plasmid DNA within surfactant vesicles may has a notable influence on cell proliferation activity and this method of investigating immunological efficacy of vaccine formulations should be further optimised.

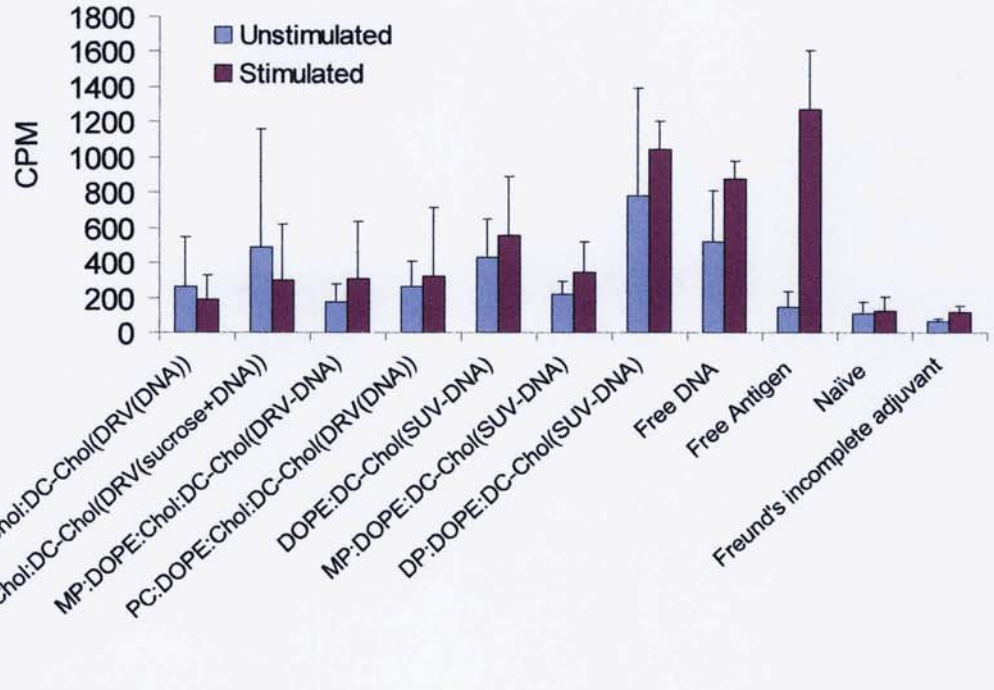


Fig. 4.6 Splenocyte cell proliferation following stimulation with recombinant hepatitis B surface antigen. CPM = counts per minute. Activity of the experimental positive control, concanavalin-A ranged from 10000 to 145000 CPM. Results represent cumulative mean of replicates ± SD, n = 5.

4.3.3.3 Cytokine quantification

As a measure of the cellular immune response elicited by different experimental groups, the production of the cytokines (such as IFN-γ, responsible for Th1 dependent, CMI immunity and IL-6 for Th2 dependent humoural immunity) released from splenocytes from immunised mice were assessed (Fig. 4.7a and b) after 24 hours post-stimulation with recombinant HBsAg.

4.3.3.3.1 Th1 responses

The level of IFN-γ secreted from spleen cells of mice immunised with recombinant HBsAg alone was moderate (Fig. 4.7a) while a similar trend in eliciting IL-6 production (Fig. 4.7b) was observed. Measurements of IgG_{2a} subclass and splenic IFN-γ are generally used to indicate a cell-mediated component of (Th1) immune responses. Immunisation of mice with MP based (DRV(DNA)) was shown to induce relatively higher levels of IFN-γ (with 4 out of 5 mice responding compared to control group (naïve)) (Fig. 4.7a) whilst immunisation with the smaller MP based (DRV(sucrose,DNA)), resulted in 2 out of 5 mice responding, the MP-based DRV-DNA, PC based (DRV(DNA)) and DOPE:DC-Chol based SUV-DNA preparation both in the presence of MP or DP along with the free DNA group, all resulted in inducing a weak response for IFN-γ production with only 1 out of 5 mice responding.

4.3.3.3.2 Th2 responses

In contrast to the Th1 responses for the MP DRV(DNA), induction of IL-6 was only moderate with 2 out of 5 mice which received this formulation responding compared to the naïve mice (Fig 4.7b). The group with MP based SUV-DNA also yielded a moderate response (with 2 out of 5 mice responding; Fig. 4.7b). Overall, with the exception of the groups receiving MP based DRV-DNA and DOPE:DC-Chol where no response was apparent, at least one mouse from each group responded compared to the naïve group, although no significant difference in responses was seen in relation vesicle formulated groups compared to the group which received free DNA.

As discussed in section 4.3.3.1 for antibody responses for formulations, when mice were boosted with recombinant HBsAg, the main type of immune response generated is the antibody response, since the exogenous antigen is mainly presented by B cells

through the MHC class II pathway to Th2 cells. In the case of DNA immunisation, part of the HBsAg generated *in vivo* is secreted and presented by B cells through the MHC class II pathway leading to an antibody response, while part of the antigen is cleaved within the antigen presenting cells and presented through MHC class I pathway, leading to a Th1/CTL response (Woo *et al.*, 2001). Generation of a dominant Th1 cytokine profile is important to facilitate eradication of hepatitis B virus infection (Geissler *et al.*, 1995; Chisari and Ferrari, 1995; Davis, 1996) and IL-6 has been shown to induce B-cell terminal differentiation (Dunkley *et al.*, 1990) as well as enhancing IgA production both *in vitro* (Beagley *et al.*, 1989) and *in vivo* (Ramsay *et al.*, 1994) and as such increased production of IL-6 may be an advantage for vaccines against pathogens such as hepatitis B virus.

4.4 CONCLUSIONS

Taken together, from the antibody responses, little or no discrimination was noticed between the various preparations incorporating MP or PC or even with a variation in the method of preparation i.e., DRV(DNA) vs DRV-DNA vs SUV-DNA especially in case of IgG and IgG1 subclass production. While MP based DRV(DNA) in the presence or absence of sucrose induced an early IgG2a response compared to the rest of the experimental groups suggesting a Th1 bias. In case of cell proliferation assay discussed in section 4.3.3.2 though a suggested superior activity of DP based SUV-DNA was seen, a clear conclusion on the proliferative responses pertaining to various groups could not be made and further method optimisation was thought to be needed. Analysis of the cytokine repertoire produced by spleen cells revealed that the although the levels of IFN-γ and IL-6 produced by all the groups were weak to moderate, MP based DRV(DNA) showed a relatively high response compared to remaining groups in the study.

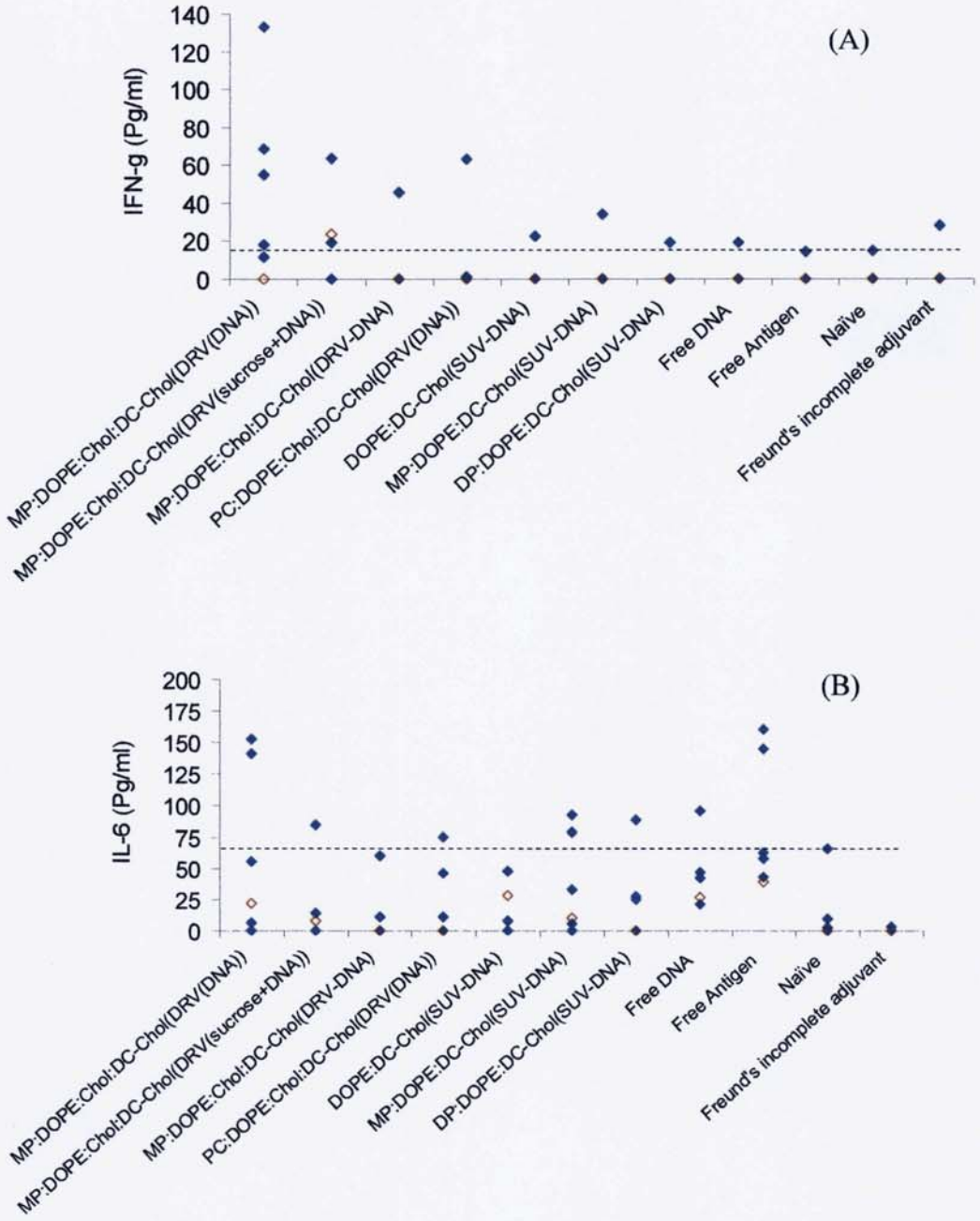


Fig. 4.7 Hepatitis B antigen-specific Th1/Th2 cytokine production in splenocytes. Splenocytes were obtained from BALB/c mice after 146 days of first injection, immunised with naked, surfactant vesicle-entrapped or complexed- DNA (pRc/CMV-HBS) described in Table 4.1, followed by a mock challenge (1 μg) with recombinant hepatitis B antigen. Pooled splenocytes from individual mice (♦) of each group were stimulated with recombinant HBsAg (5 μg/ml) or unstimulated (◊). Supernatants were collected after 24 hours and assayed for the production of interferon-γ (IFN-γ) (A), and interleukin-6 (IL-6) (B). Dashed line on the graphs represent the threshold of responders with respect to naïve mice. Results represent cumulative mean of replicates from individual mice, n = 5.

Over all the trend suggests that MP based DRV(DNA) system in the present studies (antibody and cytokine production) may be better compared to the remaining formulations. Interestingly, this system seems to possess a Th1 bias as seen from early onset of IgG2a subclass and also inducing high IFN-γ production, both indicators of a Th1 response which is important to facilitate eradication of hepatitis B virus infection (Geissler *et al.*, 1995; Chisari and Ferrari, 1995; Davis, 1996).

Previous immunisation studies have shown that cationic vesicles (both liposomes and niosomes) promote much greater antibody and cytotoxic T lymphocyte immune responses against the antigen encoded by the entrapped DNA vaccine (McCormack and Gregoriadis, 1998; Perrie and Gregoriadis, 1998; Obrenovic *et al.*, 1998) compared to naked DNA. DNA immunisation could be a promising approach for designing vaccines in situations where antigens are either ineffective or unavailable. However, naked plasmid DNA vaccines used as such are vulnerable to *in vivo* degradation by nucleases following their administration and do not normally target antigen presenting cells. Such problems can be circumvented by entrapping the DNA within cationic surfactant vesicles. Entrapped plasmid is not accessible to nuclease nor can it be replaced by other competing anionic molecules. However, considering the limitation of using hepatitis B antigen in cellular assays (especially cell proliferation assay) as noted in section 4.3.3.2, in terms of its use in low concentrations, which could limit the sensitivity of the assays, such limitations imposed by using this antigen in *in vitro* assays was overcome and potent immune responses were obtained when hepatitis B antigen was incorporated in various surfactant/lipid based vesicular formulations which are discussed in 5th chapter.

Chapter Five

Hepatitis-B Antigen Based Vaccines: Characterisation and Immunisation Studies

5.1 INTRODUCTION

5.1.1 Subunit Vaccination

Whilst most healthy adults recover completely from hepatitis B infection, a percentage of infected individuals become chronic carriers. The proportion of chronic carriers varies from 3 to 5 % for immuno-competent adults up to 40% and higher for infants, immuno-compromised adults, and allograft recipients. From patients who undergo liver transplantation due to hepatitis B induced liver damage, a significant proportion remain at risk from subsequent liver disease mediated by reinfection of the transplanted liver with endogenous hepatitis B virus. Immunological control of hepatitis B infection is dependent upon T helper cells having the capacity to drive cytotoxic T cell (CTL) clearance of intracellular virus and B cell production of antibodies capable of clearing extracellular hepatitis virus (Lebray *et al.*, 2003). Indeed, a vigorous T cell and CTL response is deemed to be crucial in the elimination of the hepatitis B virus. Vaccines able to generate potent T helper responses and in particular those that can augment T helper type 1 (Th1) responses are thought to hold the key for the generation of therapeutic vaccines against hepatitis B.

More immunogenic vaccines based on the inclusion of adjuvant moieties within an antigen delivery system represent a rational design approach to the development of more effective vaccines (Bramwell and Perrie, 2005b). Antigen delivery systems such as liposomes and niosomes have been shown to be versatile in their ability for the incorporation of a diverse range of antigens. The possibility for the use of co-adjuvants in these systems further enhances their potential as potent systems for the maximisation of immune responses (Bramwell and Perrie, 2005a). In a recent study, Holten-Andersen *et al.* identified a potent adjuvant delivery system comprising a cationic vesicle carrier made up of dimethyl dioctadecylammonium bromide (DDA), together with the

synthetic mycobacterial cord factor trehalose 6,6'-dibehenate (TDB) (Holten-Andersen *et al.*, 2004). It has been reported that the use of an adjuvant, such as the cationic amine containing [*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), can be fundamental in overcoming non-responsiveness potentially associated with hepatitis B using HBsAg based vaccines (Brunel *et al.*, 1999). Similarly, DDA is a cationic lipid that is able to form a homogenous suspension in water or buffer that has been shown to engender enhanced cellular and antibody mediated immune responses. In a previous study, the addition of monophosphoryl lipid A to this lipophilic quaternary ammonium compound facilitated a further enhanced immune response to the delivery of multiple antigens (for example, Ag85B and ESAT-6 of *Mycobacterium tuberculosis*) (Brandt *et al.*, 2000). Subsequently, the use of TDB has demonstrated significant potential for inclusion as a novel immuno-modulator suitable for enhancing antigen specific IFN-γ production against tuberculosis subunit vaccines ((Holten-Andersen *et al.*, 2004) (IFN-γ production being a strong indicator of protective efficacy in the tuberculosis model). Following these initial studies, DDA-TDB as a delivery system has been extensively studied for the optimisation of adjuvant dose, ratio of components and stabilisation of vaccine formulation characteristics (Davidsen *et al.*, 2005). A comparative study with the Ag85B-ESAT-6 fusion protein and malarial antigens (MSP1 and GLURP) has outlined the potential for alteration of qualitative aspects of the immune response by changing the composition of this system by the inclusion of the non-ionic surfactant, 1-monopalmitoyl glycerol (MP) and cholesterol (discussed in 6th chapter). This study outlined different best candidate formulations for each of the subunit antigens from within the range of adjuvant delivery systems tested. There is an increasing amount of supporting non-clinical data suggesting that variations of the DDA-TDB adjuvant

delivery system may be useful for the assessment of a number of subunit and candidate subunit vaccines in order to generate improved vaccine formulations.

5.1.2 Aims and Objectives

This study was carried out to assess the potential of cationic surfactant vesicle based delivery systems for the generation of immune responses that may be appropriate for the abrogation of non-responsiveness, or additionally, the mediation of therapeutic treatment of chronic hepatitis B infection. Various cationic surfactant based delivery systems were initially characterised in terms of their physico-chemical and morphological aspects and were employed for vaccination in order to alter qualitative aspects and increase the magnitude of immune responses including the antibody production, spleen cell proliferation and secretion of various cytokines against hepatitis B surface antigen (HBsAg) in BALB/c mice.

5.2 MATERIALS AND METHODS

5.2.1 Materials

5.2.1.1 Chemicals and Surfactants/Lipids

All chemicals and surfactants/lipids used in the present study are listed in section 4.2.1.1 and 4.2.1.2 respectively. For radio-labelling of the hepatitis B antigen, Iodo-gen® pre-coated iodination tubes from Pierce Biotechnology (Rockford, IL) were used and ^{125}I (NaI in NaOH solution) was purchased from Amersham Biosciences (Amersham, UK). Sephadex G-75 was purchased from Sigma-Aldrich, Poole, Dorset, UK. Dimethyldioctadecylammonium bromide (DDA), α,α'-trehalose 6,6'-dibehenate (TDB) were purchased from Avanti® Lipids, Alabaster, AL, USA.

5.2.1.2 Antigen

Recombinant hepatitis B surface antigen (HBsAg) (*ayw* subtype) was obtained from Aldevron, Fargo, USA and is produced in *Saccharomyces cerevisae* expressing the plasmid pCGA7 which encodes the S region subtype *ayw*; the *a* epitope generally being responsible for the majority of the observed antibody mediated response.

5.2.2 Methods

5.2.2.1 Preparation of Liposomes

MLV and DRV were prepared as outlined in sections 2.2.2.4.1 and 2.2.2.4.3 respectively.

5.2.2.2 Determination of vesicle size and zeta potential

z-average diameter and zeta potential of vesicles were measured using the methods as outlined in sections 2.2.2.4.5 and 2.2.2.4.6 respectively.

5.2.2.3 *^{125}I radiolabelling of antigen*

Radiolabelling of HBsAg was performed using the Iodo-gen® pre-coated iodination tubes (Pierce Biotechnology, Rockford, IL). Briefly, antigen was diluted with PBS buffer (0.01 M, pH. 7.4) and added to the pre-coated iodination tube. A pre-determined activity of ^{125}I (3.7 MBq) was then added to the iodination tube. This mixture was left for 15 minutes with intermittent shaking. Unlabelled antigen was removed using Sephadex G-75 gel column. In order to make the column, Sephadex G-75 (1%, w/v) was first soaked in double distilled water at 90°C for 1 hour, with stirring. The swollen gel was then packed into a 5 ml column and equilibrated with the 0.01 M PBS. Prior to separation, the reaction mixture from the iodination tube was further diluted with the PBS, and then passed through the column with PBS as mobile phase. Aliquots of the eluted solution (0.5 ml) were collected and measured for gamma radiation using a

Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA) and also for UV absorbance at 280 nm to confirm the presence of radiolabelled antigen. The appropriate aliquots were then pooled and stored at –20°C until required for further use.

5.2.2.4 Transmission Electron Microscopy (TEM)

Morphological analysis of vesicle structures was performed using TEM as outlined in section 3.2.2.10.

5.2.2.5 Immunisation of mice

Groups of five female 6 – 8 week old BALB/c mice (source of mice and ethical approval are outlined in section 4.2.2.2) were immunised subcutaneously into the scruff of the neck (0.2 ml/dose in 10% sucrose, for tonicity) using 1 ml calibrated disposable plastic syringes. Immunisations were performed three times, at two-week intervals and mice were bled from the tail vein at scheduled time intervals (Fig. 5.1) using capillary tubes coated with a 0.1% w/v heparin solution in PBS. The blood sample was then diluted 10-fold in 450 µl of PBS and centrifuged at 13000 rpm for 5 min to remove blood cells. The diluted sera e was transferred into an eppendorf tube and stored at –20°C until analysis.

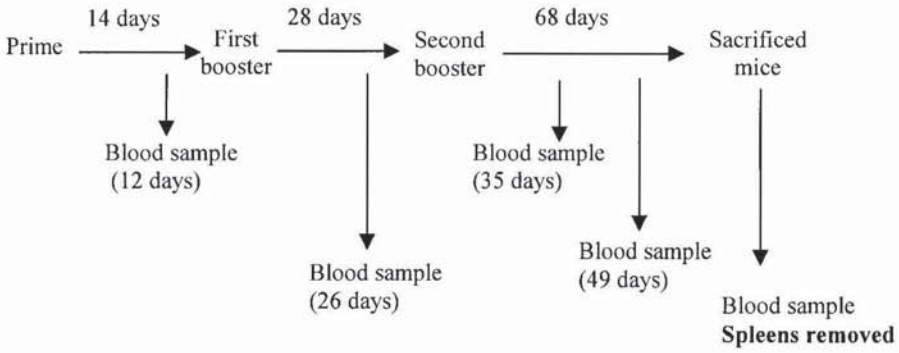


Fig. 5.1 Immunisation regimen and time plan for blood sampling.

The surfactant vesicle based vaccines were prepared by mixing (adsorbing) or encapsulating 1 μg of recombinant hepatitis B antigen (HBsAg) (S region; *ayw* subtype). The Freund's incomplete adjuvant was administered intramuscularly following emulsification of the 3 μg HBsAg, free antigen (1 μg) was administered subcutaneously. Freund's incomplete adjuvant is a mixture of 85% mineral oil and 15% mannide monooleate. The emulsified immunogen (HBsAg) when injected intramuscularly into the host generally gives a high and long lasting antibody response generating positive controls (Eyles *et al.*, 2001) that are used for the validation of ELISA experiments.

5.2.2.6 In vitro Cellular Assays

Antibody ELISA, splenocyte cell proliferation assay and cytokine ELISA for mouse IFN-γ, IL-2, IL-5, IL-6, IL-10 were performed as outlined in sections 4.2.2.3, 4.2.2.4, 4.2.2.5 respectively.

5.3 RESULTS AND DISCUSSION

5.3.1 Characterisation of formulations

DRV systems such as MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles) and its PC based counter part (for comparison) were further carried forward as the former showed promise in the plasmid DNA delivery discussed in chapter 4. This composition was chosen for encapsulation and also for surface complexation of HBsAg antigen. Vesicle system consisting of DDA:TDB (4:0.5 μmoles) was previously reported to provide an adequate immunological response to tuberculosis vaccine (Davidsen *et al*, 2005) as already mentioned in section 5.1.1 and further characterisation of this formulation by supplementing it with additional surfactants such as MP and Cholesterol (Chol) (MP:Chol:DDA:TDB, 16:16:4:0.5 μmoles) was carried out which is also further discussed in chapter 6. Results in table 5.1 show the vesicle size, zeta potential and HBsAg incorporation efficiency of various formulations tested. HBsAg encapsulated MP based system (DRV(Ag)) yielded a vesicle size of approximately a micron and was significantly ($P<0.05$) larger than its PC based counter part. (for example, 1048 ± 109

nm vs 571 ± 97 nm) without a significant variation in the zeta potential (Table 5.1), considering the relatively high HBsAg incorporation efficiency of MP based system compared to PC based system (~86 ± 1% vs 61 ± 4%; Table 5.1), this relatively high antigen loading which presumably could be embedded in the multilamellar vesicle bilayers (as seen in the TEM micrograph; Fig. 5.2a) due to the hydrophobic nature of the antigen, could possibly be one of the contributing factors to the apparent increase in vesicle size in addition to the hydrophilicity possessed by the MP molecule which yields large vesicle sizes (due to increased surface free energy) as discussed in chapter 2. Aggregation of vesicles, seen in TEM micrograph (Fig. 5.2b) could also contribute to increased vesicle size. Surface-complexation of the antigen in MP based system (DRV+Ag) did not alter the vesicle size and also the surface charge however, an approximate 10% decrease in incorporation efficiency was noticed compared to the MP based DRV(Ag) (Table 5.1). The characteristics of DDA:TDB system in terms of vesicle size and surface charge did not show a significant variation when MP and Chol were added to it, although the antigen was surface-complexed in the former as opposed to its encapsulation in the latter (Table 5.1).

Formulations	Vesicle type	Vesicle size (nm)	Zeta potential (mV)	HBsAg Incorporation (% of total)
MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles)	DRV(Ag)	1048.2 ±108.9	44.0 ± 3.0	85.9 ± 1.2
PC:DOPE:Chol:DC-Chol (16:8:4:4 μmoles)	DRV(Ag)	570.5 ± 96.7	51.0 ± 7.4	60.6 ± 4.0
MP:DOPE:Chol:DC-Chol (16:8:4:4 μmoles)	DRV+Ag	958.5 ± 160.9	43.4 ± 1.5	76.0 ± 1.3
MP:Chol:DDA:TDB (16:16:4:0.5 μmoles)	DRV(Ag)	863.0 ± 60.6	41.7 ± 4.2	76.1 ± 4.8
DDA:TDB (4:0.5 μmoles)	MLV+Ag	909.2 ± 67.9	43.0 ± 6.9	57.0 ± 8.0

Table 5.1 Vesicle size, zeta potential and hepatitis B antigen incorporation in vesicle preparations. Antigen was either encapsulated i.e., DRV(DNA) or surface complexed i.e., DRV+Ag. Results represent mean ± S.D., n=3.

However, a significant ($P<0.05$) decrease in the incorporation efficiency was noticed for DDA:TDB system (~57 ± 8%; Table 5.1) compared to its MP based counter part (MP:Chol:DDA:TDB; ~76 ± 5%). This relatively poor antigen adsorption for the DDA:TDB MLV system could be due to the higher isoelectric point of HBsAg in comparison to other proteins (Saraf *et al.*, 2006) (the isoelectric pH of HBsAg is about 5.2). In a 10% v/v sucrose medium bearing a pH of about 6.5, HBsAg is less anionic and as a consequence, the electrostatic interaction with the cationic molecules on the vesicle surface would be less. This would affect the MLV formulation in particular and perhaps less so the DRV adsorbed formulation due to a low molar concentration of DDA:TDB system (i.e., 4:0.5 μmoles; Table 5.1) used in comparison to MP based DRV in turn resulting in a limited HBsAg adsorption and the observed trend of HBsAg incorporation is similar to that expected: MP:DOPE:Chol:DC-Chol based DRV(Ag) > MP:DOPE:Chol:DC-Chol based DRV+Ag > DDA:TDB based MLV+Ag. It is also possible that the increased incorporation of HBsAg corresponds with limited vesicle aggregation and that also forms the basis of the observed differences in vesicle size. TEM micrograph depicting vesicular structure of DDA:TDB system (Fig. 5.2c) also demonstrates a partial aggregation (Fig. 5.2d) which corroborates previous morphological observations (Davidsen *et al.*, 2005) of these systems. Over all, with respect to the surface charge, incorporation of HBsAg appears to be independent of the observed zeta potential, and all vesicle systems were strongly cationic (Table 5.1).

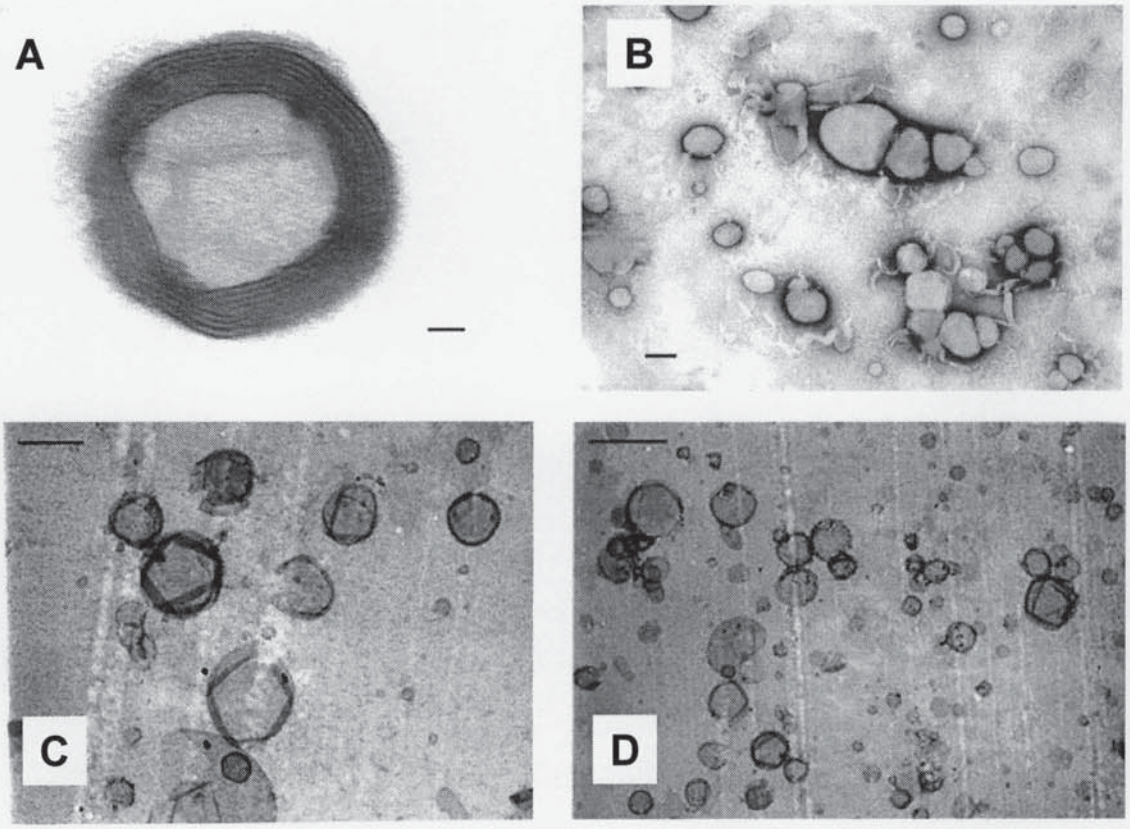


Fig. 5.2 TEM micrographs of MP and DDA:TDB based preparations. (a) A single MP:Chol:DDA:TDB (16:16:4:4 μmoles) DRV vesicle revealing the multilamellar nature of the bilayers, Bar = 50 nm, (b) aggregation of MP based preparations, Bar = 1 μm, (c) DDA:TDB vesicles, bar = 300 nm, (d) DDA:TDB vesicles showing partial aggregation, Bar = 1 μm.

5.3.2 Immunisation studies

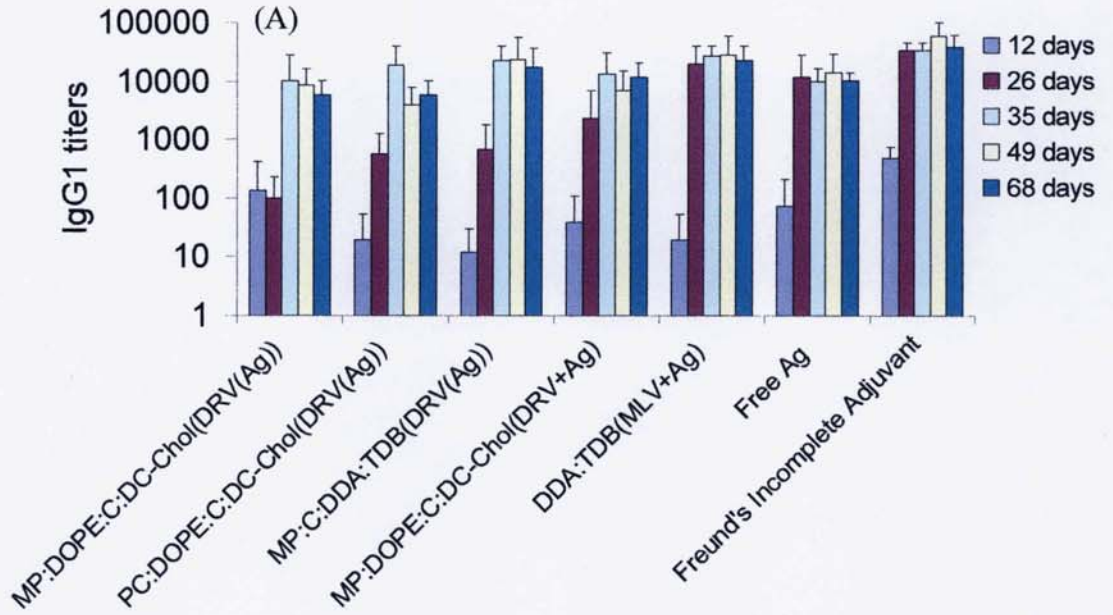
5.3.2.1 Antibody production

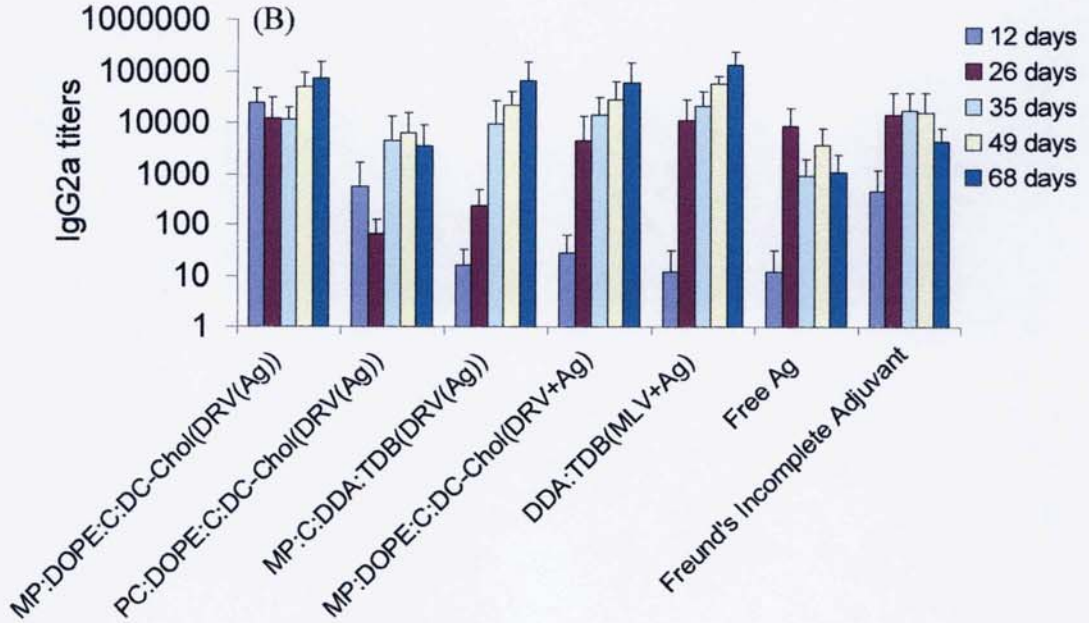
In terms of particulate delivery systems, traditional concepts such as antigen protection, delivery to antigen presenting cells and depot formation remain important aspects, whilst the inclusion of selected co-adjuvants and enhanced delivery of these moieties in conjunction with antigen now has a firm rationale (Bramwell and Perrie, 2006). Inclusion of more diverse antigenic epitopes (such as pre-S_2 and pre-S_1) represents a good strategy for the maximisation of the long term efficacy of hepatitis B vaccines, and this in itself has shown the ability to overcome non-response against the S region (Milich *et al.*, 1986). Indeed, 'third-generation' hepatitis B vaccines produced in

mammalian cells offer increased immunogenicity thought to be due to the ability of these cells to glycosylate the secreted proteins. Inclusion of pre-S_2 and pre-S_1 antigens in these systems has been shown to work well in humans for overcoming a low response to the yeast derived HBsAg (Rendi-Wagner *et al.*, 2006).

Antibody mediated HBsAg specific responses (Fig. 5.3) clearly demonstrate that the free HBsAg and HBsAg/Freund's incomplete adjuvant (IFA) are able to initiate good IgG and IgG1 antibody isotype immune responses. However, though not significant, a trend in the reduction of these responses was observed when compared to the IgG2a antibody isotype immune responses seen for majority of the cationic surfactant vesicle vaccines. These results reveal the bias towards a Th2 response shown by the un-adjuvanted HBsAg and even more convincingly for HBsAg/IFA. In previous studies, IgG2a has shown the best discrimination for the determination of skewing of the immune response towards a Th1 type response (Bramwell *et al.*, 2002). On this basis, all of the MP-containing cationic preparations offer an improved Th1 component. The inclusion of PC in the place of MP appears to limit overall IgG and reduce IgG2a mediated responses, whilst having less of an effect on IgG1 subclass antibody titres (Fig. 5.3). The DDA:TDB MLV were able to elicit high titres of all three antibody isotypes and also show strong HBsAg specific IgG2a mediated immune responses. It was previously reported (Phillips and Emili, 1992) that liposomes enhance the proportion of IgG2a/2b to IgG1 levels. The mechanism of liposome-induced humoural immunity to liposome-associated antigens, although unclear still, is almost certainly related to the liposomal fate *in vivo*, for instance, it is likely that antibody production is stimulated, at least partly, as a result of the system's function as an antigen depot supplying antigen presenting cells (APC) with released and/or entrapped antigen at rates which favour its efficient processing by the cells and eventual presentation.

Involvement of macrophages in liposomal adjuvanticity, a strong possibility in view of T cell participation (Shek, 1984; Beatty *et al.*, 1984) is supported further by experiments showing its absence in animals depleted of their macrophages (Shek, 1984; Su and van Nieuwmegan, 1989).





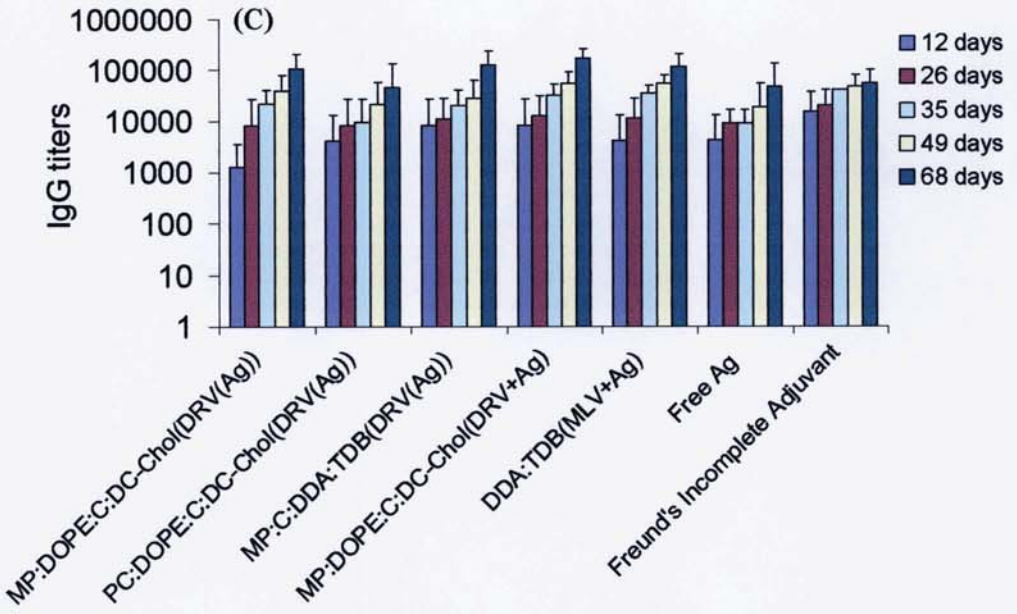


Fig. 5.3 Antibody responses in BALB/c mice to vesicle incorporated hepatitis B antigen (HBsAg). Free or vesicle entrapped (DRV(Ag)) or complexed (DRV+Ag) antigen was immunised subcutaneously and IgG1 (A), IgG2a (B) and IgG (C) subclass production was assayed by ELISA and compared with untreated control mice (naïve) at different time points. Incomplete freund's adjuvant served as a positive control. Results represent means ± S.D. of reciprocal end-point serum dilutions, (n=5).

5.3.2.2 Cell proliferation assay

Measurement of cultured spleen cell proliferation demonstrated that the DDA:TDB MLV elicited markedly increased ability to facilitate clonal expansion in response to re-stimulation with HBsAg (Fig. 5.4). From the other groups, DDA:TDB incorporating MP and Chol in DRV was also useful in effecting HBsAg specific spleen cell proliferation. The background cell proliferation was highest in the DDA-TDB group which suggests a non-HBsAg-specific response mediated by a strong adjuvant effect, or a prolonged HBsAg specific response in the spleen, providing the basis for increased immune responses in this group, either of which may be beneficial.

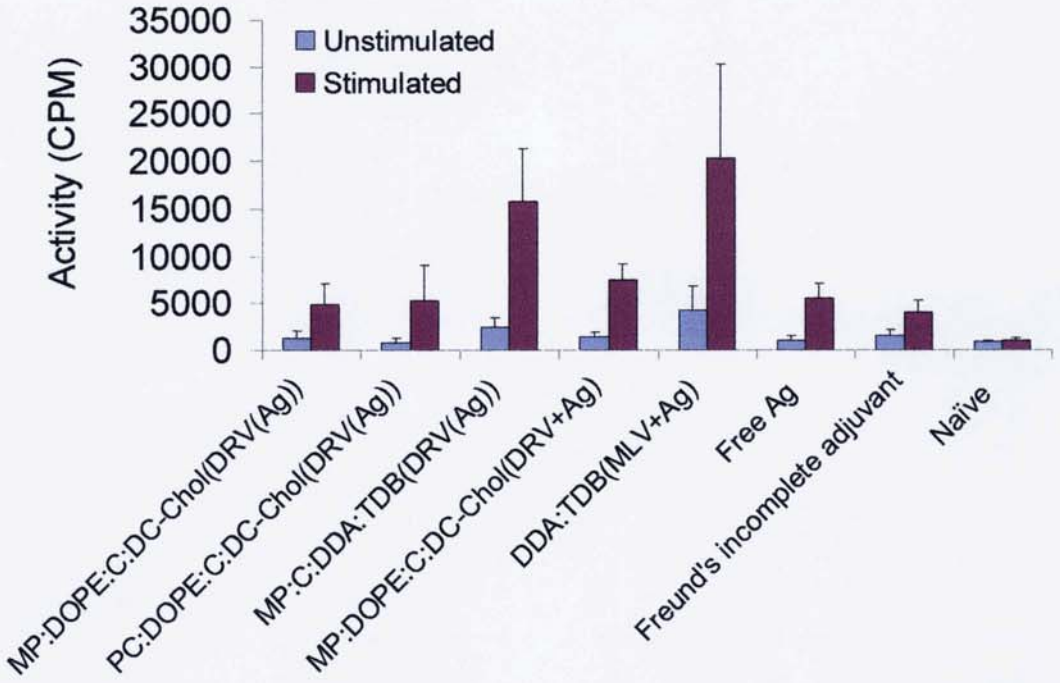


Fig. 5.4 Splenocyte cell proliferation stimulated by HBsAg (5μg/ml). Mice immunised with free or vesicle entrapped (DRV(Ag)) or complexed (DRV+Ag) antigen were sacrificed upon termination of the experiments and assayed for their spleen cell proliferation. CPM = counts per minute of incorporated [^{3}H] labelled thymidine. Activity of the experimental positive control concanavalin-A ranged from 60000 to 145000 CPM. Results represent cumulative mean of replicates ± S.D. (n=5).

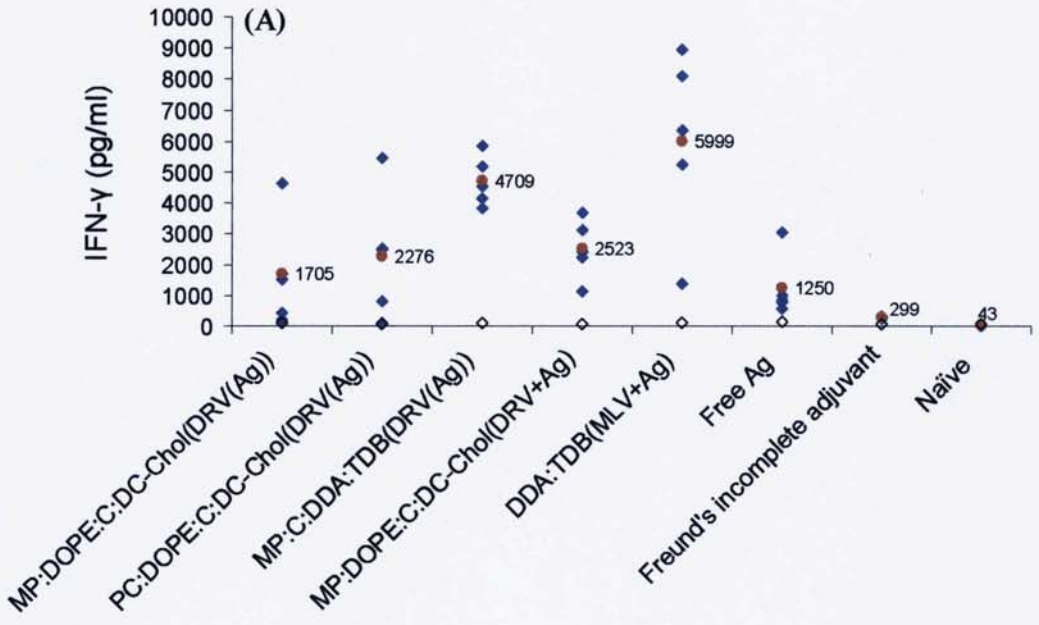
5.3.2.3 Cytokine production

Similar to the trend in cell proliferation data, concomitant cytokine production was also increased for both MP:Chol:DDA:TDB and DDA:TDB based vaccines in terms of antigen specific IFN-γ, IL-2, and IL-6 (Fig. 5.5), and although observed IL-10 levels were low, this trend was again mirrored (Fig. 5.6b). Interestingly, from the cytokines tested, none were increased in the HBsAg/IFA immunised groups. HBsAg-specific IL-5 was marginally increased for the group receiving HBsAg (Fig. 5.6a). The DRV using PC in the place of MP and DC-Chol as the cationic agent showed the best HBsAg-specific IL-5 levels (Fig. 5.6a), offering an indication of the Th2 bias by the use of this formulation. IL-5 is only associated with a Th2 response and B cell functions such as proliferation and differentiation.

HBsAg is a T cell dependent immunogen and the DC-chol associated immune responses, previously observed in mice as having potential to overcome non-responsiveness, were shown to correlate with significantly increased antigen specific IL-2 and IFN-γ production in lymphocytes from draining peripheral lymph nodes (Brunel *et al.*, 1999). IL-2 is important in the proliferation and subsequent differentiation of T cells, B cells, NK cells, lymphokine activated killer cells, monocytes, macrophages and oligodendrocytes. In terms of re-stimulation of spleen cells *in vitro*, IL- 2 is produced by T helper type I cells and therefore more likely associated with a Th1 response when examining cell recall immune responses, as it is not produced by B cells. The higher production of HBsAg specific IL-2 by in particular the DDA:TDB MLV (Fig. 5.5b) is encouraging, especially given the high levels of HBsAg specific antibody production as well as increased IFN-γ and IL-6 (Fig. 5.5a & c). IFN-γ has important Th1 functions, enhancing Th1 cellular responses, such as NK and CTL activity as well as down regulating proliferation of Th2 cells. It is interesting that the MP:Chol:DDA:TDB based DRV(Ag) were able to elicit IL-6 production comparable to the DDA:TDB group (Fig. 5.5c). IL-6 is produced by monocytes, macrophages and Th2 cells and facilitates differentiation of B cells into antibody secreting plasma cells and antibody secretion by plasma cells, but also has a role in the acute phase response. Taken together, this data suggests that potent and comprehensively orientated HBsAg specific immune responses can be generated by the DDA:TDB combination.

As noted in chapter 3, cationic vesicles containing DOPE have been used to facilitate endosomal release of plasmid DNA as a 'helper' lipid attracting much attention as a potential DNA vaccine delivery vehicle (Perrie *et al.*, 2004). However, the use of this

lipid in more conventional protein based vaccines is less common. The presence of the cationic DC-Chol was found in one study to be crucial to the initiation of effective immune responses against influenza antigens (Guy *et al.*, 2001) whilst DOPE was superior to dioleoyl phosphatidylcholine (DOPC) as a co-lipid. From the results here, it is difficult to extrapolate a specific adjuvant role for DOPE. The inclusion of TDB, however, could be seen as a potential factor in the adjuvant effect of the two vaccines containing this moiety. The dose and ratio of TDB has been optimised to some extent in the DDA carrier (Davidsen *et al.*, 2005) and this combination is the basis for the most potent adjuvant activity here. It is also interesting that the inclusion of MP and Chol in this system is able to modulate this reactivity and that the production of some cytokines may even be improved as indicated by HBsAg specific IL-6 production (Fig. 5.5c).



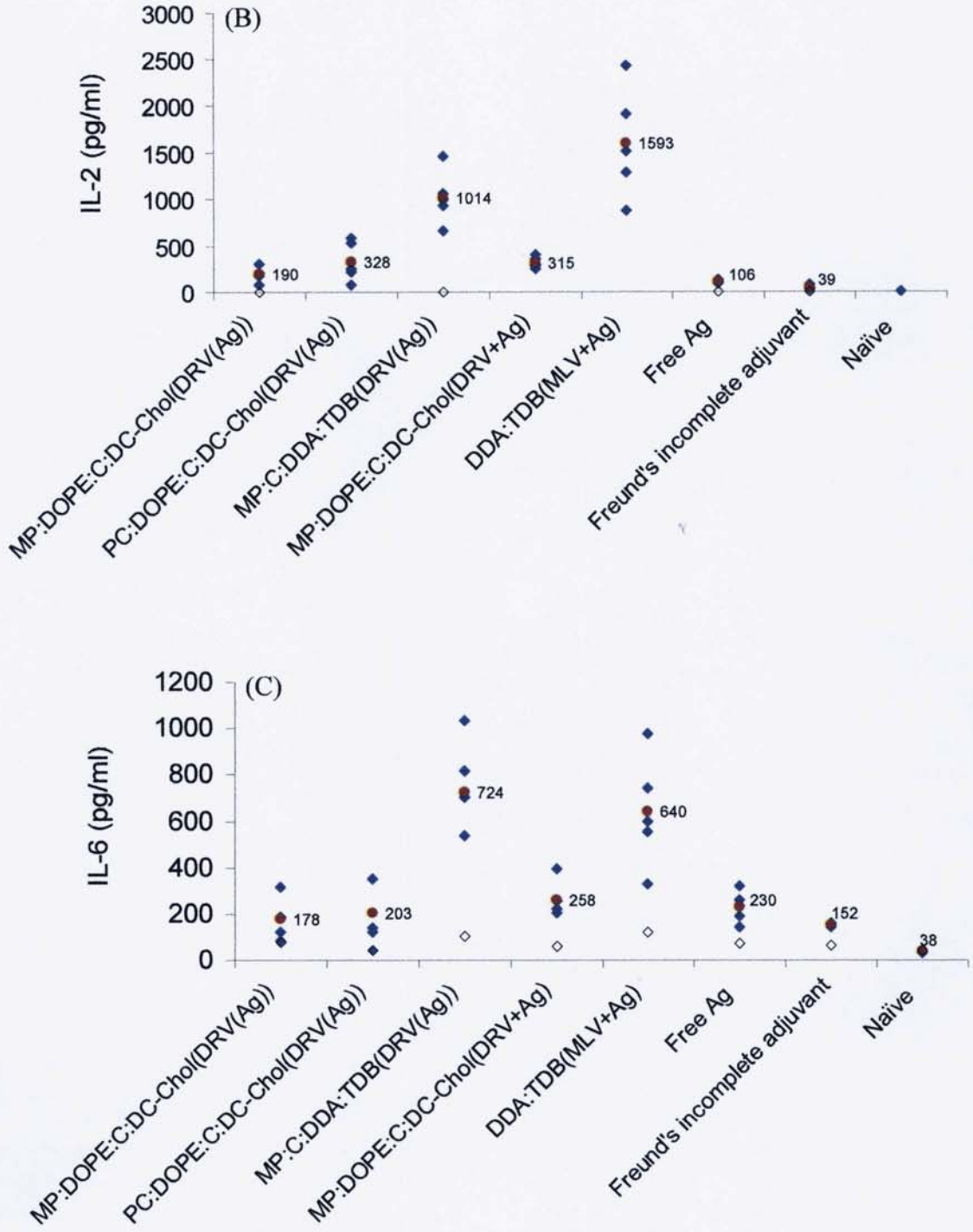


Fig. 5.5 Spleen cell cytokine production in response to restimulation with HBsAg. Mice immunised with free or vesicle entrapped (DRV(Ag)) or complexed (DRV+Ag) antigen were sacrificed upon termination of the experiments and assayed for cytokines, IFN-γ (A), IL-2 (B), IL-6 (C) production by ELISA. Measurements represent the mean of three measurements for each individual spleen (n=5). (●) represent average group value (shown numerically on the graph) of stimulated spleen cells of 5 mice (♦) and (◊) represent unstimulated spleen cells from the same group.

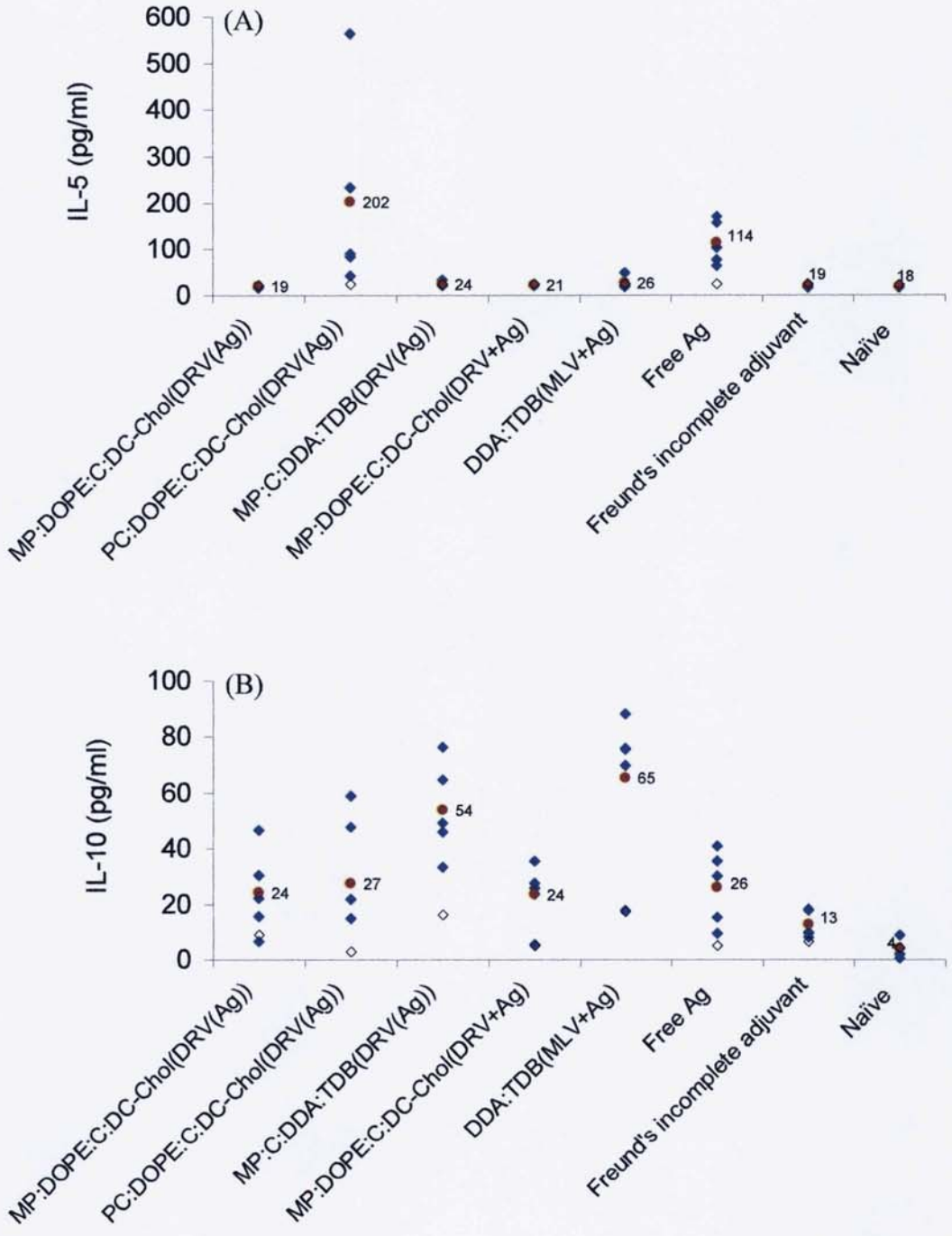


Fig. 5.6 Spleen cell cytokine production in response to restimulation with HBsAg. Mice immunised with free or vesicle entrapped (DRV(Ag)) or complexed (DRV+Ag) antigen were sacrificed upon termination of the experiments and assayed for cytokines, IL-5 (A) and IL-10 (B) production by ELISA. Measurements represent the mean of three measurements for each individual spleen (n=5). (●) represent average group value (shown numerically on the graph) of stimulated spleen cells of 5 mice (♦) and (◊) represent unstimulated spleen cells from the same group.

5.4 CONCLUSIONS

It is likely that, on the basis of present data on vesicle-mediated humoural and CMI discussed and previous reports (Gregoriadis, 1990) on related mechanisms, liposomal adjuvanticity is a reflection of the system's vesicular structure and, probably, lipid nature rather than the result of the system's lipid composition or other characteristics such as vesicle size, lamellarity, surface charge, etc. These characteristics, however, effectively control (Gregoriadis, 1988) liposomal behaviour *in vivo* and are therefore likely to play decisive roles in both qualitative and quantitative expression of immunoadjuvant activity.

The overall HBsAg specific cytokine response in this cited study was deemed to be of a balanced Th1/Th2 profile, and the ability to drive T cell responses could be crucial in initiating a therapeutic as well as a prophylactic outcome. Immune responses in chronic carriers are generally narrowly focussed, weak and monospecific. This is in contrast to strong and multispecific immune responses in patients who control the virus (Chisari and Ferrari, 1995). These observations, the strong IL-2 and IFN-γ production observed in DDA:TDB group, combined with the comparatively high antigen specific antibody titres for the IgG, IgG1 and IgG2a isotypes provide evidence for an integrated and potentially resolute immune response in terms of that required to overcome non-responsiveness or to provide the basis for an effective therapeutic vaccine. The combination of antigens with a delivery system strategy utilising judicious choice of adjuvant or adjuvant combinations for co-delivery is proposed here as providing an effective approach for the generation of effective therapeutic vaccines against hepatitis B. Moreover, these results define a useful platform technology that may be further improved by combination with more immunologically effective antigens. Some of such

aspects in using TB and malarial based subunit antigens delivered via surfactant vesicle systems are discussed in chapter 6.

Chapter Six

Tuberculosis and Malarial Vaccines: Characterisation and Immunisation Studies

6.1 INTRODUCTION

6.1.1 Particulate Adjuvant Systems

As noted in chapter 1, several colloidal lipid- and surfactant-based particulate delivery systems such as liposomes (Aramaki *et al.*, 1994), non-ionic surfactant vesicles (niosomes) (Brewer & Alexander, 1992) as well as polymeric micro- and nano-particles (O'Hagan *et al.*, 1993; Florence *et al.*, 1995) have been exploited in subunit vaccine delivery not only for their inherent adjuvanticity associated with them, that augment immune response to often poorly immunogenic yet otherwise promising vaccine candidates, but also for an array of other reasons such as, increased safety in comparison to live vaccines, protecting the antigen from *in vivo* degradation and thus allowing its administration by different routes, ability to sustain the antigen release over an extended period of time, intracellular delivery of antigen to achieve CMI response, and also targeting the antigen presenting cells (APCs). This way the particulate adjuvant systems play a broader and yet a fundamental role in vaccination, acting as carriers to target immune cells and also in some cases to form short lived depots (Sasaki *et al.*, 2003). Initial studies using mono-palmitoyl glycerol based niosomes as vaccine delivery systems (Brewer & Alexander, 1992) suggested that merely mixing antigen with empty vesicles was not sufficient for adjuvant activity, however more recent studies have demonstrated that mixing antigens such as Ag85B-ESAT-6 with empty pre-formed liposomes composed of the cationic lipid DDA and/or TDB can produce effective immune-responses (Davidsen *et al.*, 2005) suggesting the ability of vesicles to retain high amount of the associated antigen (either via electrostatic adsorption or encapsulation) and providing a greater capacity to target antigen to APCs. In the case of surfactant vesicles, and subsequently their associated antigens, macrophages are known to function as the primary APCs (Su and van Nieuwmegan, 1989) since it is known that

such vesicles are rapidly endocytosed by APCs infiltrating the site of injection or in the draining lymph nodes after local injection (Gregoriadis, 1990). However, the adjuvanticity of these surfactant vesicles also appears to be dependent on structural characteristics such as vesicle size, surface charge, lipid to antigen ratio, lamellarity of bilayers, and rigidity of the bilayer (Gregoriadis, 1990; Gupta *et al.*, 1993).

Studies have shown that addition of non-ionic surfactants to liposomes can greatly enhance the adjuvanticity of these preparations (Zigterman *et al.*, 1987; Zigterman *et al.*, 1988). Similarly, cationic liposomes formulated using the quarternary ammonium surfactant, dimethyldioctadecylammonium (DDA) has also been reported as an effective adjuvant for eliciting both cell-mediated and humoural responses (Lindblad *et al.*, 1997; Holten-Andersen *et al.*, 2004). DDA is a synthetic, self assembling (in aqueous environment), amphiphilic cationic lipid compound comprising a hydrophilic positively charged dimethylammonium head group attached to two hydrophobic 18-carbon alkyl chains. However DDA vesicles, depending on their method of preparation, salt concentration, and inclusion of other lipids, have been shown to have limitation in terms of their stability on long term storage (Carmona-Ribeiro *et al.*, 1985; Feitosa *et al.*, 2000).

6.1.2 Aims and Objectives

Present work is aimed at stabilising the DDA based system by supplementing it with several other non-ionic surfactants such as 1-monopalmitoyl glycerol (MP), cholesterol and trehalose 6,6'-dibehenate (TDB), incorporating different subunit antigens into the system and studying their physico-chemical characteristics, morphological aspects followed by assessing the adjuvant activity of these systems in tuberculosis and malarial subunit vaccination carried out in mice.

6.2 MATERIALS AND METHODS

6.2.1 Materials

6.2.1.1 Chemicals

Tris base (ultra pure) was purchased from ICN Biomedicals, Inc. Ohio, USA. Dextrose and Sephadex G-75 were obtained from Sigma-Aldrich, Poole, Dorset, UK. Alexa Fluor 350 dye kit for labelling Ag85B-ESAT-6 antigen was obtained from Molecular Probes™ Ltd. For radio-labelling of the antigens, Iodo-gen® pre-coated iodination tubes from Pierce Biotechnology (Rockford, IL) were used and ^{125}I (NaI in NaOH solution) was purchased from Amersham Biosciences (Amersham, UK). Splenocyte cultures were conducted using round-bottomed 96-well microtiter plates obtained from Nunc (Roskilde, Denmark). RPMI 1640 medium was obtained from Gibco (Invitrogen, Carlsbad, CA), 2-mercaptoethanol from Sigma-Aldrich, glutamine (Gibco), pyrovate (Gibco), penicillin-streptomycin (Gibco), Hepes (Gibco), nonessential amino acids (MP Biochemicals, Ohio, US), and 10% foetal calf serum from Biochrom AG (Berlin, Germany). ConA was obtained from Sigma-Aldrich. Plates for enzyme linked immunosorbent assay (ELISA) were from Nunc and HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 and IgG2b), was obtained from Zymed (San Francisco, CA). TMB substrate was manufactured by Kem-En-Tec (Copenhagen, Denmark). Reagents for the IFN-γ ELISA (purified rat anti-mouse and biotin-labelled rat anti-mouse) were obtained from BD Pharmingen whereas the HRP-conjugated streptavidin was from Zymed. All other reagents were of analytical grade.

6.2.1.2 Lipids and surfactants

The source of dimethyldioctadecylammonium bromide (DDA) and α,α'-trehalose 6,6'-dibehenate (TDB) is outlined in section 5.2.1.1. 1-monopalmitoyl glycerol (C16:0)

(MP), 1-Monostearyl glycerol (C18:0) (MS), 1,2-Dipalmitoyl glycerol (C16:0) (DP), and Cholesterol (Chol) were purchased from Sigma Chemical Company (UK) Ltd.

6.2.1.3 Antigens

The following antigens were used for immunisation of mice: non-His-tagged fusion protein Ag85B-ESAT-6 was produced in Escherichia coli as described previously for the His-tagged version (Olsen *et al.*, 2001), purified by column chromatography and dissolved in 10 mM Tris-buffer, pH 7.4, at a concentration of 0.5 mg/ml. His-tagged MSP1 from Plasmodium yoelii and GLURP from Plasmodium falciparum were cloned, expressed and purified as previously described (Theisen *et al.*, 1995).

6.2.2 Methods

6.2.2.1 Preparation of surfactant vesicles

6.2.2.1.1 Lipid hydration method and dehydration-rehydration method

MLV and DRV incorporating appropriate amounts of lipids and surfactants were prepared as outlined in sections 2.2.2.4.1 and 2.2.2.4.3 respectively. Antigens i.e., Ag85B-ESAT-6, MSP1 or GLURP were adsorbed or encapsulated in these preparations at a fixed concentration of 0.01, 0.05 or 0.05 mg/ml, respectively. Unadsorbed/unencapsulated antigen was removed by ultracentrifuging twice at 100,000 × *g* (BECKMAN TL-100 with TLN 100 rotor) for 20 min and the pellet resuspended to appropriate volume in 0.01 M sodium phosphate buffer containing 0.15M NaCl, (pH. 7.4; PBS). Antigen adsorption or encapsulation in various vesicle preparations was determined by measuring ^{125}I gamma radiation of the incorporated ^{125}I radio-labelled Ag85B-ESAT-6, MSP1 or GLURP antigens recovered in the resuspended pellets and in the supernatants.

6.2.2.1.2 Aqueous heat method

DDA liposomes were also prepared by aqueous heat method as described previously (Holten-Andersen *et al.*, 2004). Briefly, DDA was dispersed in 10 mM Tris-buffer, pH 7.4 at a concentration of 1.25 mg/ml and heated to 80°C for 20 min with intermittent shaking, and then cooled to room temperature to produce liposomes. Where appropriate, 0.25 mg/ml of TDB (250 µl of a 1 mg/ml solution in 0.2% triethylamine) was included, and the resulting solution was vortexed briefly. The Ag85B-ESAT-6 was added to these preformed liposomes at a concentration of 0.01 mg/ml.

6.2.2.2 Labelling of Ag85B-ESAT-6 using Alexa Fluor 350 dye

Labelling of Ag85B-ESAT-6 was initially performed using Alexa Fluor 350 dye according to manufacturer's protocol. Briefly, 1 mg of antigen (in a volume of ~ 500 µl of buffer) was added to the vial provided, which contain a premeasured quantity of amine-reactive dye and a magnetic stir bar. Reaction was allowed to take place for 20 min. Separation of free dye from the labelled antigen was accomplished on a gravity-feed size-exclusion column, which is supplied with the kit. Aliquot of the eluted flurorescent band was collected and measured at an absorbance and fluorescence emission maxima of 346 nm and 442 nm respectively and simultaneously measured for UV absorbance at 280 nm to confirm for the presence of fluorescent labelled antigen and was stored at -20°C until further use.

6.2.2.3 ^{125}I radiolabelling of antigens

Radiolabelling of the three antigens i.e., Ag85B-ESAT-6, MSP1 and GLURP was performed using Iodo-gen® pre-coated iodination tubes by following the method described in section 5.2.2.3.

6.2.2.4 Antigen release and retention studies

The amount of subunit antigen (Ag85B-ESAT-6) released from the vesicles was determined by incubating antigen adsorbed/encapsulated vesicles in 15 ml 0.01 M Tris buffer or 0.01 M sodium phosphate buffer, pH. 7.4 at 37°C in a shaking water bath. At regular time intervals, 1 ml of release medium was withdrawn and the same amount of fresh buffer was replaced. Withdrawn sample was ultracentrifuged at 100,000 × *g* for 1 hour. Antigen release was then determined on the basis of radioactivity of ^{125}I labelled antigen recovered in the resuspended pellets. At regular time intervals, a similar procedure was adopted to determine antigen retention at 4°C and 25°C.

6.2.2.5 Determination of vesicle size and zeta potential

The vesicle size and zeta potential of surfactant vesicles was determined as outlined in sections 2.2.2.4.5 and 2.2.2.4.6 respectively.

6.2.2.6 Differential Scanning Calorimetry (DSC)

Phase transitional behaviour of the formulations was studied using differential scanning calorimeter (Perkin Elmer Diamond DSC). Briefly, 50 µl of vesicle dispersion was placed on the aluminium DSC pan and sealed. Empty sealed aluminium pan was used as the reference. The difference in the heat flow between both the pans was then measured as a function of temperature. Samples were scanned at a rate of 2.5°C/min from 10 to 60°C.

6.2.2.7 Transmission electron microscopy (TEM) and environmental scanning electron microscopy (ESEM)

TEM and ESEM analysis were carried out as outlined in sections 3.2.2.10 and 3.2.2.11 respectively.

6.2.2.8 Immunisation of mice

Female C57BL/6j mice, 8 to 12 weeks old, were obtained from Harlan Scandinavia (Allerød, Denmark). The DDA based vaccines were prepared by simply mixing preformed DDA liposome dispersions in the presence or absence of TDB, with the antigen of choice to the final Ag85B-ESAT-6, MSP1 and GLURP concentration of 0.01, 0.05 or 0.05 mg/ml, respectively (2, 10 or 10 μg, respectively, in each vaccine dose). The amount of DDA and TDB in each dose was 250 μg (0.4 μmoles) and 0 or 50 μg (0 or 0.05 μmoles), respectively. The MP based vaccines were prepared as described in section 6.2.2.1.1. Mice were immunised subcutaneously with the vaccines (0.2 ml/dose) at the base of the tails three times with a two-week interval between each immunisation.

6.2.2.9 Lymphocyte cultures

Splenocyte cultures from individual spleens of immunised mice (n = 3) were obtained 3 weeks after the last immunisation by passage of spleens through a metal mesh followed by two washing procedures using RPMI. After recovery of the lymphocytes, cell cultures were established as previously described (Andersen *et al*, 1991). Briefly, cultures were performed in triplicate in round-bottomed microtitre wells (Nunc, Denmark) containing 2×10^5 cells in a volume of 200 μl RPMI supplemented with 5×10^{-5} M 2-mercaptoethanol, 1% non-essential amino acids, 1% pyruvate, 1 mM glutamine, 1% penicillin-streptomycin, 1% Hepes and 10 % fetal calf serum. Ag85B-ESAT-6, MSP1 and GLURP for re-stimulation was used in a concentration of 5 μg/ml. Wells containing medium only or 5 μg/ml of ConA were included in all experiments as negative and positive controls, respectively. Culture supernatants were harvested from parallel cultures after 72 hours of incubation in the presence of antigen, and the

amounts of IFN-γ were determined by ELISA. In brief, microtitre plates were incubated with purified rat anti-mouse IFN-γ overnight followed by the blockage of free-binding sites using 1% (w/v) BSA and 0.05% Tween in PBS. The culture supernatants were tested in triplicates and the IFN-γ detected by biotin-labelled rat anti-mouse IFN-γ followed by HRP-conjugated streptavidin.

6.2.2.10 Determination of antibody titres

Antibody titres were determined by ELISA as previously described (Rosenkrands *et al*, 2005). Briefly, plates for enzyme linked immunosorbent assay (ELISA) were coated with Ag85B-ESAT-6, MSP1 or GLURP (0.05 μg /well) in PBS, and individual mouse sera (n=3) were analysed in duplicate in five-fold dilutions. HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 and IgG2b) diluted 1/2000 in PBS containing 1% BSA were added, and Ag85B-ESAT-6, MSP1 and GLURP specific antibodies were detected by TMB substrate as described by the manufacturer. Antibody titres were then defined as the serum dilution which gives an absorbance value of 1.00 in the parallel portion of the curves as previously described (Rosenkrands *et al*, 2005).

6.2.2.11 Calculation of CPP

Work space cache® and Molecular modeling Pro® softwares were used to construct the molecules and calculate operational CPP values of the components respectively, in a vesicle system.

6.3 RESULTS AND DISCUSSION

6.3.1 Optimisation of DDA liposomes - effect of preparation method

The physico-chemical characteristics of DDA and DDA-TDB vesicles prepared by either the lipid hydration method or the aqueous heat method were studied. Results in Table 6.1 demonstrate that liposomes prepared by lipid hydration were significantly ($P<0.05$) smaller than liposomes of the same composition prepared by the aqueous heat method (~400-500 nm vs. ~800-1300 nm, respectively; Table 6.1). Further, while supplementation of DDA liposomes with TDB resulted in no significant difference in vesicle size when prepared by the lipid hydration method, the opposite was apparent when liposomes were prepared by the aqueous heat method, with DDA-TDB vesicles being larger than their DDA counter-parts (Table 6.1), suggesting that potentially the TDB is less effectively incorporated/packaged within the lipid membranes when the latter method was adopted.

	Lipid hydration method		Aqueous heat method	
Formulations	Size (nm)	ZP (mV)	Size (nm)	ZP (mV)
DDA 'empty'	488.2 ± 124.4	46.3 ± 1.6	846.4 ± 130.2	51.7 ± 2.6
DDA + Ag85B-ESAT-6	421.3 ± 25.8	52.1 ± 3.1	928.7 ± 113.2	47.2 ± 2.6
DDA-TDB 'empty'	416.2 ± 40.4	48.2 ± 5.1	1280.6 ± 133.8	46.8 ± 0.6
DDA-TDB + Ag85B-ESAT-6	485.6 ± 109.8	60.4 ± 4.7	1256.3 ± 150.2	44.3 ± 0.3

Table 6.1 Vesicle size and zeta potential (ZP) of DDA and DDA:TDB based liposomes incorporating Ag85B-ESAT-6 prepared by either the lipid hydration method or aqueous heat method in 10mM Tris buffer, pH 7.4. Results denote mean ± S.D., n = 3.

The zeta potential of liposomes can both directly influence liposome suspension stabilities and indirectly reflect vesicle surface net charge- a factor which can be used to evaluate the extent of charged lipid incorporation and head-group interaction. Zeta potential analysis of the DDA and DDA-TDB formulations showed that all liposomes

tested have a high net positive charge (44-60 mV; Table 6.1), clearly resulting from the cationic nature of DDA. Neither the method of preparation nor the inclusion of TDB within the liposome membranes was shown to make any significant difference to the surface charge, suggesting that no marked electrostatic interactions between the DDA and TDB lipids occurred at the surface of the vesicle. In addition, the adsorption of Ag85B-ESAT-6 antigen (0.01 mg/ml) to both DDA and DDA-TDB liposomes made no significant difference to the measured size or zeta potential of vesicles (Table 6.1), suggesting that at these concentrations adsorption of antigen on the vesicle surface made no significant impact on the liposome's physical attributes.

6.3.2 Optimisation of DDA liposomes - effect of medium and method

Unfortunately, DDA liposomes dispersed in small amounts of salt (i.e., in 0.01 M PBS) resulted in instantaneous aggregation (Fig. 6.1a). This physical instability could possibly be due to reduced long range electrostatic repulsion among the positively charged vesicles. Salt-induced aggregation is to some extent reversible as removing the salts restore the liposome dispersion (Carmona-Ribeiro *et al.*, 1985; Tsuruta and Carmona-Ribeiro, 1996). However, even in pure water DDA vesicles aggregate over time, indicating that electrostatic repulsive forces between the cationic liposomes were insufficient to stabilize the vesicles (Hilgers and Weststrate, 1991). Studies on stability of DDA and DDA-TDB vesicles over time prepared by both lipid hydration method and aqueous heat method in two different media i.e., Tris buffer (10 mM) and in dextrose (5% w/v) medium were conducted at 25°C (room temperature) and 4°C. Results (Fig. 6.2a, b and Fig. 6.3a, b) showed that, prolonged storage at 25°C and 4°C was not possible without the visible aggregation and precipitation of DDA liposomes in Tris buffer prepared by both lipid hydration (Fig. 6.2a, b) and aqueous heat methods (Fig.

6.3a, b). The average vesicle size of DDA liposomes increased rapidly from about half a micron to a micron during the first 7 days of storage at 4°C presumably due to aggregation or fusion of liposomes, and after 28 days of storage it was difficult to determine the size of the liposomes due to its increased vesicle size (~4.5 μm; Fig. 6.2a). In contrast, liposomes containing TDB showed significantly ($P<0.05$) less aggregation with a vesicle size of 1.5 μm after 28 days (Fig. 6.2a), indicating that the presence of TDB in the DDA liposome bilayers had stabilizing effect on the liposome vesicle sizes. A similar trend was also apparent in preparations stored at room temperature (25°C; Fig. 6.2b), with the vesicle size of DDA liposomes increasing approximately 4- fold after only 7 days, compared to DDA-TDB which showed no significant increase in size up to 14 days. However, by 28 days both formulations had shown a marked increase in vesicle size (Fig. 6.2 b). Zeta potential analysis of these formulations showed no significant changes in the surface characteristics of liposomes stored at 4°C up to 28 days with values ranging between 40 – 50 mV (Fig. 6.2a), however storage at room temperature resulted in a significant reduction ($P<0.05$) in the zeta potential of both DDA and DDA-TDB preparations on day 14 and 28, respectively (Fig. 6.2b). In case of DDA liposomes prepared by lipid hydration method in dextrose medium, in the presence or absence of TDB, vesicle sizes appeared stable at both the temperatures i.e., 4°C and 25°C with consistent values until day 14 (550-600 nm; Fig. 6.2c, d) while a similar trend in zeta potential was noticed with no significant variation in the mean values which ranged between 50 – 60 mV during this period (Fig. 6.2c, d) however, by day 28 a significant reduction in the zeta potential values was evident compared to day 14 and as a consequence a rise in vesicle size was seen in both the storage conditions (Fig. 6.2c, d). The stability data of these preparations made by aqueous heat method in both Tris buffer and dextrose medium showed a similar trend as

that of the lipid hydration method where an increased vesicle size over a 28 day period was noticed (Fig. 6.3a, b, c, d). Interestingly, however DDA preparations in the presence of TDB showed a relatively higher vesicle size compared to those prepared by lipid hydration method suggesting an inefficient packaging/incorporation of TDB with in the bilayers of the DDA liposomes when prepared by aqueous heat method as noted in section 6.3.1 and eventually resulting in poor vesicle stability. Indeed, in case of vesicles prepared by lipid hydration method, the TEM micrographs revealed that the preparations in the absence of TDB were slightly angular in shape (Fig. 6.1b) while a majority of DDA liposomes in the presence of TDB were observed to be spherical (Fig. 6.1c, d) reinforcing the notion of efficient TDB incorporation by this method.

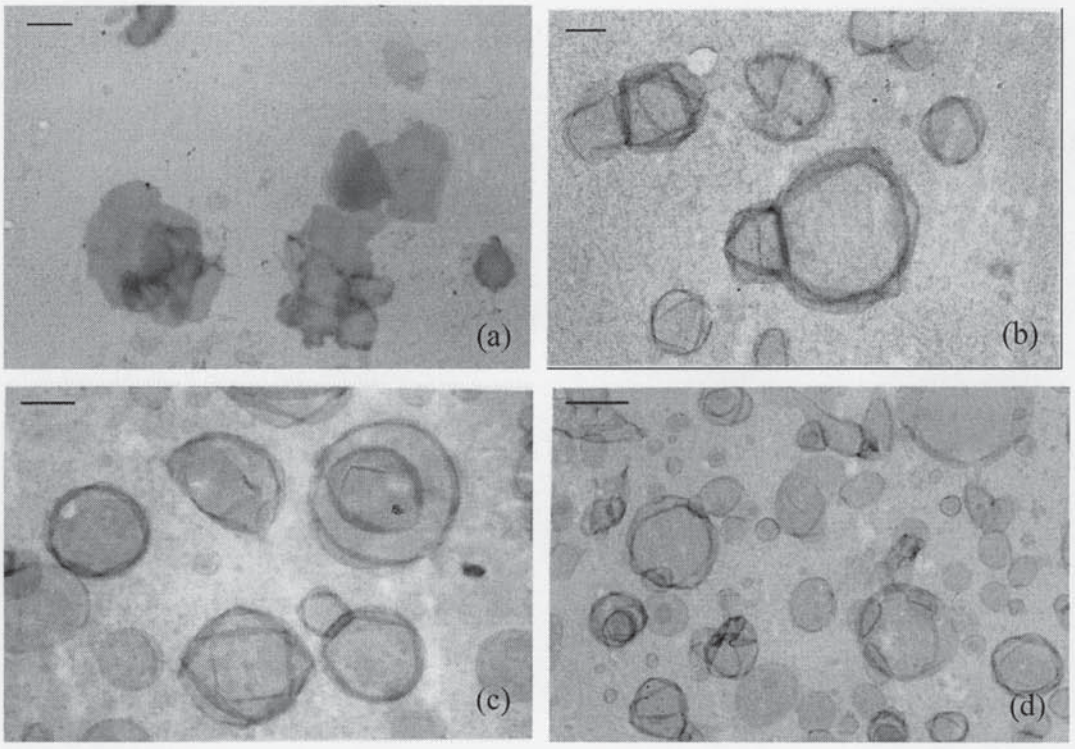


Fig. 6.1 TEM micrographs of DDA based systems prepared by lipid hydration method in PBS or dextrose medium. (a) Precipitation of DDA liposomes occurred in the presence of salts in PBS, bar = 500 nm; (b) DDA liposomes in dextrose medium were stable possessing a slightly angular shape, bar = 300 nm; (c) Incorporation of TDB into DDA liposomes (DDA:TDB) prepared in dextrose medium rendered the vesicles to be more spherical, though a few angular structures are apparent, Bar = 200 nm; (d) Presence of antigen in DDA:TDB liposomes did not effect the vesicle morphology, Bar = 400 nm.

In case of DDA based preparations prepared by aqueous heat method once again a significant ($P<0.05$) reduction in the zeta potential was observed between day 14 and 28 at both the temperatures i.e., at 4°C and 25°C in Tris buffer (Fig. 6.3a, b) while on the other hand, DDA-TDB preparations in dextrose medium showed a relatively lower zeta potential and remained unaltered until day 28. Liposomes prepared with DDA alone in this buffer did not show significant ($P<0.05$) variation in the zeta potential at both the storage temperatures (Fig. 6.3c, d). Taken together the vesicle size and zeta potential measurements, although not observed in all cases, a reduction in the zeta potential was directly correlating with the increase in vesicle size signifying an aggregation process of the vesicles due to less electrostatic repulsive forces acting between them and thereby increasing the vesicle size. However, of all the conditions, DDA-TDB system prepared by lipid-hydration method in Tris buffer stored at 4°C (Fig. 6.2a) was observed to be relatively stable in terms of vesicle size (aggregation).

One of the reasons for the stabilising effect of TDB in DDA preparations is that, it has previously been shown that double chain glycolipids, such as TDB and TDM, inhibit fusion between phospholipids vesicles (Crowe *et al.*, 1994; Spargo *et al.*, 1991). The stabilizing effect is presumably caused by the relatively large hydrophilic trehalose head-group increasing the overall hydration of the liposomal surface, preventing dehydration of the quaternary ammonium head-groups and aggregation caused by reduced charge repulsion. Alternatively, the trehalose moiety might act as a steric barrier that prevents close contact between adjacent liposomes, which is a prerequisite for the formation of aggregation or fusion of the liposomes (Crowe *et al.*, 1994; Spargo *et al.*, 1991).

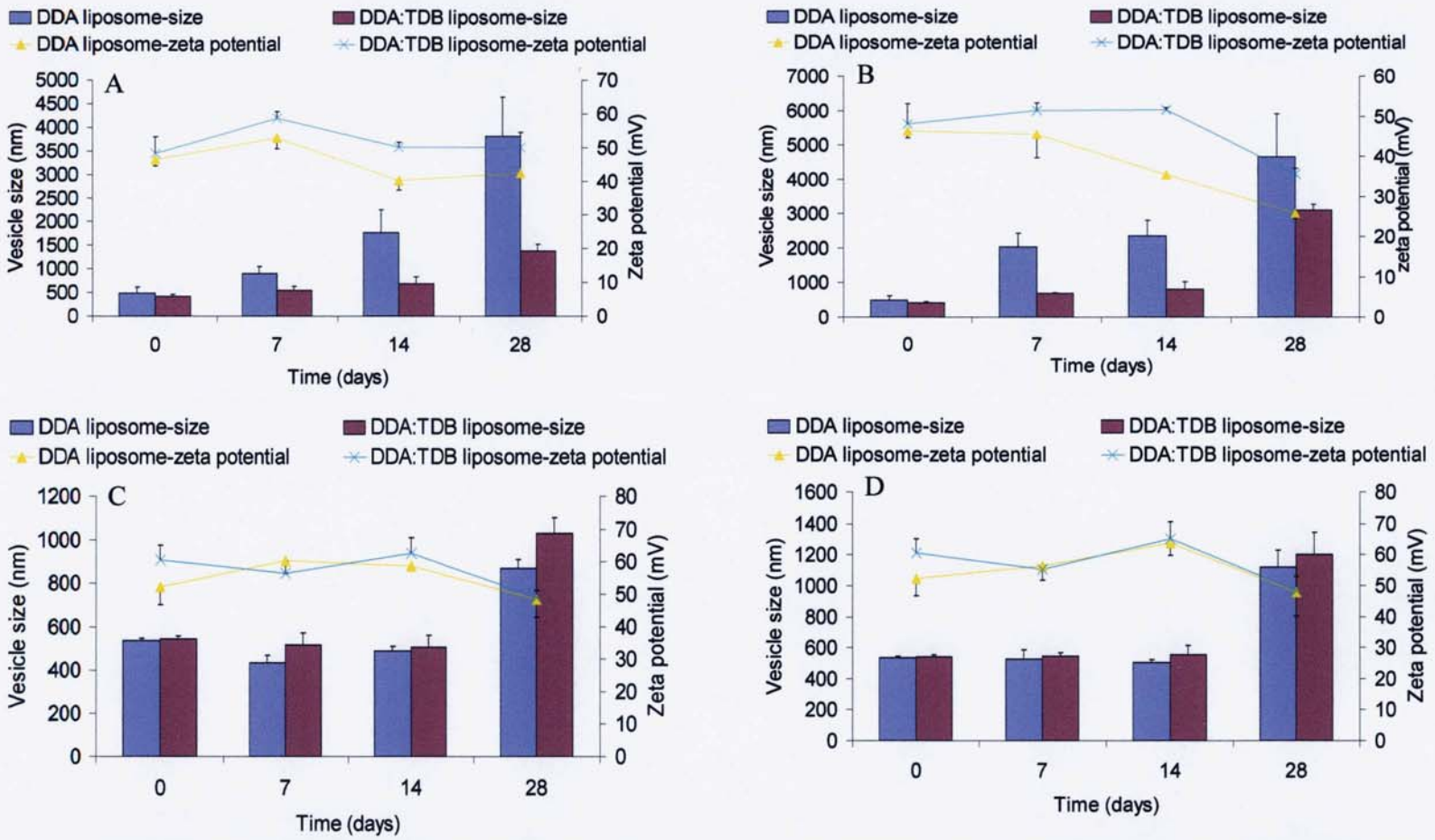


Fig. 6.2 Vesicle size and zeta potential of Ag85B-ESAT-6 incorporated DDA and DDA:TDB based liposomes over time stored at 4°C (A, C) and 25°C (B, D) prepared by lipid hydration method in 10mM Tris buffer (A, B) or in dextrose solution (5%w/v) (C, D). Results denote mean ± S.D., n = 3.

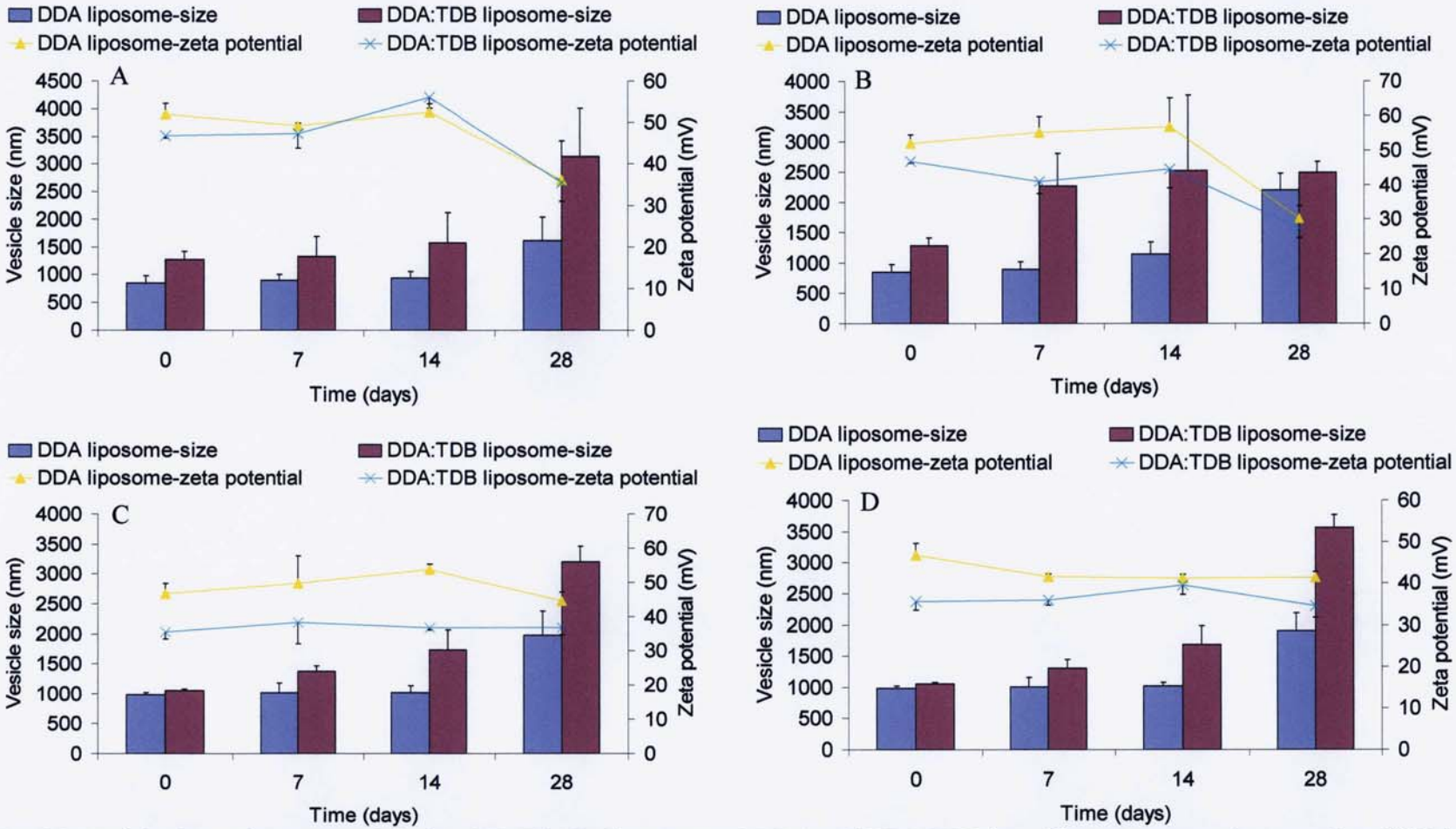


Fig. 6.3 Vesicle size and zeta potential of Ag85B-ESAT-6 incorporated DDA and DDA:TDB based liposomes over time stored at 4°C (A, C) and 25°C (B, D) prepared by aqueous heat method in 10mM Tris buffer (A, B) or in dextrose solution (5%w/v) (C, D). Results denote mean ± S.D., n = 3.

6.3.3 Morphological characterisation of DDA liposomes prepared by aqueous heat and lipid hydration methods

TEM was further used to investigate the morphological characteristics of liposomes prepared by either the aqueous heat or lipid hydration method. Fig. 6.4a shows that DDA liposomes prepared by aqueous heat method were angular and relatively large in size (>1 μm) with notable signs of vesicle aggregation. The larger vesicle size of liposomes produced by aqueous heat compared to lipid hydration method was also apparent when comparing the DDA-TDB (Fig. 6.4b vs e) and DDA-TDB/antigen (Fig. 6.4c vs f) liposome formulations. The formulation of the liposomes had less of an impact on the morphological characteristics of the aqueous heat preparations, with no notable morphological changes seen upon addition of TDB (Fig. 6.4b) and protein (Fig. 6.4c) with vesicles remaining around 1 μm in size. However, the extent of aggregation appears to be reduced when TDB is incorporated within the liposomal membranes, again suggesting the stabilizing role TDB in the vesicle construct.

In agreement with the particle size analysis, when prepared by lipid hydration method, DDA liposomes (Fig. 6.4d) were smaller (~500 nm) and less angular than those prepared by aqueous heat (Fig. 6.4a), although few large vesicles and aggregates were present. Addition of TDB to the liposome formulation (Fig. 6.4e) gave more spherical vesicles which display less aggregation compared to their DDA counterparts, possibly again due to the bilayer stabilizing effect of the longer alkyl chain TDB. Similar to aqueous heat preparations, no obvious morphological change was apparent upon addition of protein to lipid hydration liposomes (Fig. 6.4f).

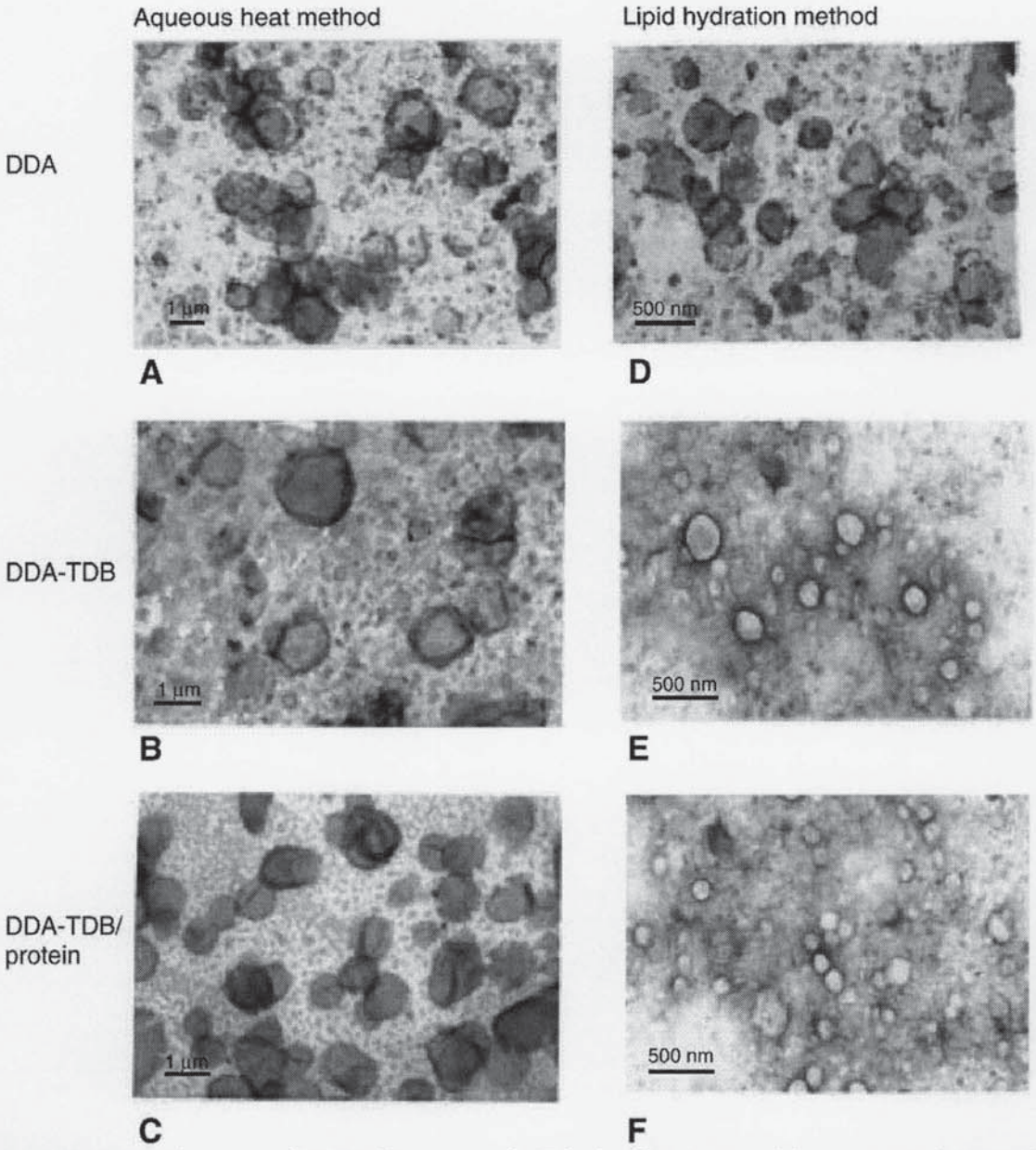


Fig. 6.4 TEM images of DDA liposomes in Tris-buffer prepared by aqueous heat and lipid hydration method. Vesicles prepared by aqueous heat method were observed to be angular, and relatively large in size, showing significant vesicle aggregation (A). No morphological changes were seen upon addition of TDB (B) or antigen (C). When prepared by lipid hydration method, DDA liposomes (D) were smaller and less angular than those prepared by aqueous heat method seen in (A), although some large vesicles were present. Addition of TDB to these DDA liposomes gave more spherical vesicles which displayed less aggregation (E). Similar to aqueous heat preparations, no obvious morphological change was apparent upon addition of antigen to lipid hydration liposomes (F). In all formulations, liposomes prepared by the aqueous heat method were larger than the lipid hydration based counterparts.

6.3.4 Optimisation of DDA liposomes: effect of non-ionic surfactants

Considering the results in sections 6.3.1 to 6.3.2, the lipid hydration method showed superior features by efficient incorporation of TDB in the DDA liposome bilayers hence this preparation method was further employed. The DDA vesicle composition was modified by supplementing with several other non-ionic surfactants including, MP (C16:0), 1-monostearoyl glycerol (MS), 1,2-dipalmitoyl glycerol (DP) in combination with cholesterol (1:1 molar ratio, an optimum ratio for production of physically stable vesicles; Uchegbu and Florence, 1995). The choice of lipids and surfactants were so made as these were examined in other immunisation studies previously with success, i.e., TDB in DDA based systems has been reported to improve the immune response to the tuberculosis vaccine fusion protein, Ag85B-ESAT-6, in mice and furthermore stabilise the formulation (Davidsen *et al*, 2005). The non-ionic surfactant, MP possessing ester linkage between the hydrophobic chain and the hydrophilic head group is reported to be ten fold less toxic than its ether linked analogue and is readily metabolized *in vivo* (Hofland *et al*., 1991) and this non-ionic surfactant based vesicle system was effective in improving the immune response in mice against bovine serum albumin (Brewer & Alexander, 1992). Two more surfactants, one varying in alkyl chain length (MS (C18:0)) and the other in number of alkyl chains (di-alkyl chained, DP (C16:0)) were also been employed in the presence of cholesterol to aid in the formation of stable vesicles.

The SUV and DRV based cationic preparations composed of MP or MS in the presence of cholesterol and DDA with a molar ratio of 4:4:1 gave stable vesicles in aqueous buffer, pH 7.4 (Table 6.2). Interestingly, similarly formulated systems using the double-alkyl chain non-ionic surfactant, DP in replace of MP or MS failed to form vesicles presumably due to either a chemical incompatibility between the

surfactant/lipid mixture used and/or as a result of variation in the critical packing parameter (CPP) of the system which could be used to predict the vesicle forming ability of surfactants (Israelachvili *et al.*, 1977). To support vesicle formation a CPP value in the range of 0.5 and 1.0 is required whereas a CPP below 0.5 (indicating a large contribution from the hydrophilic head group area) is said to give spherical micelles and a CPP of above 1.0 (indicating a large contribution from the hydrophobic group volume) would lead to precipitation. It was observed that the effective combined operational CPP value of entire components in DP based formulation was 1.13 (calculated using molecular modeling Pro® software) thus explaining for the system's inability to form vesicles when used in such combination. However it was also evident that DDA played a key role in stabilising the MP and MS based vesicles, as it was observed that omission of DDA in the formulation in an attempt to make neutral formulation again caused precipitation. Hence, the two stable cationic preparations i.e., MP and MS based vesicles were characterised in terms of vesicle size and surface charge (zeta potential) (Table 6.2).

Formulations	Vesicle type	Vesicle size (nm)	Zeta potential (mV)
MP:Chol:DDA	SUV	78.6 ± 0.1	41.0 ± 5.1
MS:Chol:DDA	SUV	100.9 ± 0.1	40.5 ± 3.4
MP:Chol:DDA	DRV	847.1 ± 43.8	46.4 ± 8.6
MS:Chol:DDA	DRV	1431.6 ± 45.0	41.6 ± 4.7

Table 6.2 Vesicle size and zeta potential of 1-monopalmitoyl glycerol (C16:0) (MP) and 1-monostearyl glycerol (C18:0) (MS) based formulations. 'empty' small unilamellar vesicles (SUV) or dehydration-rehydration vesicles (DRV) were prepared from MP or MS, cholesterol (Chol) and dimethyldioctadecylammonium bromide (DDA) (16:16:4 μmoles). Results represent mean ± S.D., n=3.

Results in Table 6.2 show that neither the choice of non-ionic surfactant (MS vs MP) nor the choice of preparation method (SUV vs DRV) made a significant difference to the positive surface charge (as indicated via measurement of the zeta potential) which

resulted from the presence of the cationic DDA. However, in comparison, the vesicle size of both SUV and DRV systems was significantly influenced by the choice of surfactant with MS based system being around 25% (101 nm vs 79 nm for SUV) and 50% (1432 nm vs 847 nm for DRV) larger in measured diameter compared to its MP counter part and systems respectively; Table 6.2). This is presumably due to the longer alkyl chain length of mono-stearyl glycerol which might be contributing to the expansion of the bilayer as the hydrophilic portion of the molecule is decreased relative to the hydrophobic portion (Israelachvili, 1993; Uchegbu and Duncan, 1997; Hao *et al.*, 2002).

6.3.5 Characterisation of DDA and MP based formulations

Based on the initial characterisation studies and on previous reports of MP based niosome preparations encapsulating bovine serum albumin (BSA) inducing high antibody production in mice compared to their MS based counter part (Brewer & Alexander, 1992), MP-based systems were further investigated and compared to cationic liposome (DDA based) systems previously demonstrated to be effective adjuvants (Lindblad *et al.*, 1997; Holten-Andersen *et al.*, 2004; Davidsen *et al.*, 2005). Thus formulations composing of DDA, DDA:TDB (4:0.5 μmol) with adsorbed antigen were compared to DRV systems composed of MP:Chol:DDA (16:16:4 μmol) and with or without inclusion of TDB (0.5 μmol) encapsulating three different antigens i.e., Ag85B-ESAT-6, MSP1 and GLURP.

The physico-chemical characteristics of different preparations in the presence or absence of antigens were studied. Results in Table 6.3 demonstrate that the sizes of empty DDA vesicles were significantly smaller ($P<0.05$) with DDA liposomes being almost half of those composing MP:Chol:DDA (421 nm vs 828 nm respectively).

Further, no significant difference in vesicle size was observed when TDB was incorporated in DDA and MP:Chol:DDA based systems. Analysis of the zeta potential of these formulations showed that the supplementation of DDA vesicles with MP and Cholesterol and/or TDB made no significant difference with all vesicles before addition of antigen having a net positive charge (50-60mV; Table 6.3), clearly resulting from the cationic nature of DDA, suggesting that no marked electrostatic interactions between the DDA and TDB or with MP and Chol occurred at the surface of the formed vesicles. No significant effect on vesicle size and zeta potential was observed upon adsorption of Ag85B-ESAT-6 on to the surfaces of DDA and DDA:TDB systems in line with previously reported results (Davidsen *et al.*, 2005).

Formulations	Vesicle size (nm)	Zeta potential (mV)	Antigen loading (% of used)
DDA	421.2 ± 26.1	52.4 ± 4.1	NA
DDA:TDB	416.3 ± 40.4	54.7 ± 4.7	NA
MP:Chol:DDA	847.7 ± 43.8	56.4 ± 1.6	NA
MP:Chol:DDA:TDB	877.0 ± 12.6	55.3 ± 2.2	NA
DDA – Ag85B-ESAT-6	488.1 ± 123.6	46.2 ± 4.6	69.9 ± 10.2
DDA:TDB – Ag85B-ESAT-6	485.8 ± 110.0	59.7 ± 4.7	67.0 ± 2.8
MP:Chol:DDA (Ag85B-ESAT-6)	1346.8 ± 140.0	57.1 ± 1.8	87.8 ± 0.5
MP:Chol:DDA:TDB (Ag85B-ESAT-6)	1031.6 ± 132.1	57.9 ± 3.4	89.3 ± 0.4
MP:Chol:DDA (MSP1)	958.6 ± 86.6	53.2 ± 1.2	60.4 ± 2.2
MP:Chol:DDA:TDB (MSP1)	1038.8 ± 89.2	52.7 ± 2.4	57.9 ± 0.8
MP:Chol:DDA(GLURP)	2547.9 ± 363.0	46.0 ± 2.9	58.5 ± 2.8
MP:Chol:DDA:TDB (GLURP)	2797.8 ± 530.6	45.7 ± 1.1	60.0 ± 3.7

Table 6.3 Vesicle size, zeta potential and antigen loading of surfactant vesicles with three different antigens i.e., Ag85B-ESAT-6, MSP1 and GLURP. Vesicles were formulated from dimethyldioctadecylammonium bromide (DDA) alone or in combination with 1-monopalmitoyl glycerol (C16:0) (MP) and cholesterol (Chol) (MP:Chol:DDA) and/or α,α'-trehalose 6,6'-dibehenate (DDA:TDB; 4:0.5 μmol, MP:Chol:DDA:TDB; 16:16:4:0.5 μmol). Antigens were either adsorbed (e.g DDA:Chol:DDA-Ag85B-ESAT6) or entrapped via the DRV method (e.g. DDA:Chol:DDA (Ag85B-ESAT-6)). Results represent mean ± S.D., n=3; NA=Not Applicable.

Encapsulation of Ag85B-ESAT-6 in MP:Chol:DDA and MP:Chol:DDA:TDB systems was shown to result in an increased vesicle size with out significantly affecting the zeta potential (Table 6.3) suggesting that the antigen may be internalized and due to its lipophilic nature could possibly be accommodated within the vesicle bilayers thus increasing the vesicle size. A similar trend in increased vesicle size yet no significant change in zeta potential was observed in case of MP:Chol:DDA and MP:Chol:DDA:TDB systems when the malaria antigen, Merozoite surface protein 1 (MSP1) was encapsulated showing vesicle sizes to be about a micron with a zeta potential of ~52 mV (Table 6.3). In contrast, when another malaria antigen, Glutamate rich protein (GLURP), was encapsulated in MP based systems both in the presence and absence of TDB, the vesicle sizes were significantly increased to over 2.5 microns. This is presumably due to high molecular weight of GLURP (~55 kDa), in addition to its high hydrophobic nature, causing it to associate with the hydrophobic regions of the vesicle bilayers and possibly encouraging a degree of vesicle fusion and/or influencing the packing arrangements of the surfactants and thus increasing the overall size of the vesicles. This interaction of the GLURP antigen with the vesicular bilayers was further reinforced by a small decrease in zeta potential (Table 6.3).

Adsorption or entrapment efficiency of the antigens in the vesicles was determined by assaying the activity of ^{125}I label of these antigens in the vesicles. Adsorption of Ag85B-ESAT-6 antigen to cationic DDA vesicles with or without TDB was almost 70% of initial amount used (0.01 mg/ml) in line with previously reported studies (Davidsen *et al.*, 2005). Entrapment efficiency of MP based systems incorporating Ag85B-ESAT-6 was observed to be about 90% (Table 6.3) while those incorporating MSP1 and GLURP had about 60%. Similar to antigen adsorption, the presence of

TDB did not affect the entrapment efficiency in all the formulations compared to their counter parts in the absence of TDB with in each of the antigens (Table 6.3).

6.3.6 Spatial location of the protein in DRV

To study the surface adsorption of the antigen (Ag85B-ESAT-6) on the MP based systems, empty DRV were incubated in the presence of fluorescently labelled antigen (Alexa Fluor® 350 (Molecular Probes) as detailed in section 6.2.2.2) at room temperature for 1 hour followed by centrifugation of the DRV. Fluorescence measurement of these supernatants revealed that approximately 90% of the antigen remained in the supernatant after incubation and only 10% was actually adsorbed on to the surfaces suggesting that the antigen do not largely have a tendency to adsorb onto the surfaces of the vesicles implying that majority of the antigen might be encapsulated in the vesicle bilayers, this observation was further reinforced in section 6.3.13. The reason for the antigen not adsorbing onto the surfaces of MP based systems is further explained in section 6.3.7.

6.3.7 Orientation of surfactant monomers at the interface

Supported by the 3-D images of the surfactant monomers with structural orientation in horizontal plain, the molecules were originally constructed using Work space cache software® and then added in to the Molecular Modeling Pro® software based on the mole ratios used in the formulations and consequently the molecules assumed a typical orientation depending upon their relative molecular affinities. Fig. 6.5a shows a typical structural arrangement of several DDA molecules in horizontal plain overlapped on each other (seen as a single molecule in the figure) whose hydrophilic head group is observed to be relatively small. Addition of TDB to DDA molecules led to an orientation depicting hydrophobic interactions between alkyl chains of these

molecules (Fig. 6.5b). Further addition of MP and cholesterol to DDA and DDA-TDB molecules in Fig. 6.5c and Fig. 6.5d respectively, revealed the projection of hydrophilic portion of the MP molecule (circled in figure) from the bulk of remaining molecules which could presumably be acting as a stearic barrier and preventing the adsorption of globular antigen, Ag85B-ESAT-6 on to the vesicle surfaces of MP based systems in accordance with previous studies (Crowe *et al.*, 1994; Spargo *et al.*, 1991) thus explaining for a poor surface adsorption of antigen as noted in section 6.3.6. The projection of the large hydrophilic head group of TDB (seen in Fig. 6.5d) would presumably not have a great impact as it is present in a very low concentration (0.5 μmol) in the formulation.

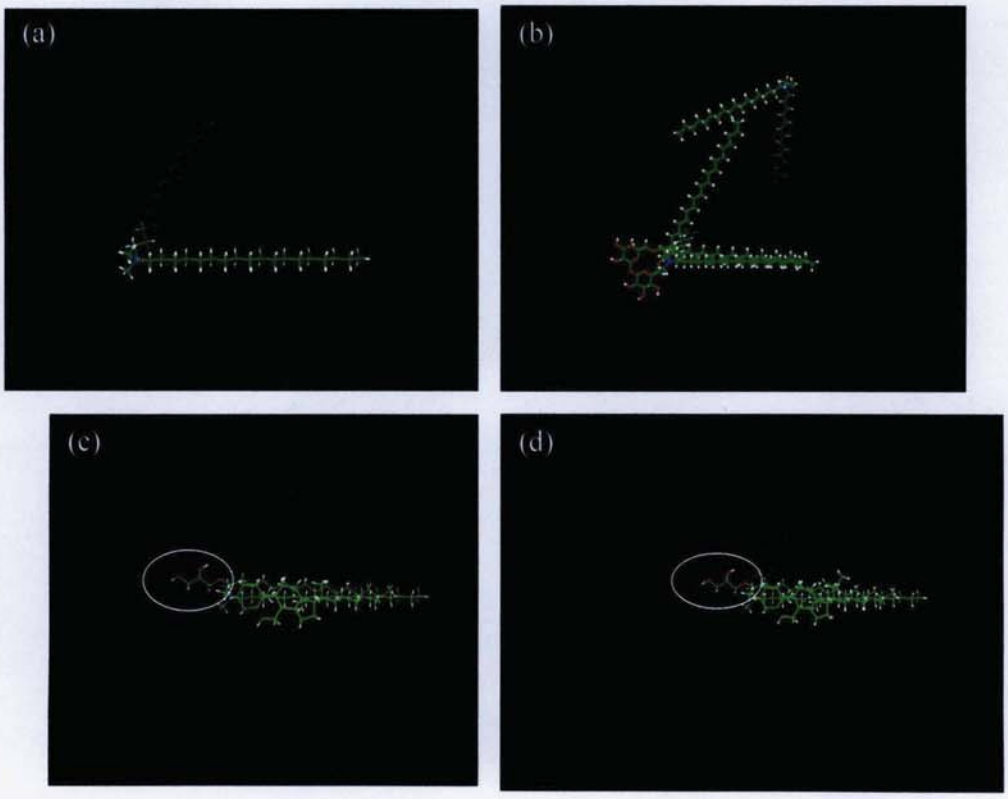


Fig. 6.5 3-D images showing the orientation of surfactant monomers at the interface. DDA molecules are aligned in a horizontal plain (a), addition of TDB molecule to DDA lead to a typical arrangement based on their individual molecular affinities (b), further addition of MP and Cholesterol to (a) and (b) lead to a structural alignment which resulted in the hydrophilic portion of the MP molecule to project outwards (circled) from the bulk of the remaining molecules (c) and (d).

6.3.8 Differential Scanning Calorimetry

DDA lipid bilayers undergo a main phase transition at a characteristic temperature (T_c) at which the lipid chains transfer from a lower temperature gel-phase dominated by ordered alkyl chain conformations to a high-temperature fluid-phase characterised by disordered alkyl chain conformations (Bloom *et al.*, 1991). The phase transition temperature (T_c) was detected as a peak in the heat capacity curve obtained by DSC. DSC thermograms for different vesicle compositions are shown in Fig. 6.6.

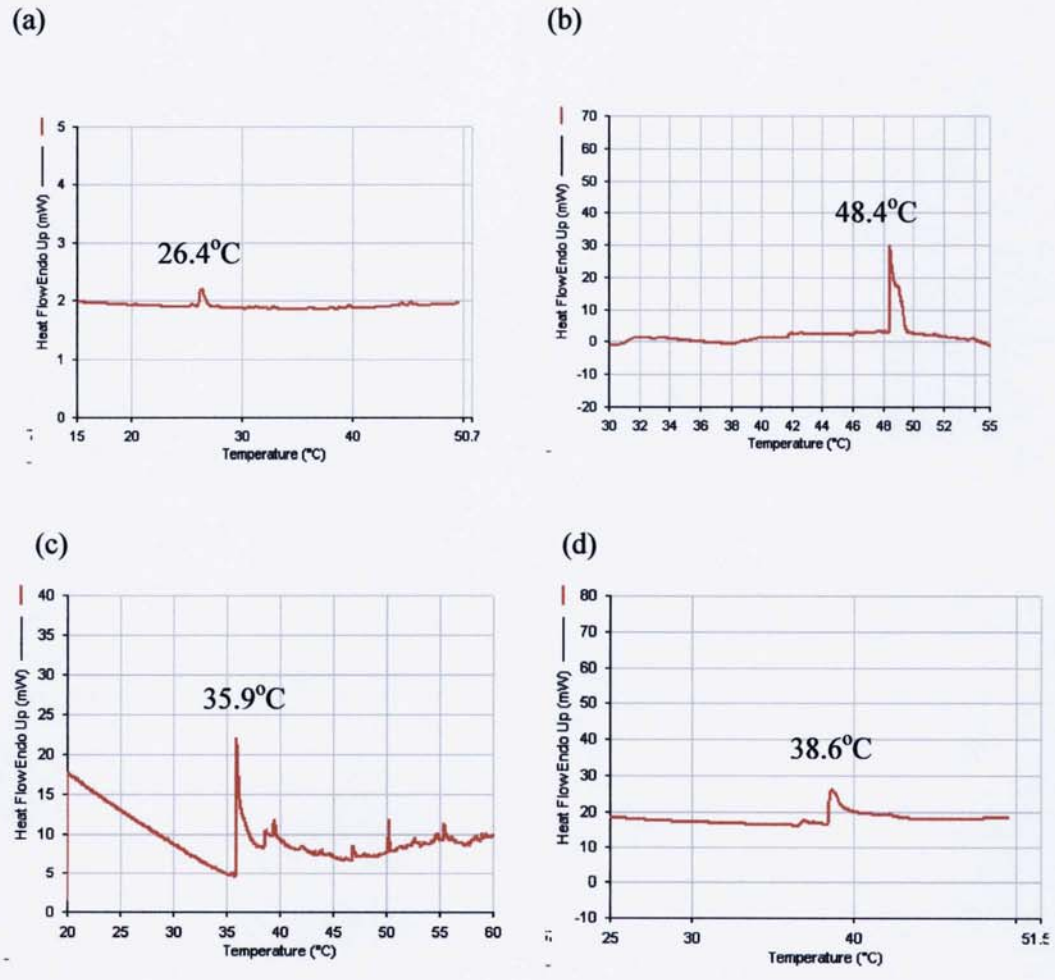


Fig. 6.6 Differential scanning thermograms of various preparations. (a) MP molecule showed a T_c at 26.4°C, measured at a scan rate of 5°C/min, (b) DDA preparations dispersed in 10 mM Tris buffer showed a T_c at 48.4°C, (c) Addition of MP and Chol to DDA preparation (MP:Chol:DDA vesicles) reduced the T_c to 35.9°C, (d) Further, addition of TDB to MP based system (MP:Chol:DDA:TDB vesicles) showed a small increase in T_c to 38.6°C, measured at a scan rate of 2.5°C/min.

The DSC scan of MP molecule was characterised with a single well defined endothermic peak possessing a T_c ~26.4°C (Fig. 6.6a) measured at a scan rate of 5°C/min in agreement with the previous report (Gehlert *et al.*, 1995). DDA liposomes in aqueous buffer, which was measured at a scan rate of 2.5°C/min, showed an endothermic peak with a T_c ~48.4°C (Fig. 6.6b), characteristic of a highly co-operative gel-fluid phase transition in agreement with DSC results for DDA liposomes reported in the literature (Feitosa *et al.*, 2000; Davidsen *et al.*, 2005). In addition, it was reported previously that a shift in T_c to ~42.4°C was noted (Davidsen *et al.*, 2005) when TDB was introduced into DDA preparations. Here, introduction of MP and cholesterol into the DDA system further lowered the T_c of the system to 35.9°C (Fig. 6.6c) measured at a scan rate of 2.5°C/min with a shoulder detected at the higher temperature side suggesting that more than one co-operative heat transition has occurred, most likely due to changes in the local structure of the vesicle bilayer resulting in a lateral phase separation and presumably led to formation of domains enriched in DDA, though it was not anticipated to have a prominent heat transition peak at 48°C which practically disappeared because of the dominance of lead surfactant MP in the system possessing a much lower T_c (26.4°C) while cholesterol not possessing a T_c of its own, is reported to have a tendency to abolish the overall T_c of the system (Horiuchi and Tajima, 2000). Further, incorporation of TDB in the MP based system, the endothermic peak slightly shifted its position towards higher side increasing the T_c to 38.6°C (Fig. 6.6d) measured at a scan rate of 2.5°C/min. The present DSC results demonstrate that addition of surfactants to DDA liposomes revealed a pronounced effect on the thermotropic phase behaviour of DDA liposomes implying that the alkyl chains of the DDA were in fact incorporated into the hydrophobic core of the MP bilayers causing a change in the lipid chain packing

parameters. It should be emphasised that reduction in the T_c of DDA by ~12°C when MP and cholesterol were added enabled the formulations to be made at lower temperatures and thus encapsulation of the antigen was facilitated due to reduced risk of antigen denaturation.

6.3.9 Stability of vesicles: comparison between DDA and MP preparations

Vesicle stability, in terms of their size and zeta potential was studied over a period of time at two different storage temperatures i.e., 4°C and 25°C. DDA vesicles in the presence or absence of TDB were initially about 500 nm (Fig. 6.7a, c). At 4°C a steady rise in the vesicle size was observed for DDA liposomes which lead to eventual precipitation by day 28. Indeed after only 7 days of storage the measured vesicle diameter had almost doubled (900 nm; Fig. 6.7a). In line with previous studies (Davidsen *et al.*, 2005), the addition of TDB to DDA liposomes was shown to stabilise the liposome formulation by having no significant increase in vesicle size during 28 days of storage at 4°C (Fig. 6.7a).

As already noted (Table 6.3), addition of MP and cholesterol to DDA formulations resulted in the formation of vesicles of significantly ($P<0.05$) larger diameter than their DDA counterparts with z-average diameters of ~1300 nm (Fig. 6.7a). After 28 days storage, the mean vesicle size of these MP based systems increased to approximately 1900 nm (Fig. 6.7a). However, unlike DDA liposomes, supplementation of the MP based systems with TDB resulted in no significant change in vesicle size being recorded over the 28 day investigation with vesicle sizes remaining at approximately 1900nm (Fig. 6.7a).

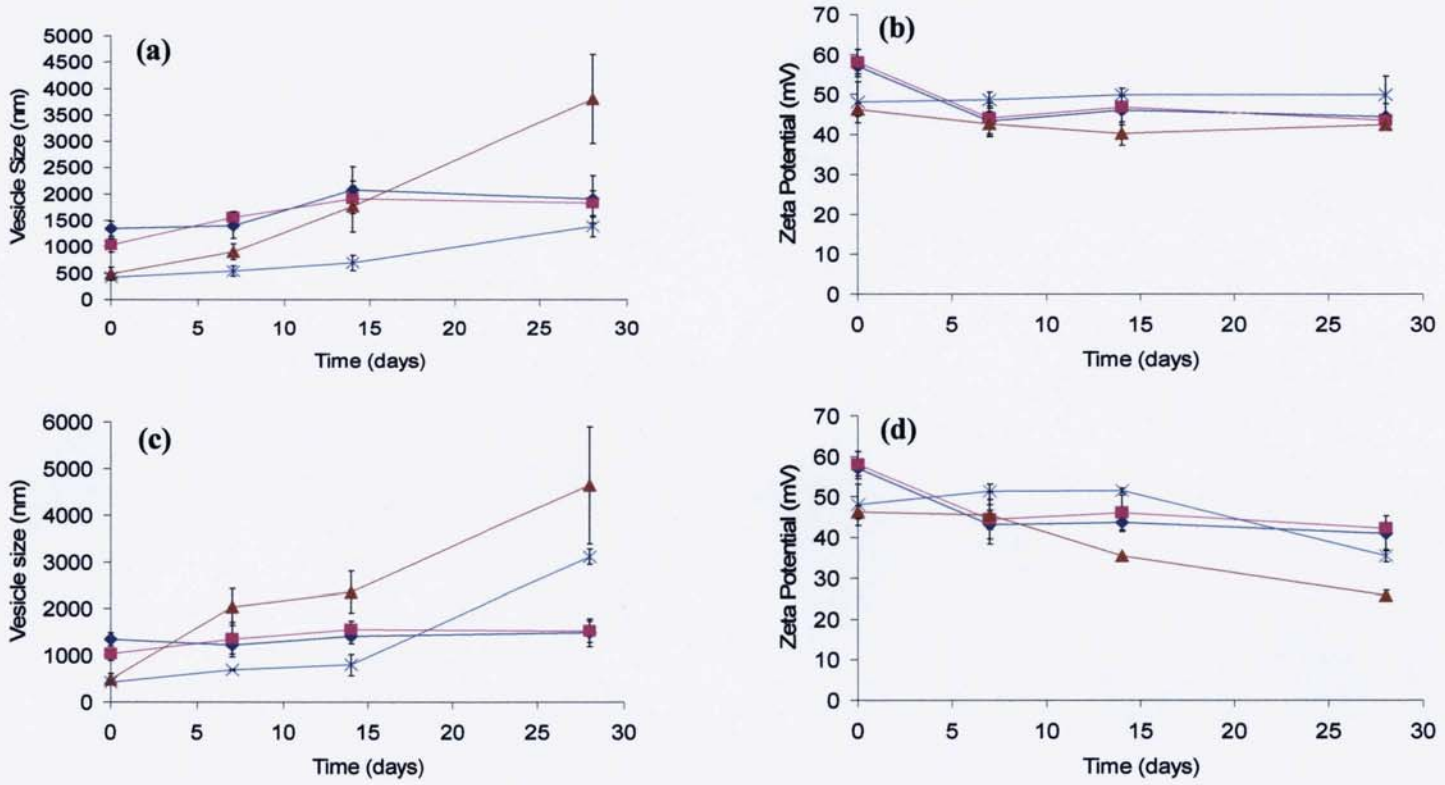


Fig. 6.7 (a) Time development of average vesicle sizes of DDA (▲), DDA:TDB (×), MP:C:DDA (♦) and MP:C:DDA:TDB (■) preparations stored at 4°C. A significant increase in size was observed in DDA vesicles after 14 days. Addition of TDB improved the stability with no significant difference compared to MP based systems after 28 days. (b) Time development of average zeta potential of vesicles stored at 4°C. Zeta potential remained fairly constant at 4°C after 7 days in all the preparations. (c) Time development of average vesicle sizes stored at 25°C. A significant increase was observed for DDA liposomes in the absence of TDB after 14 days. Addition of TDB improved the stability but to a lesser extent than MP based preparations after 28 days. (d) Time development of average zeta potential of vesicles stored at 25°C. A significant fall in the zeta potential was seen in DDA and to a lesser extent DDA:TDB preparations after 28 days.

Under these conditions the zeta potential of the DDA and DDA:TDB remained consistent (40 – 50 mV) with no significant changes measured over 28 days. Both MP based formulations displayed an initial small decrease in zeta potential after 7 days, but no significant changes subsequently were observed (Fig. 6.7b).

At 25°C DDA liposomes (without the addition of TDB) displayed a steady increase in vesicle size with eventual precipitation, as was observed with formulations stored at 4°C, while those in the presence of TDB maintained a submicron size until day 14 but increased to about 2900 nm by day 28 (Fig. 6.7c). This increased vesicle size in case of DDA based systems was in conjunction with a significant ($P<0.05$) and continued decrease in their zeta potential (Fig. 6.7d), whereas in the case of DDA:TDB a marked decrease in zeta potential was only apparent after 14 days, again coinciding with a significant increase in vesicle size (Fig. 6.7c). In contrast, at 25°C both MP based formulations (with and without addition of TDB) showed no significant change in vesicle size throughout the 28 days of storage and only an initial drop (day 7) in the zeta potential was observed, similar to the MP based vesicles stored at 4°C.

Taken together, the vesicle sizes of the MP based formulations (Fig. 6.7a & c) in relation to their phase transition temperatures (Fig. 6.6c & d), indicate that on storage above or nearer to their phase transition temperature, the vesicles remain relatively more stable in terms of vesicle size with less aggregation presumably due to surfactants existing in liquid crystalline state (Arunothayanun *et al.*, 2000). However, an increase in the vesicle size upon storage at temperatures far below T_c i.e., at 4°C could likely be due to less thermal energy present around the vesicles. This was in accordance with thermodynamic theory, as smaller vesicles require a higher input of energy to overcome the phenomenon of inherent instability of the system (Uchegbu and Vyas, 1998) that might otherwise cause vesicles in the vicinity to fuse or aggregate. T_c of DDA based

preparations was ~48°C while the T_c of MP based preparations was ~35-38°C. A strong correlation between the T_c of the preparation and storage temperature seems to exist considering a 6°C fall in T_c from 48°C to 42°C when TDB was incorporated into the DDA system (Davidsen *et al.*, 2005) could also possibly be a contributing factor in the stabilisation phenomenon of TDB. For a future vaccine based on DDA liposomes, storage will most likely take place at 4°C, and both the DDA based liposomes and MP based formulations were stable at this temperature.

6.3.10 Antigen retention studies

To characterise the antigen retention in different formulations, Ag85B-ESAT-6 was selected as model antigen. The extent of retention of antigen (% of total used; 0.01mg/ml) was determined using ^{125}I labelled Ag85B-ESAT-6. The addition of TDB had no detrimental effect on antigen retention, with both DDA and DDA:TDB formulations adsorbing approximately 70-80% of the total antigen initially added (Table 6.3). The adsorption of antigen to the formulation was most probably electrostatic in nature. The antigen is highly negatively charged and has a theoretical pI value of 4.80. In contrast, zeta potential analysis demonstrates that all four DDA based formulations had a high net positive charge of 45 to 53 mV (Table 6.3).

After initial removal of non-adsorbed Ag85B-ESAT-6 via centrifugation, antigen retention to the liposomes after storage at both 4°C and 25°C was measured. After 7 days storage at 4°C (Fig. 6.8a) antigen retention in both DDA and DDA:TDB formulations dropped to approximately 80% of the initial antigen adsorption. Subsequently at time points thereafter, retention values for DDA:TDB preparations remained constant at 80% with no further losses of antigen being measured up to 28 days (Fig. 6.8a). In contrast, further loss of antigen was associated with the DDA formulation with retention values being significantly ($P<0.05$) lower by day 14

(approximately 65% of initial adsorption values) compared to DDA:TDB formulations (Fig. 6.8a). Storage of the formulations at 25°C had no major impact on antigen retention compared to refrigerated samples over the period of analysis, however at this temperature, significant differences ($P<0.05$) in antigen retention between DDA based formulations with and without TDB were only apparent at day 28 (Fig. 6.8b). In case of MP based preparations, both the formulations in the presence or absence of TDB retained about 90% of the encapsulated antigen.

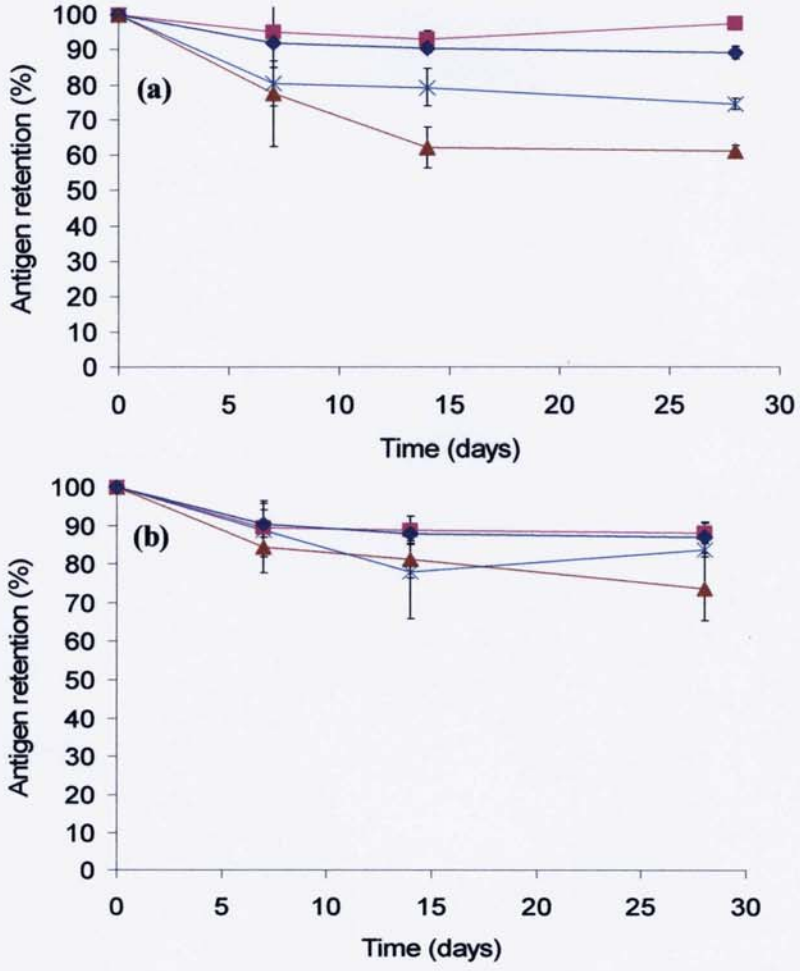


Fig. 6.8 Antigen retention over time of DDA (▲), DDA:TDB (×), MP:C:DDA (♦) and MP:C:DDA:TDB (■) preparations in aqueous buffer, pH 7.4 stored at 4°C (a) and 25°C (b). ^{125}I-labelled Ag85B-ESAT-6 was adsorbed/encapsulated and antigen retention of Ag85B-ESAT-6 was determined on the basis of ^{125}I radioactivity recovered in the suspended pellets after ultracentrifugation. Results represent percentage retention of initially loaded antigen (Table 6.3), expressed as mean ± S.D., n= 3.

6.3.11 Antigen release studies

Antigen release from the four different preparations was studied based on the activity of released ^{125}I labelled Ag85B-ESAT-6 in physiological buffer, pH 7.4 as release medium at 37°C. DDA liposomes both in the presence and absence of TDB showed an initial burst release in the first two hours of up to 35% (of the total amount) followed by a steady state of ~40% being reached after 24 hours (Fig. 6.9). Alternatively, MP based preparations in the presence of TDB displayed a more sustained release of antigen of approximately 30% (of the total amount) after 168 hours as opposed to MP:Chol:DDA system which initially had a significantly lower (P<0.05) release rates after 24 hours compared to MP:Chol:DDA:TDB system (3% vs 12%, respectively) but eventually had shown substantial rise to 65% after 168 hours, the highest release shown among the four formulations (Fig. 6.9).

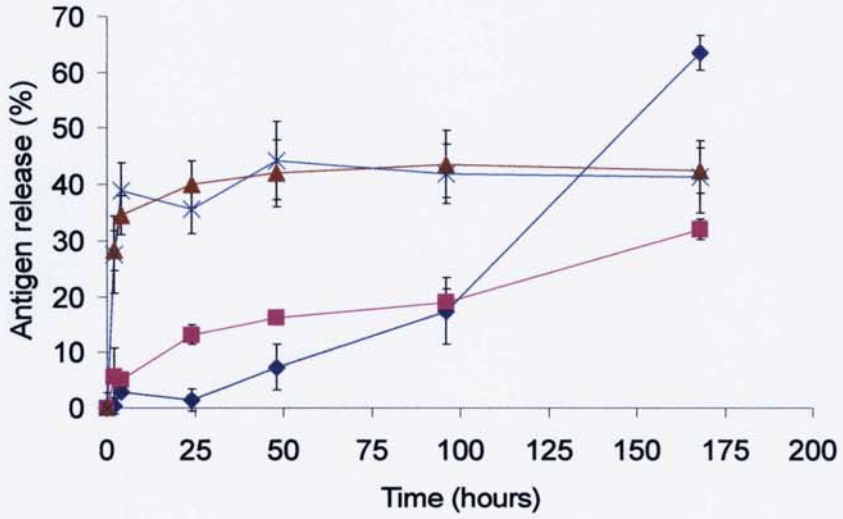


Fig. 6.9 Antigen release over time from DDA (▲), DDA:TDB (×), MP:C:DDA (♦) and MP:C:DDA:TDB (■) in aqueous buffer, pH 7.4 at 37°C. DDA preparations both in the presence and absence of TDB showed an initial burst release in the first two hours up to 35% of the total antigen and the release profile further showed a plateau after 24 hours. MP based system in the presence of TDB showed a sustained release compared to the formulation in absence of TDB. Ag85B-ESAT-6 release was determined on the basis of radioactivity of ^{125}I-labelled Ag85B-ESAT-6 recovered in the suspended pellets after ultracentrifugation. Results represent percentage release of initially loaded antigen expressed as mean ± S.D., n= 3.

6.3.12 Morphological characterisation of DDA and MP based preparations

Morphological characteristics of DDA and MP based preparations (MP:Chol:DDA, 16:16:4 μmol) when investigated using TEM revealed that, in agreement with the particle size analysis using photon correlation spectroscopy (PCS) (Table 6.3), DDA vesicles were smaller (~ 400 nm) (Fig. 6.10a) than the MP based vesicles (~ 800) (Fig. 6.10b). MP based systems revealed multilamellar nature of the bilayers (Fig. 6.10b) while the lamellarity of the DDA based vesicles was not conspicuous (Fig. 6.10a) although, these DDA vesicles were reported to be unilamellar in nature when viewed using cryo-TEM (Davidsen *et al.*, 2005). Heterogeneous population was clearly seen in MP based system with smaller vesicles adhering to the surfaces of the larger ones (Fig. 6.10c), however similar to the DDA liposomes (Fig. 6.4d) MP based systems also showed partial aggregation of the vesicles (Fig. 6.10d). TEM pictures of MP based preparations unlike DDA vesicles had less of an impact on the morphological characteristics with no notable differences observed in the presence of the TDB and also in the presence of the antigen (not shown).

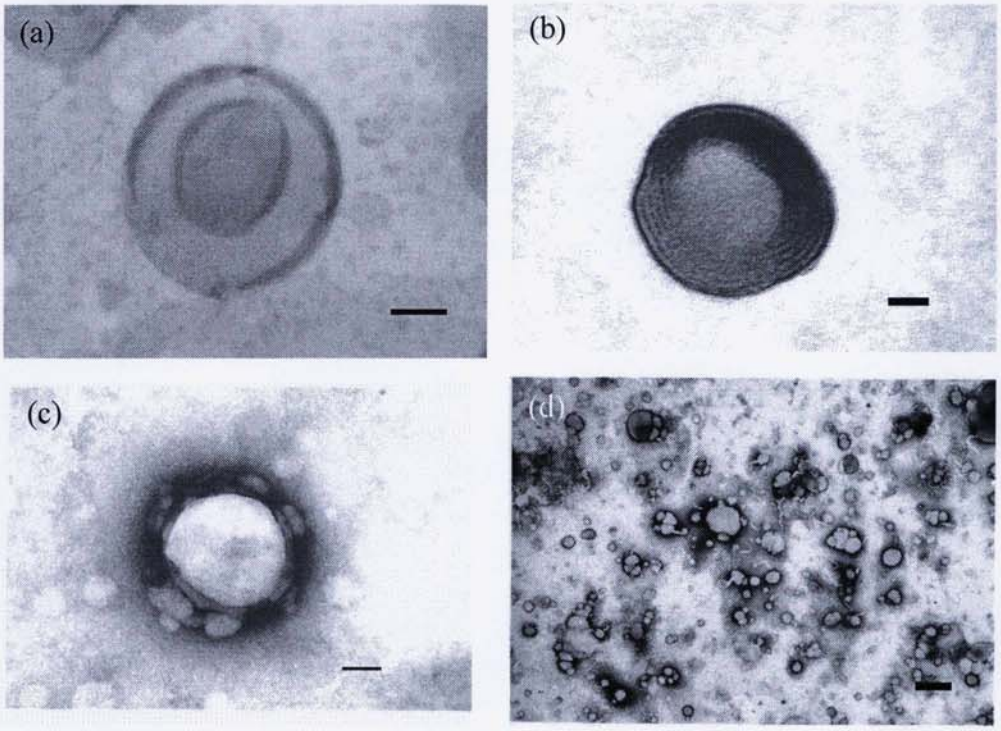


Fig. 6.10 TEM micrographs of DDA (4 μmol) and MP based preparations (MP:Chol:DDA, 16:16:4 μmol). (a) DDA vesicle-in-vesicle structure revealed a unilamellar nature of the bilayers, bar = 100 nm; (b) A single MP based vesicle revealed multilamellar nature, Bar = 200 nm; (c) MP based vesicles showed aggregation of smaller vesicles onto the surface of a single large vesicle, Bar = 200 nm; (d) MP based vesicles also showed partial aggregation in spite of a high cationic charge (Table 7), Bar = 1μm.

6.3.13 Environmental Scanning Electron Microscopy (ESEM)

MP based system consisting of MP:Chol:DDA:TDB (16:16:4:0.5 μmol) showed enhanced physico-chemical features in terms of vesicle stability and antigen retention over time and also displayed a sustained antigen release profile when compared with other preparations in the study. This system was thus chosen for assessing additional morphological characteristics and bilayer stability for the first time using Philips XL-30 field emission gun environmental scanning electron microscope. ESEM micrographs revealed giant, spherical vesicles of sizes between 10 - 30 μm (Fig. 6.11) compared with those obtained from particle size measurements where the vesicle sizes were significantly smaller (850 nm) as in the latter the values were derived as a mean of the overall distribution ratio of the sample unlike individual vesicles with a small population being towards higher size range.

A comparative study was performed between the empty vesicles (Fig. 6.11a, c, e) and antigen loaded vesicles (Fig. 6.11b, d, f). At a pressure of about 3.0 Torr, all the vesicles looked spherical and round in shape (Fig. 6.11a & b). Vesicles loaded with antigen were observed to retain their structural and bilayer integrity more efficiently than the empty vesicles as observed with reduction in operating pressure in the sample chamber. Gradual reduction of the ESEM operating pressure to 2.4 Torr resulted in the empty vesicles to coalesce (Fig. 6.11c) with a few vesicles were observed to lose their spherical shape and flatten (indicated by arrows) to form lipid masses as the buffer medium evaporated, while antigen incorporated vesicles resisted the destabilisation caused at the similar pressure (Fig. 6.11d) which lead to the reinforcement of the presumption that antigen might actually be present within the bilayers contributing to their increased rigidity. Further reduction in operating pressure to 1.9 Torr, resulted in both empty and antigen incorporated MP:Chol:DDA:TDB vesicles to coalesce

completely as evaporation of the surrounding aqueous medium continued (Fig. 6.11e & f). Salt crystallisation from the medium was visible amidst the lipid/surfactant masses.

'Empty' MP based vesicles Ag85B-ESAT-6 loaded MP based vesicles

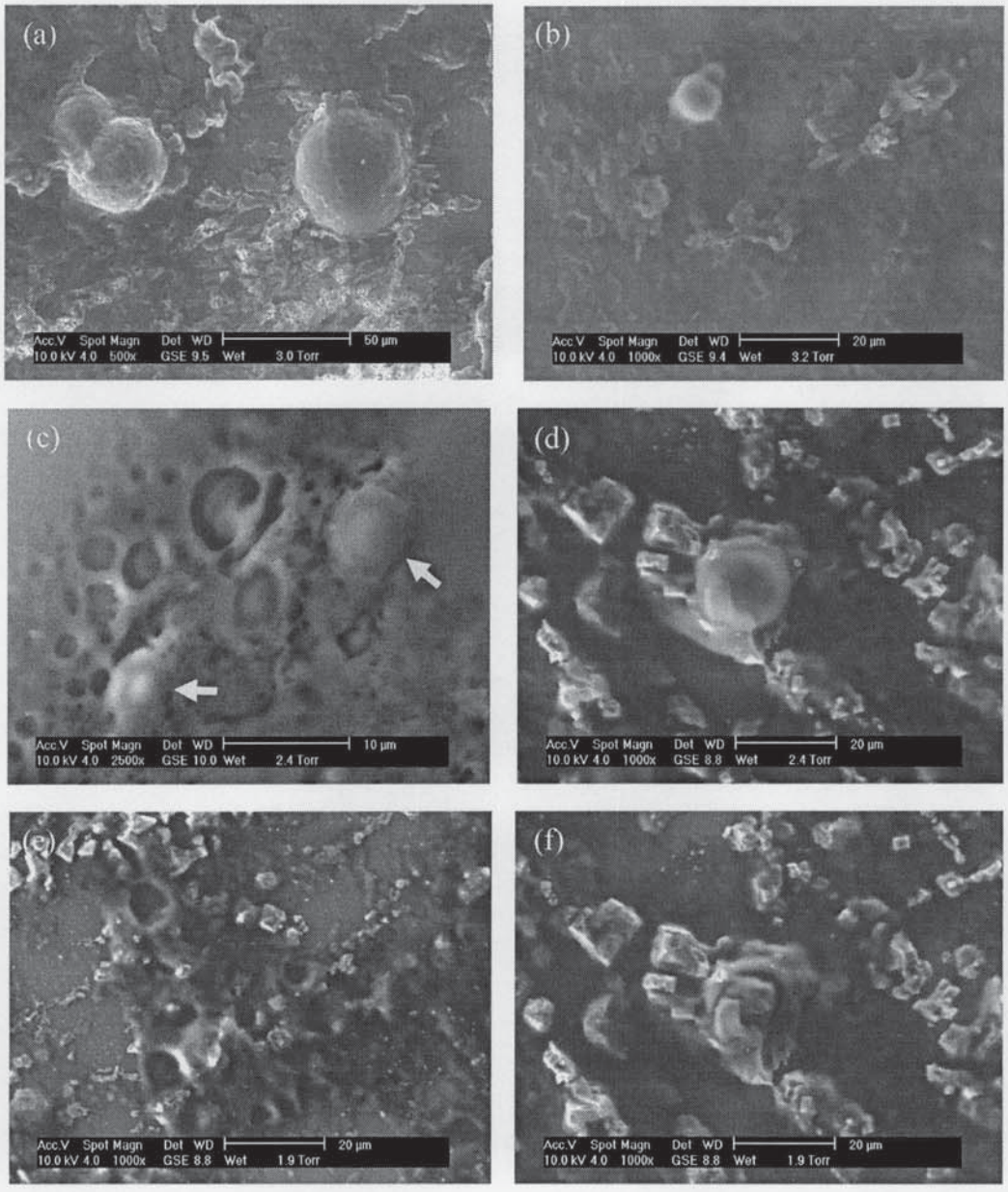


Fig. 6.11 ESEM micrographs of 'empty' and Ag85B-ESAT-6 loaded MP based vesicles (MP:Chol:DDA:TDB, 16:16:4:0.5 µmol) suspended in aqueous buffer (pH. 7.4). Vesicles appear spherical and stable at an operating pressure of 3.0 Torr, bar = 50 µm. and 3.2 Torr, bar = 20 µm (a & b respectively). Reduction of the ESEM operating pressure to 2.4 Torr resulted in the vesicles to coalesce or collapse with a couple of flattened vesicles observed (indicated by arrows) as the medium evaporated (c), bar = 10 µm, while at similar operating pressure vesicles in antigen incorporated system retained its spherical nature (d), bar = 20 µm. Further reduction of operating pressure to 1.9 Torr resulted in vesicles to completely collapse with visible lipid/surfactant masses and salt crystallisation (e & f), bar = 20µm.

ESEM images of the samples do not resemble the TEM images shown earlier due to the difference in the sample preparation (staining in case of TEM) technique, although, the ESEM analysis demonstrates here that antigen incorporation could improve the stability of the MP based systems as revealed by enhanced resistance to coalescence during dehydration compared to their empty counterparts. Increased stability of antigen incorporated vesicles may be due to the macromolecular antigen getting embedded in the spacing between adjacent lipid/surfactant chains of the vesicle bilayers and thereby increasing the rigidity of the vesicle bilayer, indeed such type of increased stability was reported previously with entrapment of hydrophobic drugs (Uchegbu *et al.*, 1996) or macromolecular prodrugs (Gianasi *et al.*, 1997) in non-ionic surfactant vesicles and in liposome systems (Mohammed *et al.*, 2004).

6.3.14 Immunisation studies

The adjuvant effect of both MP and DDA based vesicle preparations was investigated by immunising C57Bl/6j mice with the Ag85B-ESAT-6 fusion protein. The specific immune response of the spleen cells was investigated by restimulation with Ag85B-ESAT-6 *in vitro* (Fig. 6.12a) and subsequently measuring IFN-γ as an indicator of a Th1 response. All formulations induced release of IFN-γ, with the DDA and DDA:TDB vesicles giving rise to the highest responses. The ability of the various vesicle preparations to induce antibody responses after immunisation was also investigated by measuring the antigen-specific titres of the IgG1 and IgG2b isotypes by ELISA. As shown in Fig. 6.12b, the IgG1 titres determined were at the same level for MP:Chol:DDA:TDB, DDA:TDB and DDA whereas the highest IgG2b antibody titres were observed for the DDA based systems.

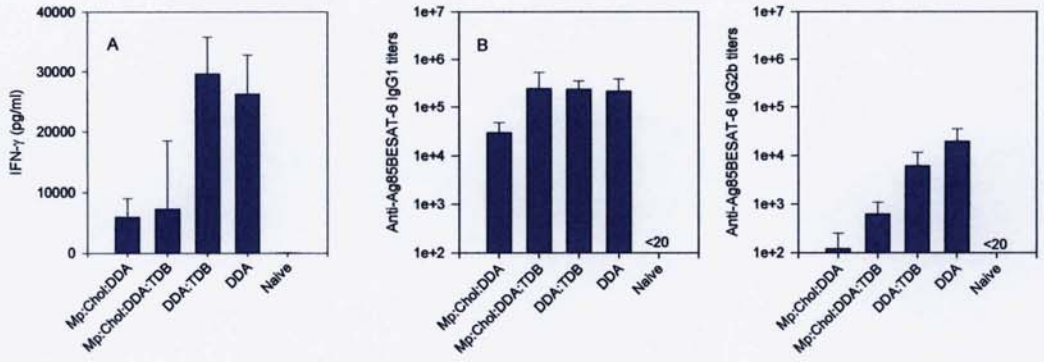


Fig. 6.12 Immune responses against the Mycobacterium tuberculosis Ag85B-ESAT-6 fusion protein generated by MP and DDA based vesicle preparations. Release of IFN-γ from spleen lymphocytes isolated from C57Bl/6j mice (n=3) immunised three times with 2 μg of Ag85B-ESAT-6 encapsulated in MP:Chol:DDA and MP:Chol:DDA:TDB vesicles or adsorbed to DDA and DDA:TDB liposomes. Splenocytes were isolated 7 weeks after the first immunisation and re-stimulated *in vitro* with the Ag85B-ESAT-6 (5μg/ml) (A). From the same mice, serum was also taken at this time point for determination of IgG1 and IgG2b Ag85B-ESAT-6-specific antibody titres (B). [*In vivo* experiments were conducted by SSI collaborators].

In contrast to tuberculosis infection where a cell-mediated Th1 immune response is essential, protection against malaria caused by Plasmodium falciparum is primarily mediated by induction of antibodies (Cohen *et al.*, 1961). The adjuvant effect of MP and DDA based vesicle preparations was thereafter investigated by immunising C57Bl/6j mice with the two malaria antigens MSP1 and GLURP. After immunisation with MSP1, high IgG1 titres were observed with all vesicle formulations (Fig. 6.13a), whereas lower IgG1 titres were observed for GLURP (Fig. 6.13c). The measured IgG2b titres induced after immunisation with MSP1 were highest for the two MP based vesicle systems (Fig. 6.13b), whereas comparable GLURP-specific IgG2b titres were observed with all four vesicle systems (Fig. 6.13d).

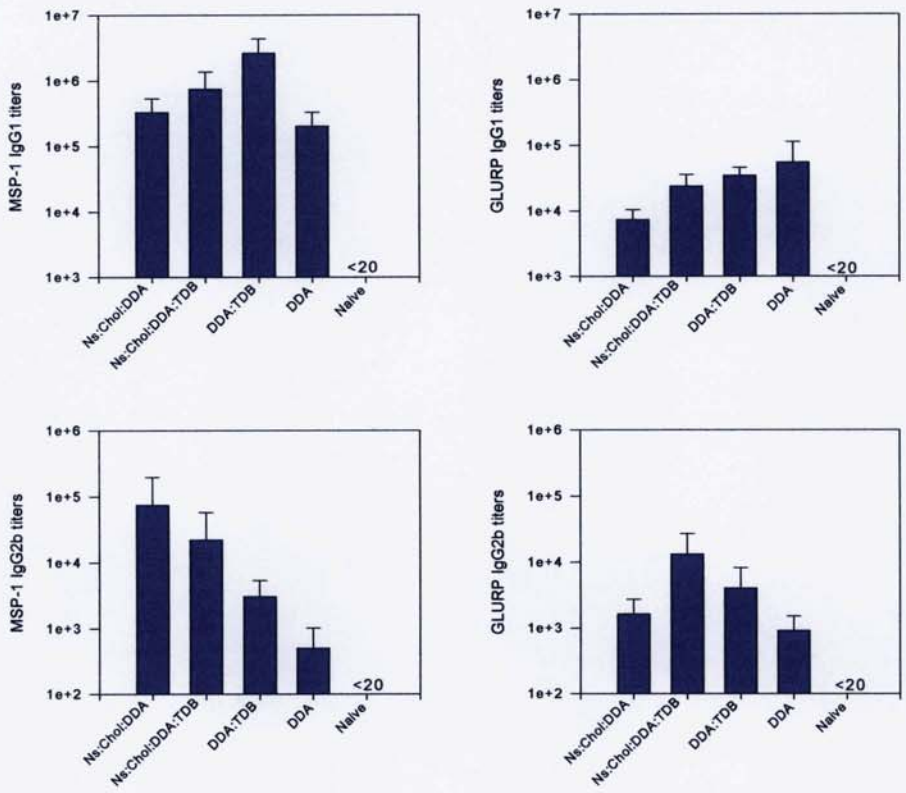


Fig. 6.13 Antibody responses against the malaria proteins MSP1 and GLURP generated by MP and DDA based vesicle preparations. C57Bl/6j mice (n=3) were immunised three times with 10 μg of MSP1 and GLURP, respectively, encapsulated in MP(Ns):Chol:DDA and MP(Ns):Chol:DDA:TDB vesicles or adsorbed to DDA and DDA:TDB liposomes. Serum was drawn 7 weeks after the first immunisation for determination of IgG1 and IgG2b MSP1- and GLURP-specific antibody titres. [*In vivo* experiments were conducted by SSI collaborators].

6.4 CONCLUSIONS

Comparison of physico-chemical characteristics (zeta potential and vesicle size) of DDA and MP based systems suggests that despite the similar cationic lipid content and hence zeta potentials of all the preparations, MP based non-ionic surfactant vesicles have measured z-average diameters approximately twice that of their DDA based liposome counterparts, this trend, apart from having relatively high surfactant content, is mainly attributed to the hydrophilicity of the lead surfactant, MP molecule (HLB, 7.2) possessing single alkyl chain with 16 carbons. Increase in vesicle size is anticipated

since the overall surface free energy of the system is said to be directly proportional to the hydrophilicity of the surfactant molecule (Wan and Lee, 1974, Yoshioka *et al.*, 1994; Uchegbu, 1994) giving rise to higher vesicle size while on the other hand opposite was true with DDA molecule (HLB, 3.6) possessing double alkyl chain with 18 carbons each, being relatively more hydrophobic in nature and hence smaller vesicle size.

In addition to these differences in physico-chemical characteristics, DDA based systems were shown to effectively enhance cell-mediated Th1 immune responses against Ag85B-ESAT-6 with high IFN-γ levels compared to their MP based counterparts while induction of antibody responses especially IgG1 titres were comparable between both the systems. A strong Th2 humoural response in terms of IgG1 titres was obtained for malarial antigens i.e., MSP1 and GLURP which were comparable between the preparations while MP based systems showed high IgG2b titres. This can be attributed to the ability of vesicles to not only protect their protein content from protease attack *in vivo* but also deliver it to APC infiltrating the site of injection or in the lymphatics. Our results suggest that both DDA and MP based systems may be useful in enhancing immunogenicity of the subunit vaccines, especially the former with the subunit antigen Ag85B-ESAT-6 against tuberculosis for which a high cell-mediated Th1 immune response is essential while the latter with malarial antigens such as MSP1 and GLURP where induction of antibody titres is vital in combating with the invading parasites.

Chapter Seven

General Conclusions

7.1 GENERAL CONCLUSIONS

Enhanced adjuvant activity by lipid and surfactant based vesicular delivery systems incorporating additional helper lipids provide useful multicomponent delivery vehicles for *in vivo* delivery of plasmid DNA and subunit antigens. In the present studies, the single-alkyl chained non-ionic surfactant, 1-monopalmitoyl glycerol (MP) based vesicle systems were extensively characterised following the initial work (Obrenovic *et al.*, 1998; Obrenovic and Gregoriadis, 1999). The physico-chemical characteristics of various MP and PC based DRV systems discussed in chapter 2, reveal some interesting aspects related to the structural properties of these molecules, for example, comparison of z-average diameter and surface charge of DRV systems suggests that despite the similar cationic lipid content and hence surface charge of comparable MP and PC based preparations, the z-average diameter of MP based DRV(DNA) was two-fold larger than its double-alkyl chained PC based counter part which is mainly attributed to the hydrophilic nature of the MP molecule and thereby increasing the overall surface free energy of the system which in turn causes a rise in the vesicle size (Yoshioka *et al.*, 1994). In addition, such MP based systems have shown to display an increased stability and increased DNA retention in the presence of competitive anions when compared to similarly formulated PC based systems. Despite these differences in physico-chemical characteristics all DRV(DNA) formulations showed high entrapment values in accordance with the previous reports (Perrie and Gregoriadis, 2000). These values predominantly reflect actual DNA entrapment within the bilayers as opposed to complexation of DNA with the vesicle surfaces which was confirmed by the gel electrophoresis. Taken together, these results suggest that similar to well established PC based DRV systems (Perrie and Gregoriadis, 2000; Perrie *et al.*, 2001; Perrie *et al.*, 2003) dehydration-rehydration technique for MP based systems in the presence of

DNA, results not only in the formation of vesicular structures (as demonstrated by the TEM micrographs) but also leads in a high incorporation efficiency. Although this DRV technique is shown to result in membrane destabilisation during freeze-drying process leading to vesicle fusion, this fusion was effectively controlled in these studies by the use of cryoprotectants such as sucrose and trehalose. Since, due to their non-eutectic nature, cryoprotectants are known to form a 'glassy' state during freeze-drying preventing the lipid fusion. They are also known to stabilise vesicle bilayers by interacting with the polar head groups of lipid molecules and in turn contributing to the maintenance of the overall vesicle size (Crow *et al.*, 1986). Indeed, a pronounced reduction in the z-average diameter of MP based system by approximately 3 fold has resulted yielding submicron sizes when sucrose was incorporated in the vesicle composition with out significantly affecting the surface charge and incorporation efficiency.

Generation of immune responses via DNA vaccination could represent an important advancement in the treatment of both genetic and acquired diseases. Thus, there has been an increased attention focused on the development of gene transfer vectors along side suitable adjuvant systems. Although several attempts has been made to use neutral or negatively charged liposomes for gene delivery, the limited efficacy of plasmid DNA-liposome interaction consequently lead to the low levels of transfection (Lasic and Templeton, 1996). The cationic vesicle systems incorporating DNA in the present studies showed a high cationic surface charge with positive zeta potential values satisfying the criteria for an efficient gene transfer vector (Behr, 1993) which necessitates the requirement of a synthetic vector to not only condense DNA but also mask its anionic nature. A positive surface charge for surfactant vesicle-DNA

complexes has also been reported to directly correlate with *in vitro* gene-transfer (Takeuchi *et al.*, 1996). Indeed, this phenomenon was observed in the present studies discussed in chapter 3, where a higher cationic lipid/DNA charge ratios yielded optimum transfection efficiency, although this efficiency was also largely influenced by the nature of the cationic lipid and also the choice of helper lipid. As it was observed that incorporation of cationic lipid, DC-Chol in the vesicle complex resulted in 6 fold increase in transfection compared to other cationic lipids such as DOTMA, DOTAP, DPTAP and DDA while the helper lipid DOPE possessing fusogenic properties induced higher levels of transfection when incorporated along with the cationic lipid DC-Chol, at similar molar ratios (1:1) compared to its cholesterol counter part highlighting the essential role played by the nature of cationic lipid, helper lipid and also the cationic lipid/DNA charge ratios of the complex. DNA displacement from the complexes in the presence of anionic molecules was observed to be lower with increase in the cationic lipid/DNA charge ratio as anticipated in gel electrophoresis studies implicating the protection offered to the DNA by the complex. Morphology of these complexes was also shown previously (Sternberg *et al.*, 1998) to have a great impact on the transfection efficiency. Some of the morphological aspects of these complexes are shown in the present studies (chapter 3) using TEM and ESEM techniques highlighting the complex structures in relation to their transfection efficiency. Incorporation of non-ionic surfactants varying in alkyl chain number i.e., mono, di or tri palmitoyl glycerol compounds in the DOPE:DC-Chol systems at various molar ratios altered the transfection efficiency dramatically although further characterisation of these systems is needed to understand the interplay between various surfactant moieties-DNA-cell membrane-cellular components.

It is well accepted that a correlation between the transfection efficiencies *in vitro* and *in vivo* is always a challenge (Lasic, 1997), since the transfection process in the biological milieu is much more complicated due to the differences in the biology, functionality and complexity between cell cultures and animals models and also due to the changes in the colloidal properties of the cationic lipid-DNA complexes upon their interaction with cells and biological fluids. Gene delivery mediated by lipoplexes is strongly dependent on their physico-chemical properties and on the cellular internalisation mechanisms. Several obstacles, including the cytoplasmic, the endosomal and the nuclear membranes are recognised to restrict the successful application of cationic liposomes to mediate transfection. However, previous DNA immunisation studies involving cationic vesicle systems were shown to promote high antibody and CMI responses against the encoded antigen (McCormack and Gregoriadis, 1998; Perrie and Gregoriadis, 1998), DNA immunisation studies in the present studies conducted in BALB/c mice, showed a little or no discrimination when compared between various vesicle compositions such as MP or PC based systems, or preparation methods i.e., DRV vs SUV or DNA encapsulated in the vesicles vs surface adsorption in spite of an antigen boost, thus making it difficult to draw a clear conclusion from these studies. However, a trend in better IgG2a responses and production of IFN-γ levels by MP based DRV(DNA) suggested a Th1 bias induced by this system which is essential for hepatitis B infection.

Despite low immune responses with hepatitis B encoded DNA vaccination, hepatitis B subunit vaccination through various vesicle systems in the present studies yielded a very potent antibody and CMI responses. Among different vesicle systems employed, all the systems showed promise compared to unadjuvated antigen and induced both antibody and cytokine (IFN-γ, IL-2, IL-6 and IL-10) responses, with DDA:TDB based

vesicular system being much more superior. The physico-chemical characterisation and adjuvant activity of this DDA:TDB system incorporating mycobacterial fusion protein Ag85B-ESAT-6 and two malarial subunit antigens MSP1 and GLURP was further studied in chapter 6 in which this system was supplemented by incorporating additional surfactants such as MP and cholesterol whose vesicle stability overtime, release characteristics and antigen retention overtime were analysed highlighting the efficiency of MP based systems in terms of stability, antigen release and retention. ESEM analysis of this MP based system (MP:Chol:DDA:TDB, 16:16:4:0.5 μmoles) conducted for the first time revealed an increased stability of the antigen incorporated vesicles compared to their 'empty' vesicle counter parts. In the immunisation studies, both MP and DDA based systems induced comparable antibody responses for all the three subunit antigens i.e., Ag85B-ESAT-6, MSP1 and GLURP while on the other hand, only DDA based systems induced powerful CMI responses against these antigens. Thus, suggesting that both the MP and DDA based systems could be useful in combating malarial infection where a strong antibody response is vital, while the DDA based system could be useful against the tuberculosis infection where an enhanced cell-mediated response is deemed to be important. Overall, the current work investigates various physico-chemical properties of surfactant vesicle systems as noted in chapter 2 and their efficacy in mediating antigen encoded plasmid DNA vaccination both *in vitro* and in mouse model as discussed in chapters 3 and 4 respectively. Further, subunit antigen based vaccination mediated by surfactant vesicle systems was studied highlighting the role of these delivery systems in enhancing immune responses compared to unadjuvated antigens which are discussed in chapters 5 and 6, where the efficient adjuvanticity of surfactant vesicle systems in generating strong immune responses against the respective antigen was emphasised.

7.1.1 Future work

In the DRV method, freeze-drying of surfactant vesicles in the presence of cryoprotectants such as sucrose resulted in a significant reduction in vesicle size in present studies while retaining a high DNA incorporation efficiency. The effect of cryoprotectants on phase transition temperature of the surfactant/lipid components could be analysed by differential scanning calorimetry (DSC) which would provide information on the stability of such systems. Knowledge of physico-chemical interaction between different vesicle components and the DNA or subunit antigen is essential, this could be studied effectively using langmuir trough by monitoring surface pressure isotherms with respect to time. *In vitro* transfection studies could be further conducted in an appropriate macrophage cell line or dendritic cells as a preliminary screening criteria for different vesicle compositions and also obtain a better correlation with immunisation experiments. Surfactant based interaction may lead to alteration of the immunological properties of hepatitis B antigen (HBsAg) as has been observed previously using a non-ionic surfactant specifically for that purpose and it has been found that delipidation using surfactant improved T cell responses, but treated HBsAg lost most of its B cell antigenicity. It is likely that vesicle interaction with the HBsAg may have had a marked effect on the immunogenicity of the HBsAg in our study discussed in chapter 5. Partitioning of lipid and surfactant components at the molecular level may occur in these systems and therefore further analysis in terms of physicochemical aspects, antigen localisation, stability and immunological characterisation of discrete epitope reactivity could supply more information on the suitability of such promising systems, their mode of action and effects on antigen recognition. Incorporation of co-adjuvants such as interleukin-2, muramyl dipeptide derivatives and lipopolysaccharides into surfactant vesicles together with the antigen

may be carried out to further enhance immune responses. In addition, receptor mediated targeting of antigen-containing vesicles to immunocompetent cells may serve as a means to induce cell-specific immunity. Recent approaches such as incorporation of oligonucleotides containing CpG sequences which are shown to have adjuvant activity could be incorporated into the vesicle systems both for protein antigens and for DNA vaccines and study their potential in increasing the immune responses.

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APPENDICES

I. Graphs

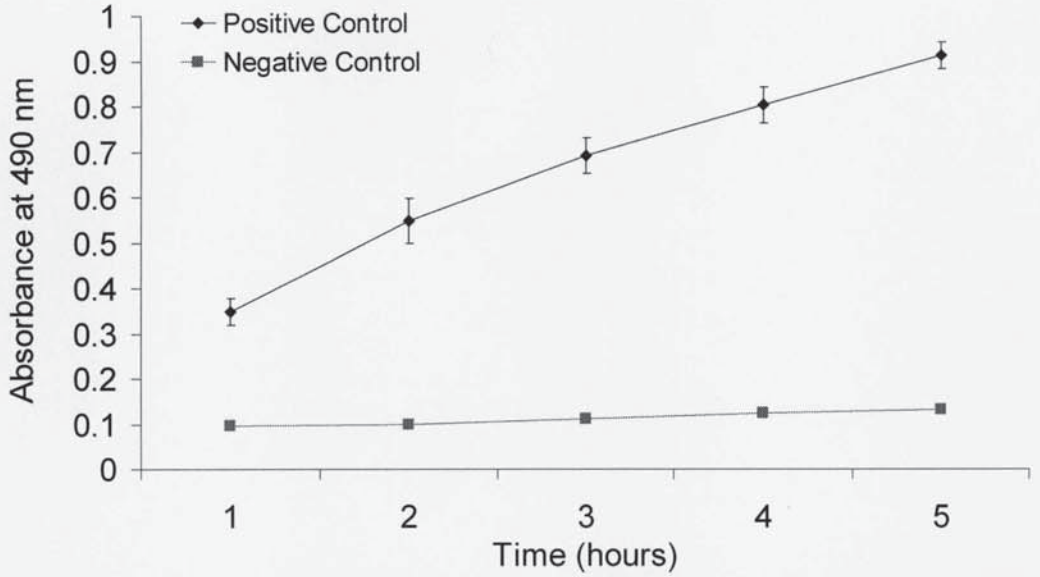

Fig. A1 Optimisation of incubation time for cell proliferation studies. In a preliminary study, the duration of incubation time was optimised. Duration of 2.5 to 5 hours was observed to be ideal for the measurement so that the absorbance of the soluble formazan produced by cellular reduction of MTS is between 0.6 to 1.0 at 490 nm which is read on 96 well plate reader.

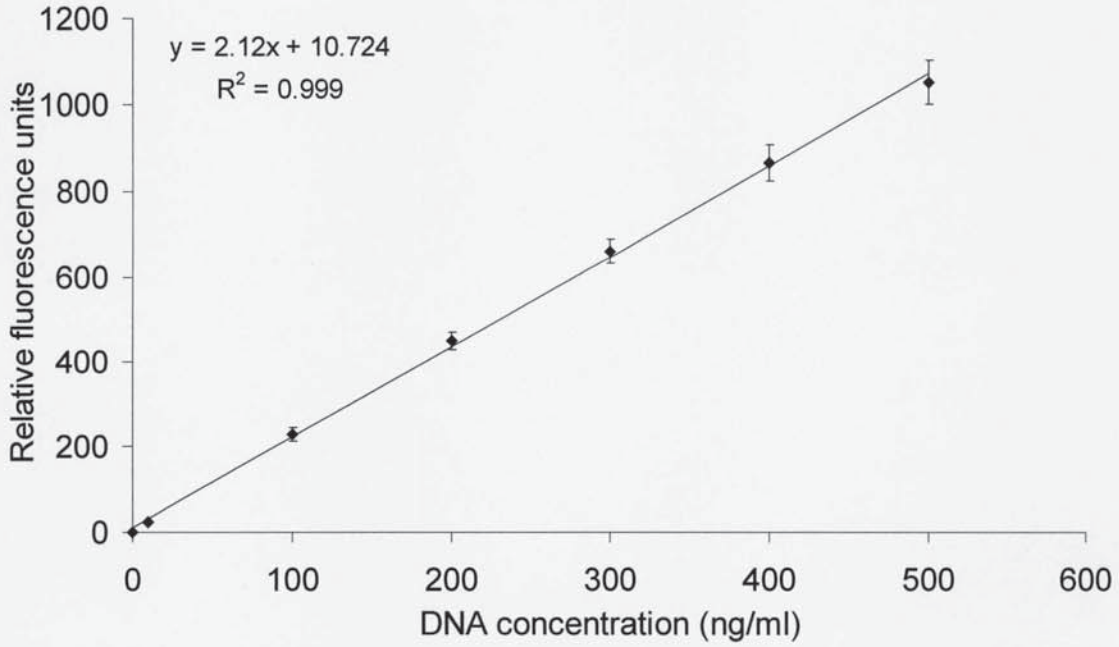

Fig. A2 Calibration curve of Plasmid DNA-PicoGreen double stranded (ds) DNA quantitation reagent.

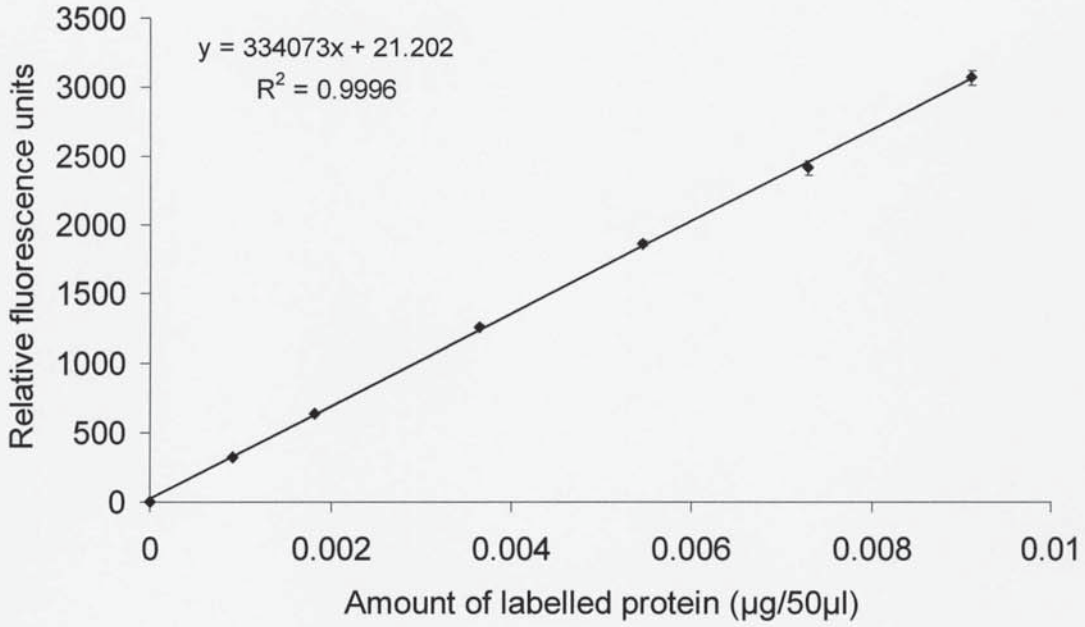

Fig. A3 Calibration curve of fluorescent labelled Ag85B-ESAT-6.

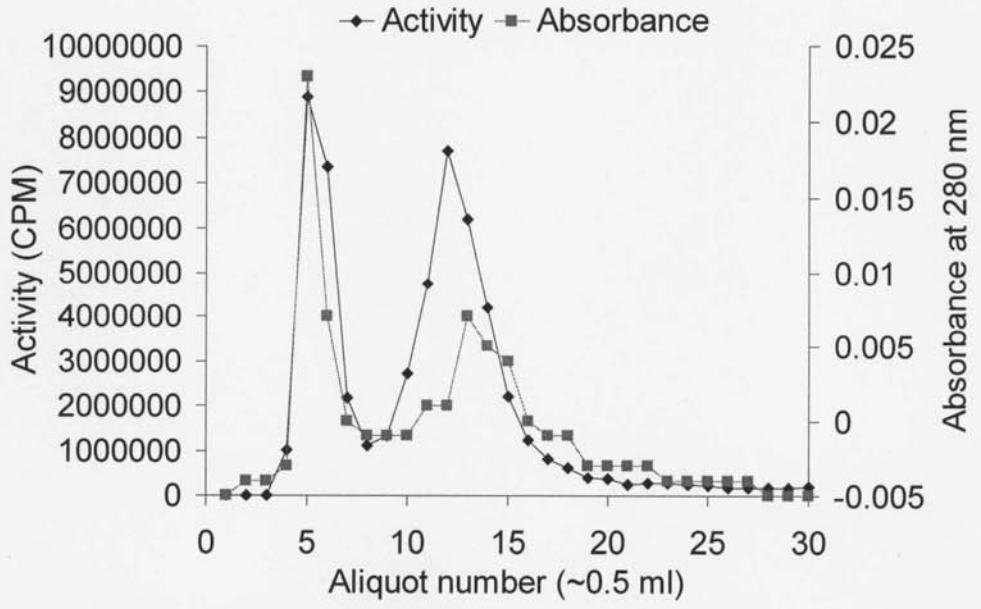

Fig. A4 ^{125}I radiolabelling of recombinant Hepatitis B antigen.

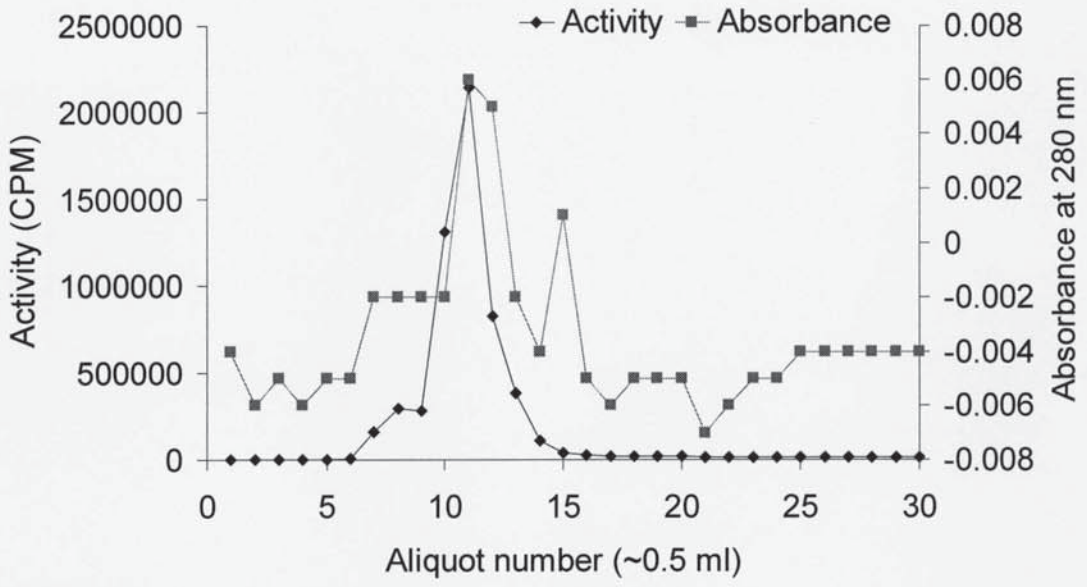

Fig. A5 ^{125}I radiolabelling of GLURP antigen.

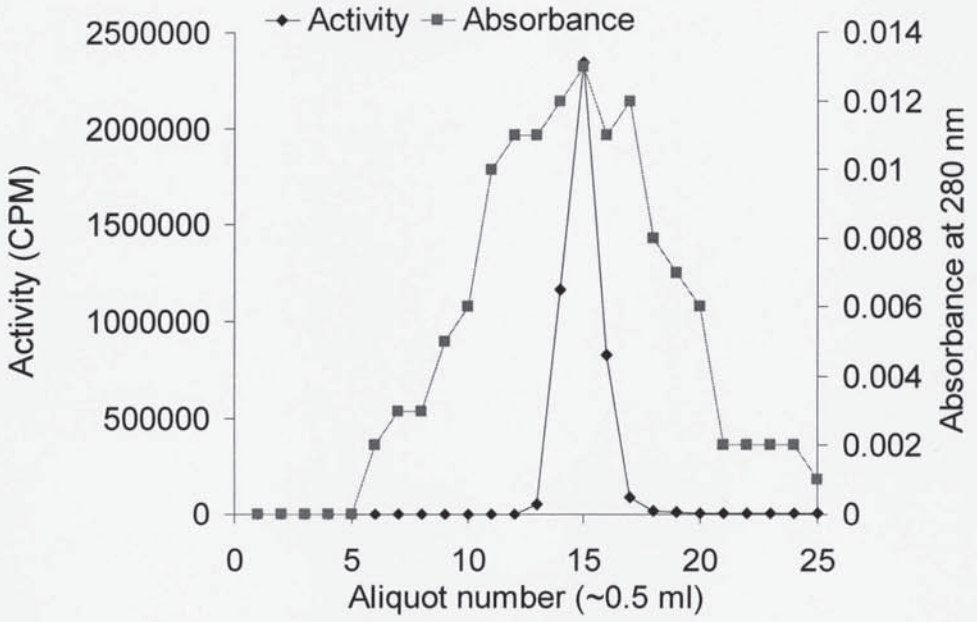

Fig. A6 125I radiolabelling of MSP1 antigen.

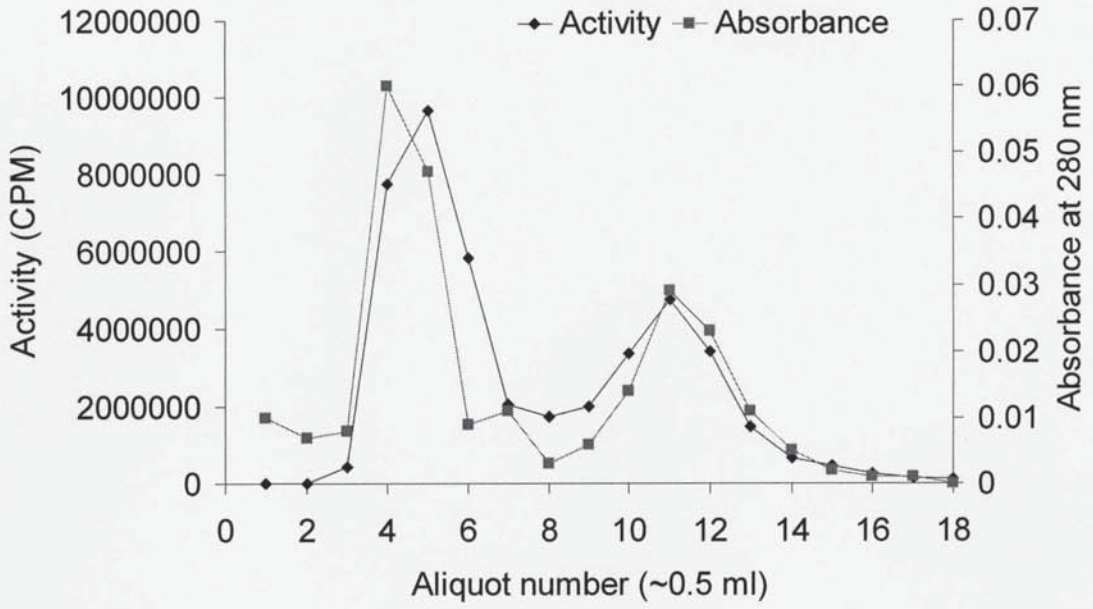

Fig. A7 ^{125}I radiolabelling of Ag85B-ESAT-6 antigen.

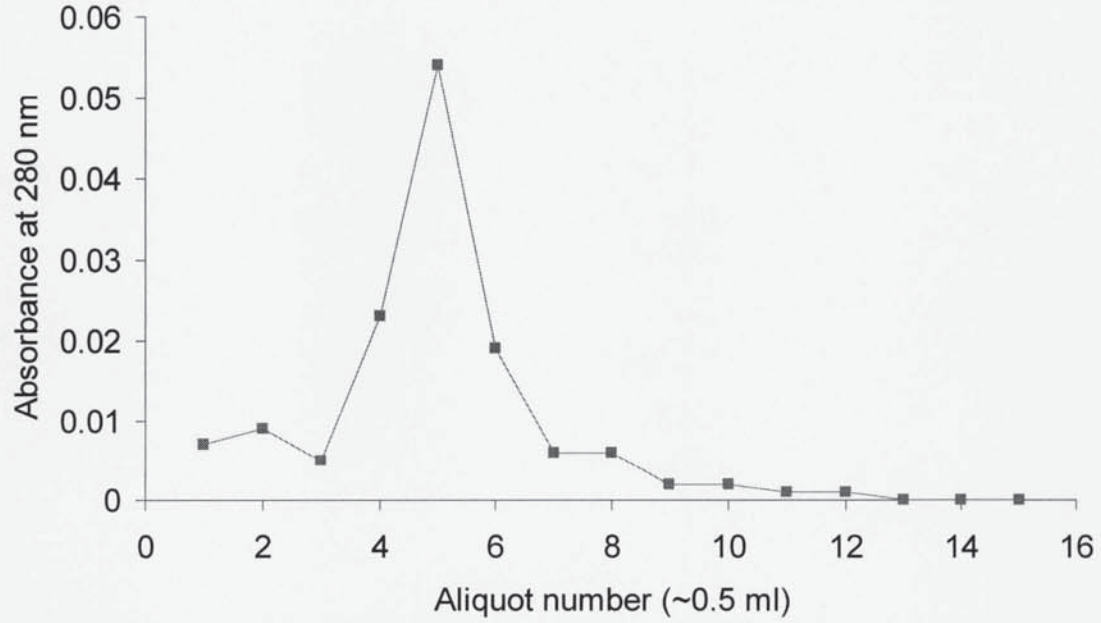

Fig. A8 Determination of Ag85B-ESAT-6 antigen peak using unlabelled antigen in the column. Out of the two peaks obtained in ^{125}I radiolabelling of Ag85B-ESAT-6 antigen (Fig. A7), the first peak was identified as the labelled antigen peak which was confirmed by passing the unlabelled antigen through the column.

II. CHEMICAL STRUCTURES OF LIPIDS AND SURFACTANTS

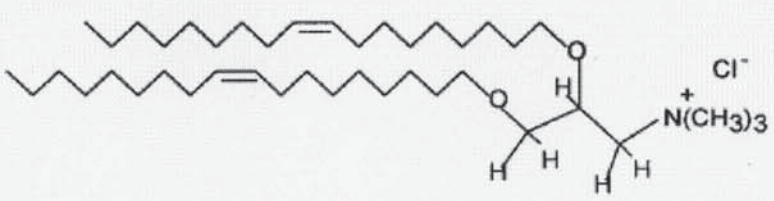

Fig. A9 *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA, 18:1), M.W. 669.50 (Source: Avanti Polar Lipids).

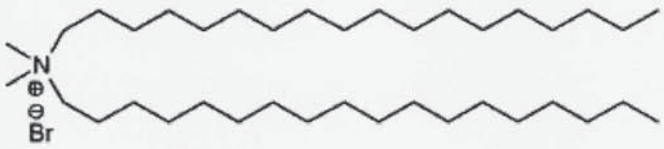

Fig. A10 Dimethyldioctadecylammonium (bromide salt) (DDA, 18:0), M.W. 630.96 (Source: Avanti Polar Lipids).

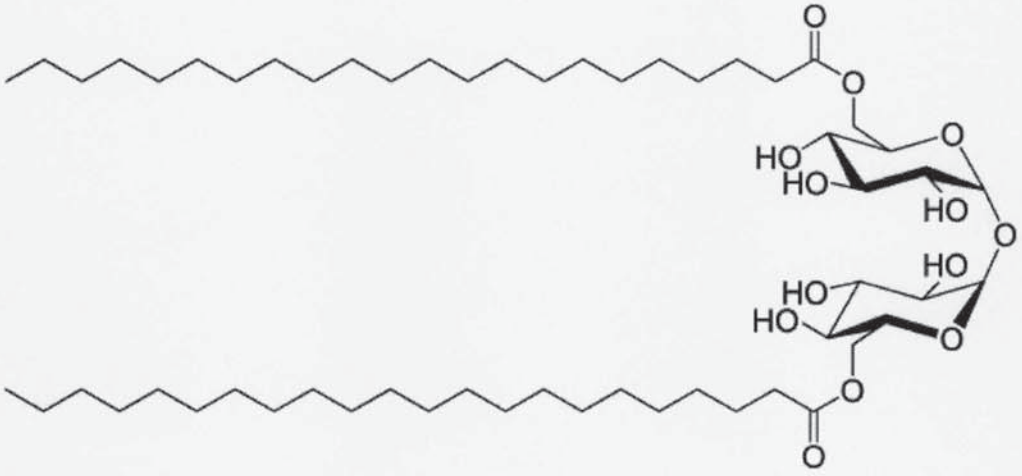

Fig. A11 D-(+)-trehalose 6,6'-dibehenate (TDB, 22:0), M.W. 987.43 (Source: Avanti Polar Lipids).

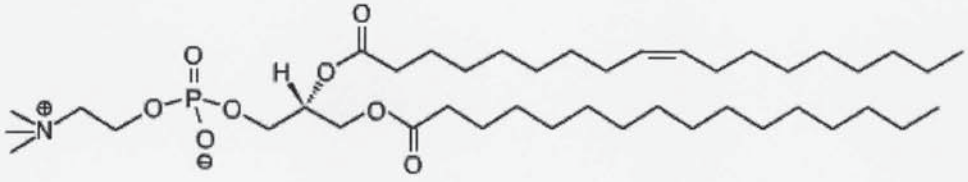

Fig. A12 L-α-egg, phosphatidylcholine, M.W. 767.0 (Source: Avanti Polar Lipids).

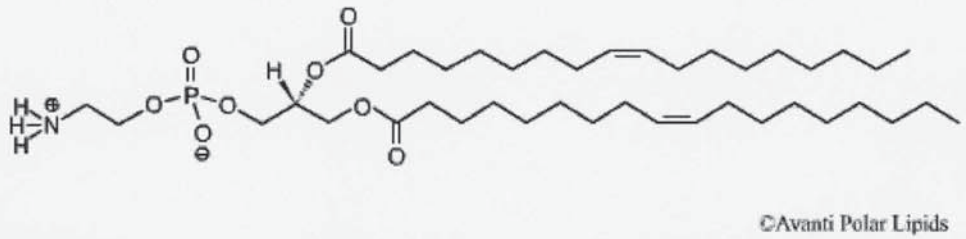

Fig. A13 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 18:1), M.W. 744.05 (Source: Avanti Polar Lipids).

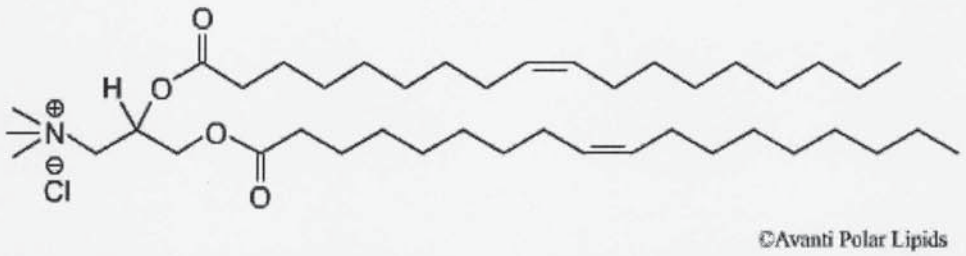

Fig. A14 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP, 18:1), M.W. 698.55 (Source: Avanti Polar Lipids).

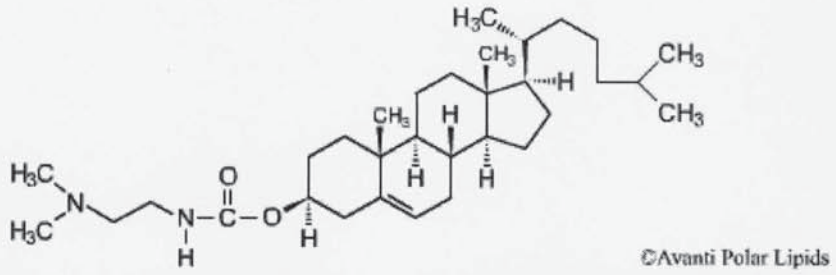

Fig. A15 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), M.W. 537.27 (Source: Avanti Polar Lipids).

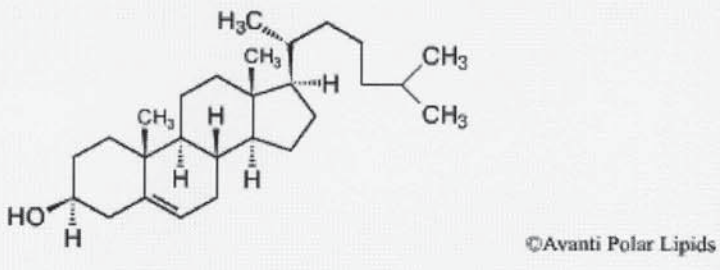

Fig. A16 3β-Hydroxy-5-cholestene 5-Cholesten-3β-ol (Chol), M.W. 386.66 (Source: Avanti Polar Lipids).

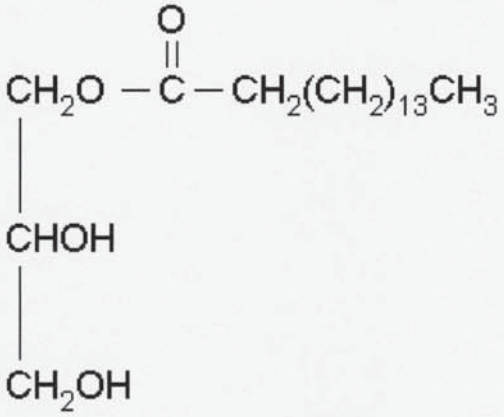

Fig. A17 1-monopalmitoyl glycerol (MP, 16:0), M.W. 330.50 (Source: Sigma-Aldrich).

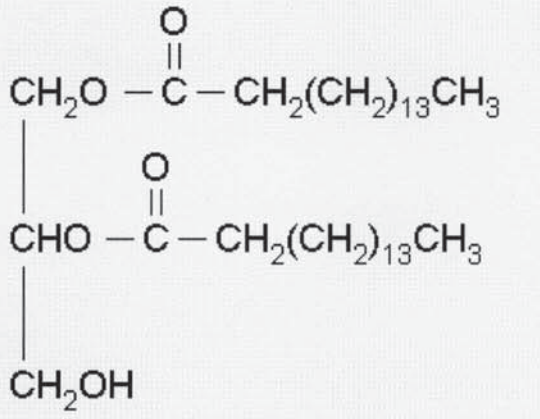

Fig. A18 1,2-dipalmitoyl glycerol (DP, 16:0), M.W. 568.91 (Source: Sigma-Aldrich).

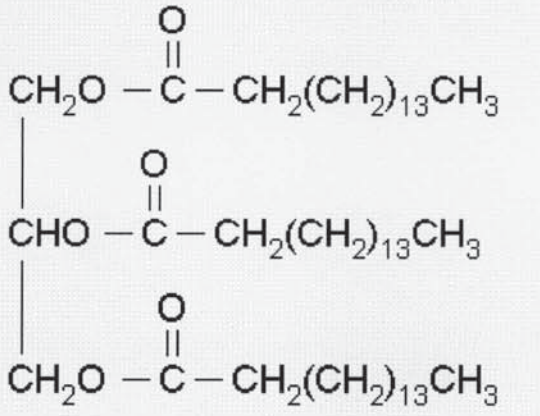

Fig. A19 1,2,3-tripalmitoyl glycerol (TP, 16:0), M.W. 807.32 (Source: Sigma-Aldrich).

III. BUFFERS AND REAGENTS COMPOSITION

I. PBST

40 g NaCl, 1 g KCl, 1 g KH_2PO_4, 7.2 g $Na_2HPO_4(2H_2O)$. 5 litres double distilled filtered water. Approx. 0.4 ml Tween 20.

II. Citrate buffer

1.02 g citric acid , 1.46 g Na_2HPO_4, 200 ml double distilled filtered water, pH 5.0.

III. Sodium carbonate buffer

0.318 g Na_2CO_3, 0.58 g $NaHCO_3$, 250 ml double distilled filtered water, pH 9.6.

IV. Block buffer for antibody ELISA

1% BSA or Marvel in PBS

V. Block buffer for cytokine ELISA

1% BSA in PBS with 0.05% NaN_3.

VI. Reagent diluent for IFN-γ and IL-2 cytokines

0.2 g BSA, 0.484 g Trizma base, 1.753 g NaCl and 0.1 ml Tween 20 in 200 ml double distilled filtered water, pH 7.2 – 7.4.

VII. Reagent diluent for IL-5, IL-6 and IL-10 cytokines

1% BSA in PBS, pH 7.2 – 7.4.

VIII. Tris-borate- EDTA (TBE) buffer 5x solution

54 g Tris base, 27.5 g boric acid, 20 ml ethylenediaminetetraacetic acid (EDTA) 0.5 M solution, makeup the volume to 1 litre, pH 8.0.

IX. Tris-EDTA (TE) buffer 1x solution

472.8 mg Tris-Cl, 87.66 mg ethylenediaminetetraacetic acid (EDTA), makeup the volume to 300 ml, pH 8.0.

RESEARCH PUBLICATIONS

Anil Vangala, Vincent W. Bramwell, Sarah McNeil, Dennis Christensen, Else Marie Agger, Yvonne Perrie (2006) Comparison of vesicle based antigen delivery systems for delivery of hepatitis B surface antigen. *Journal of controlled Release* (submitted).

Sarah McNeil, **Anil Vangala**, Peter Hanson, Yvonne Perrie (2006) Parameters influencing the transfection efficiency of liposome-DNA complexes in vitro: the effect of bilayer mechanics. *Biophysical chemistry* (submitted).

Yvonne Perrie, Afzal U.R. Mohammed, **Anil Vangala**, Sarah E. McNeil (2006) Environmental scanning electron microscopy offers real-time morphological analysis of liposomes and niosomes. *Journal of liposome Research* (accepted).

Anil Vangala, Daniel Kirby, Ida Rosenkrands, Else Marie Agger, Peter Andersen, Yvonne Perrie (2006) A comparative study of cationic liposome and niosome-based adjuvant systems for protein subunit vaccines: characterisation, environmental scanning electron microscopy and immunisation studies in mice. *Journal of Pharmacy and Pharmacology* **58**: 787-799

Jesper Davidsen, Ida Rosenkrands, Dennis Christensen, **Anil Vangala**, Daniel Kirby, Yvonne Perrie, Else Marie Agger and Peter Andersen (2005) Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6' –dibehenate) – a novel adjuvant inducing both strong CMI and antibody responses. *Biochimica et Biophysica Acta* **1718**: 22-31

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

Perrie, Y., McNeil, S., **Vangala, A**. (2003) Liposome-mediated DNA Immunisation via the Subcutaneous Route. *Journal of Drug Targeting*, **11(8-10)**: 555-563

ABSTRACTS AND ORAL PRESENTATIONS

Anil Vangala and Yvonne Perrie (2006) Application of Surfactant Vesicle Based Carrier Systems in Subunit Vaccination: Optimisation and Stability Studies, *12th United Kingdom and Ireland Controlled Release Society Symposium 2006:*12.

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A. Vangala, N. Weston and Y. Perrie (2005) Characterisation of protein antigen loaded surfactant vesicles as subunit vaccine adjuvant system: ESEM analysis of stability, *J. of Pharmacy and Pharmacology*, Abstract no. 008; **57**: S-19. *British Pharmaceutical Conference* 2005, Manchester.

Anil Kumar Vangala, Sarah McNeil and Yvonne Perrie (2005) Lipoplexes in Gene Delivery – Physico-Chemical Parameters and Analysis using ESEM, *Controlled Release Society 2005 Miami, Florida, USA.*

Anil Kumar Vangala and Yvonne Perrie (2005) Effect of Sugars on Physicochemical Characteristics of Freeze-dried Niosomes Encapsulating Plasmid DNA, *11th United Kingdom and Ireland Controlled Release Society Symposium 2005: Skin Drug Delivery: The Appliance of Science.*

D.J. Kirby, **A.K. Vangala** and Y. Perrie (2005) Immuno-modulating vesicular carriers for TB vaccines: Formulation and characterization, *11th United Kingdom and Ireland Controlled Release Society Symposium 2005: Skin Drug Delivery: The Appliance of Science.*

A.K. Vangala and Y. Perrie (2004) DNA loaded DRV niosomes: stabilization of surfactant bilayer using disaccharides, *J. of Pharmacy and Pharmacology*, Abstract no. 022; **56**: S-8. Oral Presentation at *British Pharmaceutical Conference* 2004, Manchester.

D.J. Kirby, **A.K. Vangala** and Y. Perrie (2004) Formulation of lipid-based carrier for TB vaccines, *J. of Pharmacy and Pharmacology*. Abstract no. 050; **56**: S-18.

Anil Kumar Vangala and Yvonne Perrie (2004) Surfactant vesicles - Carriers for DNA vaccines, *10th United Kingdom and Ireland Controlled Release Society Symposium 2004: Macromolecular Drug Delivery: Big Molecules Big Problems.*

Anil Kumar Vangala and Yvonne Perrie (2003) Non-Ionic Surfactant Vesicles- A potential DNA delivery vehicle, *Proceedings of the Sixth International Conference, Liposome Advances: Progress in Drug and Vaccine Delivery*, p. 134.

A.K. Vangala and Y. Perrie (2003) Preparation and Characterisation of DNA loaded non-ionic surfactant vesicles, *J. of Pharmacy and Pharmacology*. Abstract no. 101; **55**: S-41.

AWARDS

Commended Oral Presentation certificate and cash prize in short talks on "New developments in the pharmaceutical sciences" *British Pharmaceutical Conference 2004*, Manchester, UK.