LIPOSOME-BASED SUBUNIT AND DNA HEPATITIS B VACCINES

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By JYH-CHYANG YANG

Centre for Drug Delivery Research,
The School of Pharmacy, University of London,
29-39 Brunswick Square, London, WC1N 1AX.

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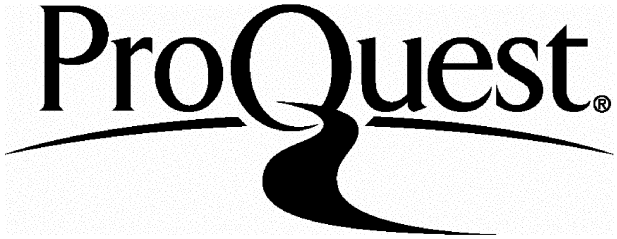

To My Parents and Sister

There are only two ways to live your life.

One is as though nothing is a miracle.

The other is as though everything is a miracle.

- Albert Einstein (1879-1955)

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Through these years' working for this PhD study, there are a lot of things must be mentioned and devote my honest thanks. At first, the financial support from the Ministry of Education in Taiwan, Republic of China makes this research be possible. At the same time, I must give a deep slate to my supervisor, Professor Gregoriadis, for giving me this opportunity to try whatever I want to try. Then, I should thank all the people had helped me in these years, and their kindness will keep in my mind forever. The colleagues in The School of Pharmacy, especially in Centre for Drug Delivery Research, had given me so much help in these years. Janny, my senior classmate from China, had helped me to pass through the most difficult stage for adaptation. Yvonne, Mia and Ibrahim helped me in the animal works. The friendships from all staffs in Centre for Drug Delivery gave me warm feeling in these years. They shared the experience with me and helped me in language expression through this thesis. In addition, the staffs in the animal house took care of mice for me. This made the animal works be processed well without any troubles. Mr. McCarthy and the staffs in the electron microscopy unit supplied the excellent technique to catch liposomal morphology so wonderfully, and the staffs in the computer unit helped me to prepare the presentation and thesis. The suggestions from my examiners, Dr. Alpar and Dr. Taylor, made this thesis be more readable. All these contributions made this job be done. Outside the School, I shared a lot of friendship from my Taiwanese friends in London. They made my life here be more versatile and comfortable. Finally, I must thank my teachers that teach me during my education process to make me have the ability to finish this project. Gods, my parents and sister gave me the psychological support and encouraged me to face the challenge.

ABSTRACT

It has been shown that liposomes have immunological adjuvant properties and earlier evidence suggested that liposomes could play a role in vaccination against hepatitis B. The objective of this thesis is to optimize the liposomal formulations for hepatitis B vaccines and to investigate the effect of these formulations on antibody induction by hepatitis B surface antigen (HBsAg). In the present studies, the work on liposomes as a carrier for a hepatitis B vaccine was carried out and promising formulations were selected for further research. The HBsAg and plasmid pRc/CMV HBS DNA were entrapped into liposomes and these formulations were evaluated in terms of preparation procedures, characteristics, stability upon storage and *in vitro* release behaviours. Recombinant HBsAg and a plasmid DNA (pRc/CMV HBS) encoding for HBsAg (S region) were entrapped into liposomes by the dehydration-rehydration method yielding dehydration-rehydration vesicles (DRV). The effect of lipid composition, vesicle size, protein characteristics, routes of administration and direct or indirect immunisation were studied. To investigate the adjuvant effect of liposomes, the mice were immunised with a variety of formulations. HBsAg-containing DRVs composed of DSPC/Chol (1:1 molar ratio) elicited stronger IgG responses in mice than liposomes composed of PC/Chol (1:1 molar ratio). Furthermore, microfluidised HBsAg-containing DRVs (smaller vesicle size) did not influence the IgG responses. However, HBsAg-containing DRVs prepared in the presence of sucrose, which have a similar size to microfluidised DRVs, were found to enhance the IgG responses to HBsAg. In addition, mannosylated HBsAg changes the adjuvant effect of liposomes. Cholesterol-containing DRVs did not enhance the IgG responses to mannosylated HBsAg, but DSPC/DOPE DRVs induced stronger IgG responses in mice immunized with mannosylated HBsAg. There was improvement of immunological adjuvant properties of liposomes by the oral route by conjugating cholera toxin B (CTB) to HBsAg-containing DRVs. Furthermore, a novel method was produced to prepare the liposomal formulations of DNA by entrapping calcium phosphate-DNA complexes into the liposomes (capisomes). Capisomes incorporating pRc/CMV HBS plasmid were evaluated in mice in terms of humoral immunity to the encoding antigen. From these studies, liposomes were shown to be versatile as immunological adjuvants for protein or DNA vaccines, or as a carrier for a combination of the two types of vaccines to develop single-dose vaccines against hepatitis B. HBsAg-containing DRVs prepared in the presence of sucrose can enhance the antibody responses to HBsAg. CTB-conjugated DRVs provide an opportunity to deliver antigen orally and capisomes offer another delivery system for DNA vaccination against hepatitis B. Further studies for these formulations are recommended.

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Abbreviations

APC	antigen presentation cell
BCA	bicinchonic acid
BSA	bovine serum albumin
CaPi	calcium phosphate
Chol	cholesterol
CMI	cell-mediated immunity
CMV	cytomegalovirus
CT	cholera toxin
CTB	cholera toxin B subunit
CTL	cytotoxic T lymphocyte
dATP	deoxyadnosine 5'-triphosphate
DC-Chol	3((N-(N',N')-dimethylaminoethane)-carbamoyl) cholesterol
DNA	deoxyribonucleic acid
DOPE	dioleoyl phosphatidylethanolamine
DRV	dehydration-rehydration vesicle
DSPC	distearoyl phosphatidylcholine
DSPE	distearoyl phosphatidylethanolamine
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbant assay
FCA	Freund's complete adjuvant
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBS	hepes buffered saline
HDL	high-density lipoprotein
HI	humoral immunity
HLA	histocompability leukocyte antigen
IFN	interferon
IgG	immunoglobulin G
IL	interleukin

IM	intramuscular
ISCOM	immune stimulating complexes
kD	kilodaltons
LUV	large unilamellar vesicle
LPS	lipopolysaccharide
MDP	muramyl dipeptide
MPL	monophosphoryl lipid A
MTP-PE	muramyl tripeptide phosphatidylethanolamine
MHC	major histocompatiability complex
MLV	multilamellar vesicle
OD	optical density
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
RES	reticulo-endothelial system
SAF	syntex adjuvant formulation
SDS	sodium dodecyl sulfate
sIgA	secretory immunoglobulin A
SMCC	(4-N-maleimidomethyl)-cyclohexane-1-carboxylic acid-N-hydroxysuccinimide ester
SPDP	3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester
SUV	small unilamellar vesicle
TCA	trichloroacetic acid
TDM	trehalose dimycolate
TEM	transmission electron microscopy
Th	T-helper
SEM	scanning electron microscopy

Chapter 1

General Introduction

1.1 LIPOSOME TECHNOLOGY

The ability of liposomes to entrap solutes to which they were selectively permeable made the system a formidable model for cell membrane, and heralded a proliferation of studies on cell biophysics. This sequestration of solutes also formed the basis of the liposome drug-carrier concept that was proposed and demonstrated in the early 1970s by Gregory Gregoriadis and Brenda Ryman (Gregoriadis and Ryman, 1971). Subsequent work by Gregoriadis's group established the feasibility of using the system with biomolecules, hydrophilic and hydrophobic drugs, and also its ability to potentiate immune responses to entrapped antigens (Allison and Gregoriadis, 1974).

The most common areas of application for liposomes are in cosmetics and medicine. When cheaper liposomes become available, other applications, such as food, ecology, agriculture, and coating industries may also benefit. The use of liposomes in medical applications had been studied in drug delivery, diagnostic imaging agents, vaccine adjuvant and gene therapy. In the ensuing years, liposomes were adopted by numerous researchers as the vehicle of choice for drug and vaccine delivery and targeting (Gregoriadis, 1990). Tailoring the structural characteristics of liposomes to take advantage of certain properties of the biological environment has led to the production of a variety of vesicles for optimal drug transport and action. There are many proposed therapeutic applications which are based on successful results with experimental animal models of disease (Gregoriadis, 1988).

Extensive studies on the behaviour of liposomes and its control within the biological environment, as well as great leaps in liposome technology, have recently culminated in the development of several injectable liposome-based formulations that have been licenced for use in humans (Table 1.1). In terms of licenced products, no such progress has as yet been made with the plethora of other drug-delivery systems, including the exquisitely specific monoclonal antibodies. This may be attributed to the innocuous nature of liposomal components, and the structural versatility of the system. However, the most promising applications rely either on the spontaneous uptake of liposomes by the reticulo-endothelial system (RES), or on the extended retention of small liposomes in the circulatory system for intravascular or extravascular access to cells, such as cancer or infected cells. Over the past 20 years, there has been a substantial investment in the research and development of liposome (or lipid-based) formulations destined for such applications. Some of the resulting formulations are already licenced for use incertain European countries and elsewhere, including the USA; some are awaiting approval, and others are in phase I-III clinical trials (Table 1.1).

Table1.1: Liposome-based products developed or under development.

Product	Composition	Drug	Disease	Status
Therapeutic Agents				
Liposomal nystatin	unavailable	Nystatin	fungal infection	Phase II
Liposomal tretinoin	unavailable	retinoic acid	leukaemia	Phase II
Liposomal annamycin	unavailable	Annamycin	Kaposi's sarcoma	Phase II
AmBisome	HSPC/Chol/DSPG	Amphotericin B	fungal infection	Approved in Europe & USA
DaunoXome	DSPC/Chol	Daunorubicin	Kaposi's sarcoma, breast cancer	Approved in UK Phase II

Product	Composition	Drug	Disease	Status
MiKasome	HSPC/Chol/DSPG	Amikacin	bacterial infection	Phase II
VincaXome	DSPC/Chol	Vincristine	solid tumours	preclinical trial
Doxil	HSPC/Chol/PEG-DSPE	Doxorubicin	Kaposi's sarcoma; other cancers	Approved by FDA
Amphocil	Cholesteryl sulphate	Amphotericin B	fungal infection	Approved in Europe
D99	Egg PC/ Chol	Doxorubicin	breast cancer	Phase III
ABLC	DMPC/DMPG	Amphotericin B	fungal infection	Approved in UK
C53	Egg PC	Prostagladin E_1	systemic inflammory diseases	Phase II
Vaccines				
Newcastle disease vaccine	Novasomes (Cetech-2, Chol, DCP)	Killed Newcastle disease virus	Newcastle disease (chicken)	Licenced by USDA
Avian rheovirus vaccine	Novasomes (Cetech-2, Chol, DCP)	Killed Avian rheovirus	rheovirus infection (chicken)	Licenced by USDA
E. coli 0157:H7 vaccine	Novasomes (glycerol monostearate, β-sitosterol, soybean oil, oleic acid, polysorbate 60)	Killed E. coli 0157:H7	E. coli 0157 infection	Phase I
Shigella flexneri 2A vaccine	Novasomes (glycerol monostearate, β-sitosterol, soybean oil, oleic acid, polysorbate 60)	Killed S. flexneri 2A	S. flexneri 2A infection	Phase I
Epaxal-Berna vaccine	IRIV liposomes Egg PC, PE and H1N1 influenza virus phospholipids	Inactivated hepatitis A virons	hepatitis A	Approved in Switzerland
Influenza-Berna vaccine	IRIV liposomes Egg PC, PE and H1N1 influenza virus phospholipids	Haemagglutin & neuraminidase	influenza	Phase II
HAV/HBs-IRIV combined vaccine	IRIV liposomes Egg PC, PE and H1N1 influenza virus phospholipids	HAV and HBsAg	hepatitis A and B	Phase I
Diphtheria/tetanus/hepatitis A combined vaccines	IRIV liposomes Egg PC, PE and H1N1 influenza virus phospholipids	Diphtheria and α and β tetanus toxoids; HAV	Diphtheria, tetanus, hepatitis A	Phase I
Hepatitis A and B/ diphtheria/ tetanus/ influenza combined vaccine	IRIV liposomes Egg PC, PE and H1N1 influenza virus phospholipids	HAV; Diphtheria and α and β tetanus toxoids	hepatitis A and B, Diphtheria, tetanus, and influenza	Phase I

(Adapted from Gregoriadis, 1995)

Eventually, the successful evolution of liposomes from experimental tools to industrially manufactured products for clinical and veterinary use depends upon efficient entrapment of substances in vesicles of a narrow size distribution using simple, reproducible and inert methods. In this respect, there has been considerable success and well defined formulations containing a variety of active agents can now be produced in a stable form. However, several of the methods developed, although highly efficient, had the drawbacks of being uneconomical, applicable only to drugs of low molecular weight or of requiring detergents, the use of sonication or organic solvents. All the developed methods of liposomal preparation are limited to the research level and pose difficulties for approval in pharmaceutical production. Therefore, optimizing the preparation method of liposomes for adaption to pharmaceutical regulations plays a critical role toward the formulation of a medical product.

1.1.1 Preparation of liposomes

Liposome preparation techniques have been described in a number of review articles and books, and the general pattern in the different preparation procedures can be discerned : (1) the lipid must be hydrated, then (2) liposomes have to be sized, and finally (3) the non-encapsulated substance has to be removed. In some preparation schemes, the hydration and sizing steps are combined.

The first stage, hydration, is the process in which lipids are dispersed in water, and can be operated by mechanical methods, methods based on replacement of organic solvents by aqueous media, methods based on detergent removal, and methods based on size transformation

and fusion or on pH adjustment. A certain degree of particle size control over the liposome dispersions can be achieved by proper selection of lipid film thickness, and nature of the lipid. Upon hydration of the freeze-dried liposomes in small volume of water, large liposomes are generated again, and high encapsulation efficiencies are encountered (Kirby and Gregoriadis, 1984). The material to be encapsulated can be added before freeze-drying (Kirby and Gregoriadis, 1984) or in the rehydration medium (Ohsawa *et al.*, 1984). As cryoprotectants tend to protect biomembrane structures, their presence may interfere with the formation of the desired liposome structure during freeze-drying/rehydration procedure. The liposomes formed by dehydration/ rehydration in the absence of cryoprotectants are heterogeneous in size.

The next stage, sizing is the procedure to control the vesicle size in liposomal preparation. The vesicle size can be reduced by mechanical methods including sonication (Saunders *et al.*, 1962), high-shear homogenization (Talsma *et al.*, 1989) and extrusion (Olson *et al.*, 1979). Sonication had been used to reduce liposome sizes since the 1960's, and probe sonicators are more effective than bath sonicators because higher energy power densities can be reached. Generally, these methods produce vesicles in the size range below 0.2μm.

The final stage for the preparation of liposomes is removal of nonencapsulated material, which can cause the side effects or physical instability (Nicolay *et al.*, 1985). For removing the nonencapsulated material, the following techniques can be applied: (a) dialysis and ultrafiltration, (b) ultracentrifugation, (c) gel chromatography, (d) ion exchange. Following removal of non-encapsulated materials, the entrapment efficiency of substance into the liposomes and subsequent

biological studies can be carried out *in vivo*.

1.1.2 Entrapment of antigens and other substances into liposomes

An assortment of molecules, including proteins and DNA, have been entrapped in liposomes, which can be administered by different routes. Therapeutic agents, such as drug molecules, proteins and DNA , can be encapsulated in the aqueous space or intercalated into the lipid bilayer; the exact location of the substances in the liposome will depend on its physicochemical characteristics and the composition of the lipids. To develop liposomes as the pharmaceutical dosage form, considerable attention must be given toward the characterisation of liposomes and reproducibility.

The entrapment of therapeutic substances in liposomes has been studied for thirty years. The factors influencing encapsulation efficiency, which have been investigated, include the characteristics of liposomes, preparation method, addition of complex agents with substances and the characteristics of entrapped substances (Kulkarmi *et al.*, 1995). To obtain optimum encapsulation of the substances into a liposomal preparation, parameters influencing both the liposomes and the substances need to be carefully considered during the preformulation stage.

Regarding the type of vesicles, multilamellar vesicles (MLVs) are suitable for the encapsulation of bilayer-interacting hydrophobic substances, and less appropriate for hydrophilic substances. For hydrophilic drugs, large unilamellar vesicles (LUVs) are convenient because of their large entrapped aqueous volume, whereas sonicated unilamellar vesicles(SUVs) have a

very small encapsulation volume (Stamp and Juliano, 1979). However, encapsulation of highly hydrophobic substances is affected to a lesser extent by vesicle type, since they remain entrapped within the phospholipid bilayers.

The choice of phospholipids is often limited to the family of phosphatidyl choline (PC) and phosphatidyl glycerol (PG), mainly due to toxicological considerations, the availability and the cost of pure compounds. Lipids with a negative charge are often used along with PC in order to improve the loading efficiencies. However, the fluidity of bilayers is also affected by addition of detergents or surface active agents (Downing *et al.*, 1993). Furthermore, incorporation of glycolipids in phospholipid bilayers affects encapsulation of some hydrophilic substances into MLVs or LUVs. Most of the liposomes are permeable and not sufficiently rigid, which is the primary reason for leakage during storage period. Frequently, cholesterol is incorporated into lipid bilayers to impart rigidity. In the liposomal formulations for DNA, dioleoyl phosphatidylethanolamine (DOPE) is well-known to contribute the induction of humoural and cell-mediated immune responses to the encoded antigen by plasmid DNA-containing liposomes.

In the preparation methods of liposomes, the effective hydration of the bilayers can be achieved by the formation of a thin film of lipid with larger surface area. The film hydration time, quantity of water used for hydration, and conditions of agitation are important factors for obtaining maximal encapsulation in a reproducible size range of MLVs. The preparation of pro-liposomes offers high surface area of dry lipids and the support material dissolves to yield a large lipid surface for hydration. The entrapment efficiencies of substances in liposomes produced by

this method are influenced by the nature and the particle size of the support materials. Dehydration-rehydration method (Kirby and Gregoriadis,1984) is the other way to prepare the liposomes with high capture volume. In this method, a suspension of SUVs is lyophilized with the entrapment substances and rehydrated with water to form MLVs with a high capture efficiency.

Most of the liposome-producing techniques have drawbacks, with some being applicable only to certain drugs of low molecular weight and others requiring the use of detergents, sonication or organic solvents in the presence of the drug destined for entrapment. The DRV method produces multilamellar vesicles by a process based on the dehydration of a buffer, or water-loaded SUVs in the presence of free drugs. Subsequent rehydration leads to the formation of multilamellar vesicles (dehydration-rehydration vesicles; DRVs) entrapping 80% or more (Figure 1.1). Further, after freeze-drying in the presence of a cryoprotectant such as sucrose, DRVs retain most of the substance content within the vesicles that re-form on reconstruction with water (Zadi and Gregoriadis, 2000). Recently, a combination of DRVs and the technology for size controlling can generate liposomes of different vesicle size composed of a variety of lipids. These vesicles contain one or more antigens and co-adjuvants. Also, the vesicles with the defined average diameters are suitable for a wide range of applications in vaccinology.

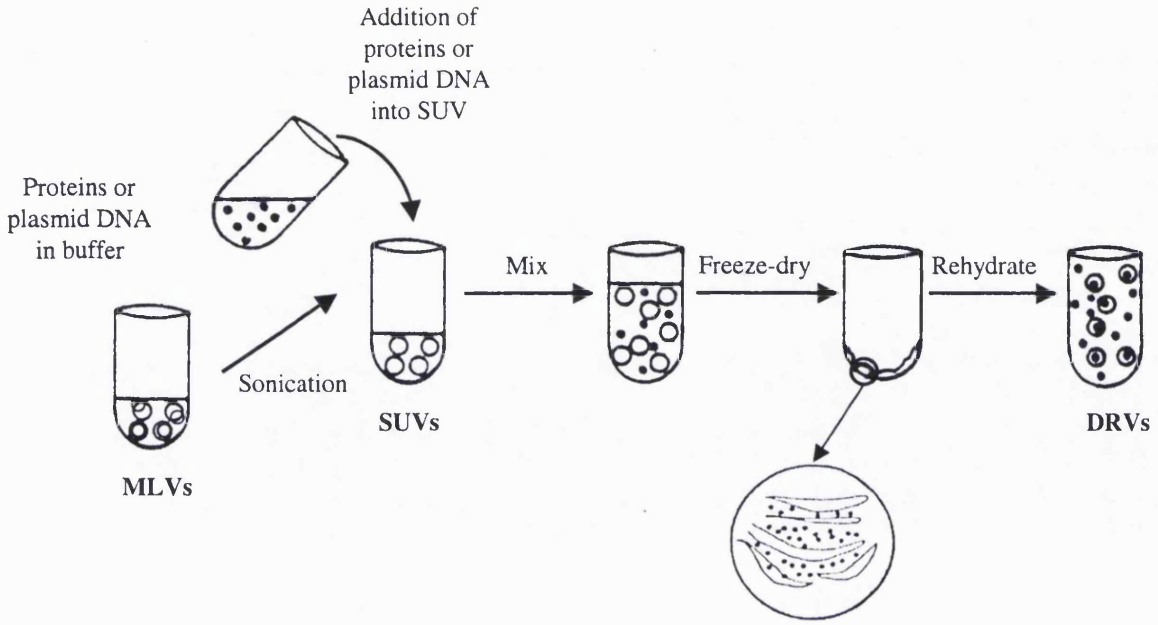

Figure 1.1: Procedures of protein and plasmid DNA entrapment into dehydration-rehydration vesicles. A solution of molecules (proteins or DNA) is mixed with a suspension of SUVs. Subsequently, the mixture is freeze-dried. The inset shows intimate contact of flattened liposomal membrane structures and drug molecules in a dry environment. Liposomes formed upon controlled rehydration contain much of the original molecules in the entrapped form (Kirby and Gregoriadis,1984).

1.1.3 Stability of liposomal formulations

The majority of published studies concerning liposomes were conducted at research level, and the liposomes were typically discarded after a few weeks. Liposome stability is a complex issue and encompasses the physical and chemical stability of the liposome preparation as well as their stability upon application, such as administration into human body. Despite the fact that, with current advances, liposomes can be physically stable for years but their stability *in vivo* may be limited.

One of the obstacles to formulating liposomes commercially is their physical stability (Fildes,1981). For pharmaceutical applications, acceptable liposomes should be stable for eighteen to twenty-four months, preferably at room temperature. Possible stability problems include leakage of entrapped substances, change of liposomal structure, which includes size and aggregation/ fusion, and sedimentation. In addition, the stability of colloids is governed by DLVO (Derjaguin, Landau, Vervey and Overbeck) theory (Kayes, 1977). This theory introduced the fundamental idea that the understanding of complex colloidal phenomena could be based on the concept of long-range forces, both attractive and repulsive, acting between assemblies of atoms, molecules or particles. Consequently, the electrostatic repulsion force from the polar group on the liposomal surface will balance with the attraction from the vander Waal or hydrophobic forces. This balance facilitates the stabilisation of liposome suspension in the media. Therefore, measuring and controlling the electrostatic properties of lipid vesicles is crucial for the basic understanding of the colloidal stability of liposomes. The most convenient method for the electrostatic characterisation of liposomes to date is measurement of their zeta potential.

Lyophilization (freeze-drying) is an excellent method to extend the shelf life of compounds with stability problems in aqueous media. These compounds are stored and distributed in a dry form for reconstitution immediately prior to use (Sandra and Pagano, 1979). Lyophilization and reconstitution of liposomes can be achieved without a significant effect upon liposome size, release properties, ion gradients or trapping ability. Cryoprotectants, such as sugars, are normally required, and preserve liposome structure by replacing the water molecules normally bound to the phospholipid headgroups in the anhydrous state. When lyophilized

liposomes are reconstituted, water quickly replaces the sugar molecules, and the liposomes appear to reseal before significant leakage of the entrapped contents can occur. Taking this into account, when dry storage of liposome is absolutely necessary, the development of a freeze-dried formulation appears to be highly feasible.

The stability of liposomes after intravenous injection is affected by blood. Plasma high-density lipoproteins (HDL) destabilize liposomes, probably by the removal of phospholipid from the bilayers. This, however, can be curtailed by rendering bilayers more packed through the presence of cholesterol. Interestingly, cholesterol-rich liposomes are found to be more stable in whole blood than in the serum, which may due to a HDL-erythrocyte interaction that takes preponderance over HDL-liposome interaction in turn reducing the amount of HDL available (Hui and Harmony, 1979). On the other hand, it is also possible that cholesterol transfers from erythrocytes to liposomes (Jones and Nicholas, 1991), which renders the later cholesterol-rich. These are known to be more stable in the circulating blood.

Depending on the route of administration and *in vivo* distribution, liposomes can be engulfed by the macrophage and transported elsewhere *in vivo*. Macrophages, one of the most important components of the complex immune defence system, play a major role in clearance of foreign particulate matter, including liposomes and other colloidal particles, from the blood circulation. On the molecular level, liposomal clearance consists of two steps: opsonization by blood proteins followed by macrophage uptake of these opsonin-marked liposomes. Opsonins are antibodies or products of complement activation in blood serum (Devine and Marjan, 1997)

that causes bacteria or other foreign particles to become more susceptible to phagocytosis. These opsonins have different properties and affinities for cholesterol-rich and cholesterol-free egg phosphatidylcholine liposomes (Maghimi and Patel, 1988). Liposomes exhibiting increased protein-binding values *in vivo* were shown by immunoblot analysis to bind more immune opsonins, leading to a higher probability of phagocytic uptake (Chonn *et al.*, 1992). The macrophages take up liposomes via complement-dependent and independent mechanisms depending on the cholesterol content of the liposomes (Huong *et al.*, 1998). This uptake can be referred to as passive targeting. Consequently, liposomes can be applied in parasitic diseases of the mononuclear phagocytic system, vaccination, or drug-mediated activation of macrophages into anti-tumour, antimicrobial or antiviral therapy.

According to the properties of liposomes overviewed, liposomes have the advantage of delivering antigens *in vivo* for the induction of immune responses. Also, the dehydration-rehydration method shows the possibility of entrapping proteins and plasmid DNA encoding protein. The stability of these formulations *in vitro* and *vivo* must be monitored to evaluate their acceptability in practical application. Furthermore, the biological processing of liposomes will be the determining factor for their adjuvanticity for vaccine applications.

1.2 LIPOSOMES AS IMMUNOLOGICAL ADJUVANTS

The structural versatility of liposomes as well as their biodegradable, innocuous nature and similarity to biological membranes are the basis of their use (Gregoriadis, 1976). Subsequently, a lot of drugs, including antitumour and antimicrobial agents, chelating agents,

peptide hormones, enzymes, other proteins, vaccines and genetic material were incorporated into the aqueous or lipid phase of liposomes of various sizes, compositions and other characteristics by an ever increasing number of technologies (Vemuri and Rhodes, 1995). As a result, liposomes have been studied extensively in terms of both behaviour *in vivo* following administration enterally or parenterally, and pharmacological effect exerted by their drug contents either in experimental animals or clinically (Lopez-Berestein and Fidler, 1989).

The application of liposomes as immunological adjuvant in this studies involved looking at different aspects. The properties of liposomes as immunological adjuvants, the immunoadjuvant activities of this system and the attempts to improve such activity were overviewed.

1.2.1 Immunological adjuvants

The term"adjuvant" is derived from the Latin word *adjuvare* which means to help. Any material that helps the antigens or increases the humoral or cellular immune response to an antigen is referred to as an adjuvant. Adjuvants help antigens to elicit an early, high and long-lasting immune response using a smaller quantity of antigen, thus saving on vaccine production costs. In recent years, adjuvants have been required urgently because of the development of purified , subunit and synthetic vaccines which are poor immunogens and require adjuvant to evoke the immune response. With the use of adjuvants, the immune response can be selectively modulated to the major histocompatibility complex (MHC) class I or MHC class II and Th1 or Th2 type, which is very important for action against diseases caused by intracellular pathogens

such as viruses, parasites and bacteria.

Many agents, although structurally unrelated, augment immune responses to weak antigens, such as aluminium hydroxide (alum), polymers, bacterial products and liposomes. Most immunological adjuvants currently available can, however, be toxic and induce diseases (Singh and O'Hagan, 1999). Even alum, the only adjuvant licenced for use in man, is not always effective and increases cell-mediated immunity (CMI) only slightly. Efforts are, therefore, being made for the development of safe and effective adjuvants to meet the challenges of new generation vaccines. Such new-generation adjuvants are expected to comply with a number of criteria which include inexpensive raw materials, simple manufacture, stability under storage, availability in freeze-dried form when necessary, biodegradability and non-immunogenicity. Ideally, they should be able to induce both CMI and humoral immunity (HI) to antigens administered by a variety of routes and, preferably, to act synergistically with other adjuvants. Many of these criteria are satisfied by liposomes which are best known for their potential and actual uses in vaccine formulations.

A number of problems are encountered in the development and application of adjuvants for human vaccines (Gupta and Siber, 1995). The biggest issue with the use of adjuvants for human vaccines, particularly routine childhood vaccines, is the toxicity and adverse side effects of most of the adjuvant formulations. At present, the choice of adjuvants for human vaccination reflects a compromise between a requirement for adjuvanticity and an acceptable low level of side effects. Other problems with the development of adjuvants include restricted adjuvanticity

of certain formulations to a few antigens, use of aluminum adjuvants as reference adjuvant preparations under sub-optimal conditions, non-availability of reliable animal models, use of non-standard assays and biological differences between animal models and humans. This leads to the failure of promising formulations to show adjuvanticity in clinical trials.

During the last twenty years, much progress has been made on development, isolation and chemical synthesis of alternative adjuvants. Other areas in adjuvant research which have received much attention are controlled release of vaccine antigens using biodegradable polymer microspheres (Kuntz and Saltzman, 1997) and reciprocal enhanced immunogenicity of protein-polysaccharide conjugates (Karzon,1991). Biodegradable polymer microspheres are being evaluated for targeting antigens on the mucosal surfaces and for controlled release of vaccines with an aim to reduce the number of doses required for primary immunization. Reciprocal enhanced immunogenicity of protein-polysaccharide conjugates will be useful for the development of combination vaccines. However, the most common adjuvants for human use today are still aluminum hydroxide and oil emulsions, although they are far from ideal. To develop liposomes as vaccine adjuvants, the basic immunology of vaccines was reviewed first and the way to apply liposomes in vaccine formulations was investigated next.

1.2.1.1 Vaccination

Vaccination as a method of preventing infectious disease has had, arguably, the greatest impact on human health of any medical intervention. Vaccine development encompasses technologies ranging from the old approach of modifying pathogens to advanced genetic

manipulation of the immune system itself. However, all vaccines have in common the intention of inducing an immune response designed to prevent infection or limit the effects of infection. Both humoral (antibody-mediated) and cellular arms of the immune system can contribute to the pathogen-specific acquired response that distinguishes vaccine protection afforded by phagocytes, cytokines and physical barriers. Another critical element to vaccines is memory. Because immunization takes place many years before exposure to the pathogen, a long-lived immune response is called for.

The immune system can be divided into two broad functional categories: humoral immunity (HI) and cell mediated immunity (CMI). HI is effected by antibodies, bivalent protein molecules that can be cell-bound or cell-free. Antibodies bind to antigenic determinants, usually on the surface of viruses, bacteria or parasites, and inactivate these infectious agents. By contrast, CMI is mediated by effector cells that destroy infected cells of the host by direct cell-to-cell contact, or by the release of molecules that possess killing activity. Both CMI and HI require a special type of T cell to initiate or enhance cellular and humoral responses (Vitetta *et al.*, 1989). These cells are called T-helper (Th) and produce cytokines which are responsible for the helper effects. Thus, both the HI and CMI components of the immune system are dependent on Th, which actually comprises part of the cellular arm of immunity.

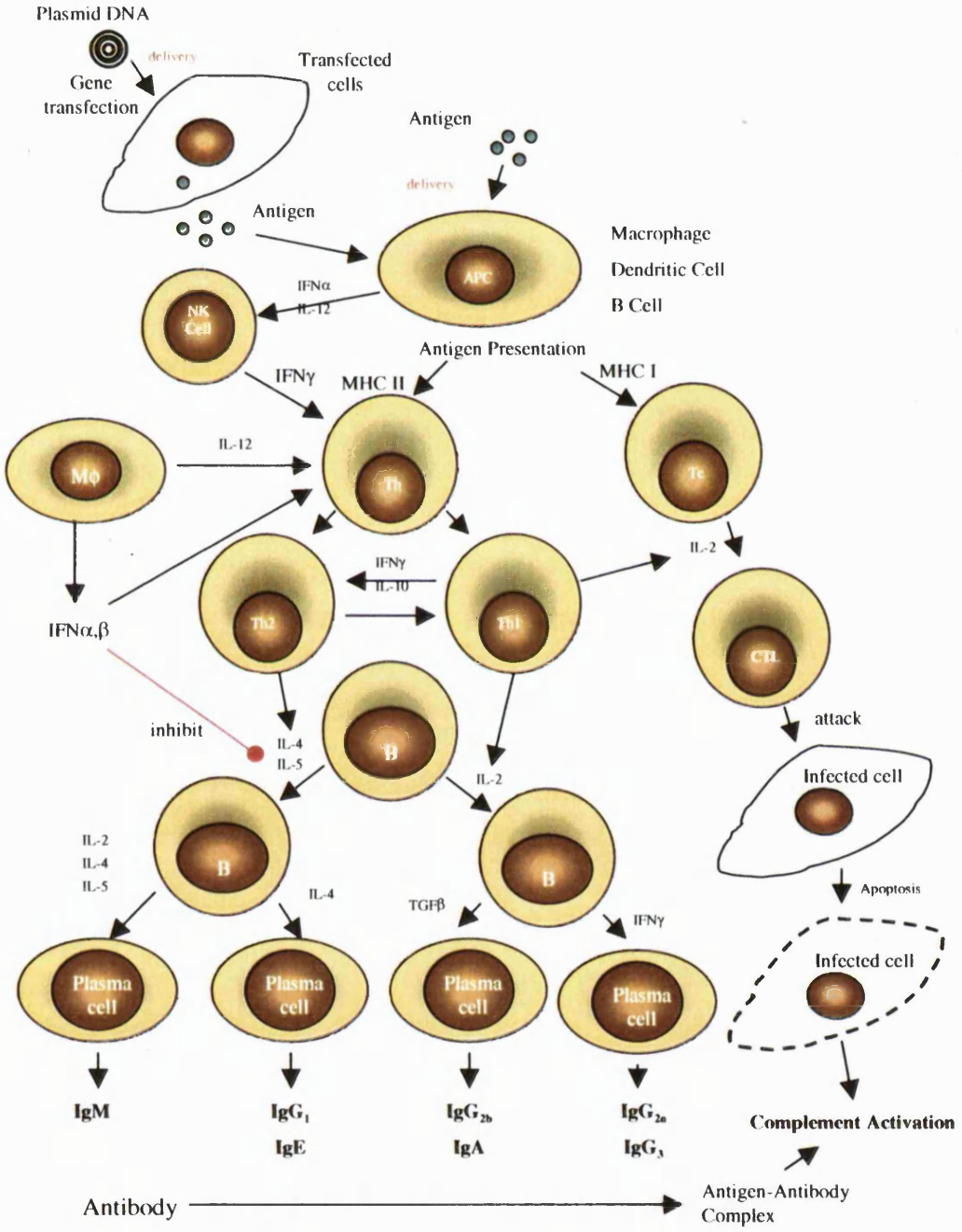

Figure 1.2: Immune responses induced by vaccination (the figure is a shorten version of Figures 11-21, 12-12, 14-1 and 14-4 in "*Kuby Immunology*" (Goldsby *et al.*,2000 ed)). Interactions of numerous cytokines with B cells generate signals required for proliferation and class switching during the differentiation of B cells into plasma cells (Figure 11-21), regulation of Th subsets by cytokines (Figure 12-12), generation of effector CTLs (Figure 14-1) and the stages in CTL-mediated killing of target cells (Figure14-4).

Using cell cloning techniques, two functionally-distinct Th subsets were identified in the mouse (Mosmann and Coffman,1989). These cloned helper cells, called Th1 and Th2 cells produced, respectively, interferon-γ (IFN-γ) and interleukin-4 (IL-4), and provided helper activity primarily for CI and HI. Similar findings were subsequently made in the human. They also found that Th1 and Th2 responses were regulated not only in a positive but also a negative way such that cellular responses were augmented by IL-2 and IFN-γ but decreased by IL-4 and IL-10. In contrast, antibody responses were enhanced by IL-4 and IL-10, but were regulated by IFN-γ and another cytokines, IL-12, which enhances IFN-γ and is produced by monocytes (Figure 1.2). Because these regulatory cytokines can be produced by different cell types and the *in situ* human immune system is more complex than a clone of T cells, the Th1/Th2 hypothesis had been interpreted and expanded to a type 1/type 2 model that includes all cell types that produce these cytokines. Therefore, the immune response of an individual on the basis of whether there is a dominant cellular or humoral response can be characterized with the associated production of cytokines.

Vaccine strategies have not considered the design of vaccines that would preferentially elicit either CMI or HI. As reported previously, low dose immunization can result in a dominant cellular response without appreciable antibody production (Fadel and Sarzotti, 2000). Immunization with higher doses of antigen results in both CMI and HI, with HI often persisting after CMI has waned. The type of antigen-presentation cells (APCs), preparation of the vaccine, route of immunization and co-stimulatory signals can all influence dominant cellular or humoral responses. It is generally considered that APCs of the monocyte/macrophage lineage present

antigens that favour dominant CMI, whereas B cells present antigens that elicit dominant HI. Genetic background and age of the animals immunised may also influence CMI and HI. Several different types of vaccine preparations have been used for immunization. Any of these vaccines may be able to elicit dominant CMI, HI or both under appropriate conditions, depending on the antigen dose, route of immunization, cytokine profile of the vaccines and adjuvants.

1.2.1.2 Classification of immunological adjuvants

Adjuvants come in many different forms, so their classification can be difficult. One method of classifying adjuvants is by their mechanism of action. Adjuvants such as alum, stearyl tyrosine, and biodegradable polymer microspheres provide sustained release of immunogen. Others, such as emulsions, liposomes, saponins, monophosphoryl lipid-A, and muramyl tripeptide phosphatidylethanolamine, can be classified as surfactant-like. Unfortunately, this simple approach to adjuvant classification is misleading because may adjuvants work through more than a single mechanism and many have overlapping mechanistic properties but unique physical properties. According to their physical properties, there are several classes by which adjuvants can be classified, including mineral compounds, oil emulsions, lipid derivatives, amino acid derivatives, surfactants with their complexes, and particular adjuvants (Audibert and Lise, 1993). Liposomes are a kind of particular adjuvant, but have shown the qualifications to satisfy the criteria for new generation adjuvant.

Table 1.2: Classification of vaccine adjuvants.

Catalogues of Vaccine Adjuvants	Examples
Mineral compounds	Aluminium phosphate Aluminium hydroxide Calcium phosphate Calcium chloride Cerium nitrate Colloidal iron hydroxide Zinc sulfate
Oil emulsions	Adjuvant 65 Freund's complete adjuvant Freund's incomplete adjuvant MF-59 SAF-1
Lipid derivatives	Lipopolysaccharide (LPS) Lipid A MPL MTP-PE Ribi Adjuvant TDM
Amino acid derivatives	Murabutide Threonyl-MDP
Surfactants with their complexes	ISCOM Quil-A Q-21 Saponin
Particles	Liposomes Microspheres Nanoparticles Niosomes

(Adapted from Gupta and Siber, 1995)

1.2.2 Liposomes as vaccine adjuvants

Early works dealt with attempts to circumvent immune responses to injected foreign therapeutic enzymes entrapped in liposomes. Contrary to expectations, studies revealed that antibody titres to the model protein (diphtheria toxoid) were much higher than those obtained with the free protein(Allison and Gregoriadis, 1974). The immunological action of liposomes was subsequently confirmed and extended to include a large variety of antigens from sources as diverse as bacteria, protozoa, viruses, tumours, spermatozoa and venoms (Table 1.3). Elicited immunity is protective in several of the animal systems and there are no liposome-induced side reactions such as granulomas (Gregoriadis, 1990).

Table 1.3: Liposome-incorporated antigens.

Antigen	
Microorganisms	
Bovine herpesvirus 1	Streptococcus Pneumonia
Killed Bacillus subtilis; Killed Bacille Calmette-Guerin	Rubella virus rosettes
Encephalomyocarditis virus	Inactivated Semliki Forest virus
Measles virus	Denatured influenza A/Taiwan/1/86 virus
Proteins	
Ovalbumin	Haemophilus influenzae outer membrane protein
Diphtheria toxoid	Neisseria meningitidis outer membrane protein
Tetanus toxoid	HIV envelope protein rgp 120
Cholera toxin	Bordetella pertussis filamentous hemagglutinin
Mycobacterium leprae soluble antigens	Bovine herpesvirus type-1 glycoprotein D
Neisseria gonorrhoeae outer membrane proteins	Mycobacterium leprae antigenic protein
Proteus mirabilis membrane proteins	Mycobacterium tuberculosis 70kDa protein
Streptococcus sobrinus ribosomal protein	Crotoxin
Streptococcus carbohydrate-protein conjugate	Influenza virus glycoproteins (A/Taiwan/1/86 strain)

Antigen	
Cryptococcal culture filtrate antigen	Bovine serum albumin
Toxoplasma gondii membrane protein	Plasmodium falciparum merozoite surface protein 1
Nippostrongylus brasiliensis antigens	Bovine rhinotracheitis virus proteins
Leishmania mexicana gp63 and lipophosphoglycan	Influenza virus glycoproteins (A/Texas/H3N2 strain)
Leishmania major soluble antigens	Brucella melitensis antigen
Hepatitis B surface antigen	Salmonella enteritidis outer membrane protein
Influenza virus glycoproteins (A/PR8/34 strain)	HIV envelope 2-3SF2 protein
Influenza virus glycoproteins (A/PR/8 strain)	Influenza virus glycoproteins (A/Hong Kong/68 strain)
Adenovirus type 5 hexon	Plasmodium falciparum circumsporozoite protein
Herpes simplex virus type 1 antigens	Porphyromonas gingivalis fimbrial proteins
Herpes simplex virus type 2 rgD antigen	*Echis ocellatus* venom
Epstein-Barr virus glycoprotein	Ricin A-chain immunotoxins
Rabbies glycoprotein	*Tityus serrulatus* venom
Hybrid protein of HTLV 1	Nigeria *Echis carinatus* venom
Measles virus haemagglutin	18kDa heat-shock proteins
Malaria antigens	Yersinia pestis F1 antigen
Human carcinoembryonic antigens	Tumour (L_2C) antigens
Gross virus cell surface antigen	Tuberculin
Fibrosarcoma surface antigens	Rat colon tumour-associated antigens
Peptides	
Plasmodium falciparum synthetic peptide	HBcAg peptide 129-140
Spermatozoal polypeptide	HIV immunodominant peptide
Muramyl tripeptide analogues	HIV gp120 V3 loop peptide
Muramyl dipeptide analogues	Thymic hormone thymopoietin oligopeptide
Murine AIDS 10-mer peptide (P12-10)	Hepatitis A VP1 peptide
Listeriolysin 91-99 peptide	Ovalbumin peptides
HBsAg peptides	Lipopeptide JBT3002
Nucleic Acids	
Therapeutic cytokine DNA	HIV pCMV 160/REV plasmid DNA
HIV-1 DNA	HIV-1 with IL-12 and GM-CSF DNA
HBV pRc/CMV HBS DNA	Leprosy pCMV 4.65 DNA
Schistosome VR 1020 DNA	Measles N6 DNA
RSV M2 DNA	

Antigen	
Polysaccharides	
Rift Valley fever virus lipopolysaccharide	Neisseria meningitidis lipopolysaccharide

(Adapted from Gregoriadis, 1995 and Gregoriadis, 1990).

It appears that physical association between lipid and antigen in liposomes is a prerequisite for immunoadjuvant action to occur (Therein and Shahum, 1989). Liposome-antigen association can be in the form of antigen entrapment within the aqueous phase of the vesicles, electrostatic adsorption of antigen onto the bilayer surface or hydrophobic insertion into the lipid phase (Gregoriadis, 1990). In the latter case, for instance, certain viral subunits with membrane solubility formed well-organized structures in conjunction with liposomal phospholipids (Almeida *et al.*, 1975). In view of these formulations, it appears that adjuvanticity of liposomes does not depend on any specific composition or size. Also adjuvanticity does not seem to be especially related to the mode of immunization and the choice of antigen, liposomal lipid dose, route and frequency of injections have often been arbitrary.

1.2.2.1 Immunological adjuvant properties of liposomes

Approaches to liposomal antigen formulations and immunization protocols have been largely empirical. Yet, liposomal adjuvanticity was observed against the vast majority of antigens studied with T-cell dependency of antigens having been demonstrated in several cases. It has been shown already that liposomal adjuvanticity occurs during primary immunization and is observed with most IgG subclasses with no apparent shift in subclass responses seen with the free antigen (Davis *et al.*, 1987). Recent work with three antigens also suggests that liposomes

enhance the proportion of $IgG_{2a}/_{2b}$ to IgG_1 levels (Phillips and Emili, 1992). However, the mechanism of liposome-induced HI to liposome-associated antigens, although unclear at present, is almost certainly related to the liposomal fate *in vivo* as already outlined. It is likely that antibody production is stimulated as a result of the systemic function as an antigen depot supplying antigen presenting cells with released and/or entrapped antigen at rates which favour its efficient processing by the cells and eventual presentation. The involvement of macrophages in liposomal adjuvanticity, a strong possibility in view of T cell participation is supported further by experiments showing its absence in animals depleted of their macrophage (Su and van Rooijen, 1989). Thus, liposome entrapped antigens can supply the antigen as a depot and make the antigen presentation be more efficient.

An additional important feature of liposomal adjuvanticity is induction of cell-mediated immunity. Related evidence includes positive DTH reactions (Manesis *et al.*, 1979), data from lymphocyte proliferation tests and induction of cytotoxic T-lymphocytes in lymph nodes. Liposome-induced CMI is unlikely to occur as a result of the antigen-depot mechanism: adjuvants such as oil emulsions and alum acting in this way, induce only or predominantly HI. A more plausible explanation is antigen presentation in a hydrophobic microenvironment in the same way that proteins conjugated to lipids induce DTH in proportion to the lipid's hydrophobicity (Dailey and Hunter, 1977). This increases uptake of the complexes by APCs and improves antigen presentation to T cells. These events may also be favoured by the efficient localization of liposomal antigens into the regional lymph nodes (Tumer *et al.*, 1983). Recent studies *in vivo* of liposomal adjuvanticity at the subcellular level suggest that, following vesicle

localization in lysosomes, degraded liposomal antigen is recycled to endosomes and then presented to T cells by the association with MHC class II molecules (Harding *et al.*, 1991). At the same time, liposomes do not engender class I processing of the entrapped antigens *in vitro* (Harding *et al.*, 1991) although this can apparently occur by using vesicles with lipid composition that renders them unstable in the acid milieu of the endosomes.

pH-sensitive liposomes (Yatvin *et al.*, 1985) are prepared by mixing DOPE molecules with deprotonated acidic lipids such as N-palmitoryl-L-homocysteine, oleic acid, dipalmitoyl succinyl glycerol, cholesteryl hemisuccinate and N-succinyl oleyl phosphatidylethanolamine. Lowering the pH will destabilize the DOPE/DOPE interactions, as well as the DOPE/ acidic lipid interactions, thus increasing the leakage of entrapped substances. Previous study suggested that the mechanism of leakage might be a result of lipid-packing defects due to the nonbilayer properties of DOPE and protonation of the acidic lipid (Nayar and Schroit, 1985). Because of the fusogenic property of DOPE, such pH-sensitive liposomes probably fuse with the endosomal membrane and release their antigen contents partly into the endosome and partly in the cytosol (Connor and Huang, 1985).

Although much of the liposomal antigen is delivered to the macrophages and processed via the lysosomotropic pathway, a portion of it is likely to escape into the cytoplasm. As already discussed (Nair *et al.*, 1992), the usefulness of pH-sensitive liposomes in this respect may be limited to *in vitro* systems only. The study *in vivo* has now shown that both pH-sensitive and conventional formulations are capable of inducing antigens-specific $CD8^+$ CTL. When antigens

are delivered into the cytosol, they have to be unfolded and then threaded into the cavity of the proteasome to be cleaved. The generated peptides are then translocated into the endoplasmic reticulum (ER) by the transporters associated with antigen processing (TAP) (Hombach *et al.*, 1995). Once constituted, peptide-class I MHC complexes exit the ER and are rapidly transported to the cell surface following the secretory pathway (Peters *et al.*, 1991). It has been suggested that some of this antigen in the cytoplasm gains access to dendritic cells which are responsible for promoting recognition in the specific CTL precursors (Reddy *et al.*, 1992). It is also feasible that dendritic cells (Nair *et al.*, 1992), expected to acquire some of the antigen *in vivo* (Gregoriadis, 1992), may retain the antigen more efficiently than macrophages. Furthermore, owing to the increased density of class I molecules on their surface, dendritic cells require much less antigen for efficient presentation.

1.2.2.2 Strategies towards the optimisation of liposomal adjuvanticity

Further improvements on the immunoadjuvant action of liposomes have been achieved through receptor-mediated targeting to macrophage (Garcon *et al.*, 1988), the use of a variety of co-adjuvants (Alving, 1991) or the modification of vesicle's structural characteristics (Gregoriadis, 1990). For example, liposomes coated with a mannose-terminating ligand gave a far greater IgG immune response against the entrapped tetanus toxoid in mice than ligand-coated vesicles to the macrophages which express mannose receptors on their surface (Garcon *et al.*, 1988). Responses to liposomal antigens are also augmented through the administration of antigens in liposomes together with other adjuvants. Cholera toxin (CT) or its B subunit (CTB) have been found to promote immune responses to antigens when they are co-administered via

mucosal routes, and recent works have reported the CT-conjugated liposomes may be a useful system for targeted delivery (Harokopakis *et al.*, 1995). In addition, liposomal adjuvanticity is a reflection of the systemic vesicular structure and it is probably due to lipid nature rather than lipid composition or other characteristics (e.g., vesicle size, lamellarity and surface charge). Indeed, there have been numerous studies on the extent to which bilayer fluidity (Davis and Gregoriadis, 1987), number of lamella in vesicle (Shek, 1984), vesicle size (Francis *et al.*, 1985) and surface charge (Kraaijeveld *et al.*, 1984), lipid to antigen mass ratio (Therien *et al.*, 1991) and mode of antigen localization (Shahum and Therien, 1988) within liposomes influence adjuvanticity. Most of these parameters were shown to have an effect but conclusions as to their role have often been contradictory.

Whilst differences in protocols of experiments may account for some of the aforementioned contradictions, others could be attributed to the diversity of interactions between membrane or water-soluble antigens and APC *in situ*. Such interactions are potentially variable as they depend on the state of the vesicles after injection. They may thus include supply of free antigen transfer to APC following disintegration of liposomes locally, direct antigen transfer to the membranes of APC on contact of liposomes with and their endocytosis by, the cells or, migration of fragments to the APC membrane following antigen catabolism intracellularly. The diversity of these events may be further amplified by the variation of liposomal structural characteristics. For example, studies on the effect of the bilayer fluidity of liposomes on immune responses to membrane-soluble antigens have shown that liposomes made of 'high melting' phospholipids (e.g., DSPC), elicit strong antibody responses to such antigens (Bakouche and

Gerlier, 1986; Kinsky, 1978). However, when a water soluble antigen was used, responses were strong with liposomes made of 'low melting' phospholipids and nil or minimal with DSPC liposomes (Gregoriadis *et al.*,1987). In contrast, the effect of DSPC on responses consisting of the two types of antigens have been attributed to the direct transfer of membrane proteins from their liposome carrier to the plasma membranes of APC where they may associate with MHC molecules, without being first processed.

A variety of factors, including an appropriate lipid to antigen mass ratio, are known to influence the balance attained *in vivo* among a number of mechanisms that lead to liposomal adjuvanticity (Davis and Gregoriadis, 1987). The rapid targeting of massive doses of antigenic material to antigen-presenting cells, especially macrophages, has often been invoked as the principal source of liposomal adjuvantity. However, the rapidity of antigen targeting is not the sole reason for liposome adjuvantity and the role of liposome as antigenic depot is probably important to sustain substantial activation through successive restimulations (Fortin and Therien, 1993). Such balance between these mechanisms is expected to reflect primarily the structural profile of a given liposomal vaccine formulation. For instance, although the lipid composition of vesicles and their average size dictate the rate and extent of liposome degradation *in situ* and the subsequent release of antigen, they also control the vesicle uptake by the lymphatics, mode of interaction with APC, as well as intracellular fate. To add to the complexity of factors influencing liposomal adjuvanticity, these events may also be affected differently by different antigens because of variability in the antigen's structural characteristics, its spatial arrangement within liposomes and the way antigens may interact with lipid components.

1.3 LIPOSOMES AS IMMUNOLOGICAL ADJUVANTS FOR HEPATITIS B VACCINES

Hepatitis B is a global public health problem, with an estimated 300 million carriers world-wide. A large reservoir of infection is maintained predominantly by the transmission of hepatitis B virus to infants born to carrier mothers in highly endemic areas. Because of the sharing of antigenicity between hepatitis B virus and 22 nm HBsAg particles, this antigen can be used as a source of immunogen. The primary objective of this study is to formulate liposomal based hepatitis B vaccines, so the basic concepts of hepatitis B vaccination and the previous studies that have been done in this work are overviewed and summarized in this section.

1.3.1 Hepatitis B vaccines

When Hippocrates described epidermic jaundice more than 2000 years ago, the syndrome included hepatitis B as well as other viruses capable of infecting the liver. Hepatitis B is an ancient disease and the outbreak was described by Lürman in Germany (Lürman, 1885). Then, the Willowbrook hepatitis studies, begun in the mid-1950s, confirmed and extended previous observations of the natural history and epidemiology of hepatitis B infections (Krugman *et al.*, 1971). Furthermore, the discovery of Australia antigen and its subsequent association with hepatitis B led to the identification of sensitive and specific markers of hepatitis B virus (HBV) infection. This technology was used to develop assays for diagnosis and detection of immunity, to clarify the natural history of the disease, and to develop vaccine against hepatitis B virus infection.

1.3.1.1 Immunology of hepatitis B

The hepatitis B virus is a noncytopathic enveloped virus with a small, circular double-stranded DNA genome that causes acute and chronic necroinflammatory liver disease and hepatocellular carcinoma (Chisari and Ferrari, 1994). Most studies suggest that HBV is not directly cytopathic, for the infected hepatocyte (Chisari *et al.*, 1989). Instead, a great deal of evidence suggests that the cellular and humoral limbs of the immune response are required for viral clearance, that the cellular response is involved in disease pathogenesis, and that noncytolytic intracellular viral inactivation by cytokines released by virus-activated lymphomononuclear cells could play an important role in the clearance of this virus without killing the infected cells.

At the effector level, apparently several pathways can be activated to eliminate the virus, either by killing the infected cells or by eliminating the virus from the cell without killing it. The first pathway is via the CD8+ cytotoxic T cell that recognizes endogenously synthesized HBV antigens presented by HLA class I molecules at the hepatocyte membrane. The second and third pathways involve antigen recognition by CD4+, HLA class II restricted T cells. While the second pathway is not likely to be very important, because endogenously synthesized viral antigen does not associate efficiently with HLA class II molecules inside of the cell, intrahepatic macrophages express high levels of HLA class II, and they can efficiently process and present phagocytosed antigen to the class II restricted T cells which could then release their own set of cytokines as discussed above. According to this notion, the third pathway, reminiscent of a classical delayed-type hypersensitivity reaction, is probably quite active in the infected liver. This suggests that the

class II restricted T cell may contribute to viral clearance and immunopathology in much the same way as do the class I restricted CTL, except for their direct cytopathic effect, which actually appears to contribute very little to the overall severity of liver disease.

However, the protection against HBV correlates (Howard and Allison, 1995), at least in part, with the appearance of antibodies to HBsAg (anti-HBs) which is the envelope of the Hepatitis B virus. The antibody response to HBV envelop antigens is a T-cell dependent process (Milich and McLachlan, 1986). Antibodies play the critical role in viral clearance by binding with free viral particles and removing them from circulation or possibly by preventing their attachment and uptake by susceptible cells (Chisari and Ferrari, 1995). In addition, they also contribute to the pathogenesis of the extrahepatic syndromes associated with HBV infection by forming antigen-antibody complexes (Moriarty *et al.*, 1985). Thus, immunization with HBV envelop antigens will have the advantage of preventing hepatitis B virus infection.

1.3.1.2 Hepatitis B surface antigen

Hepatitis B surface antigen (HBsAg) particles consist predominantly of a glycoprotein of 226 amino acids which bears the B-cell epitopes important for the induction of protective antibody responses in humans. According to its primary sequence, the HBsAg protein consists of large hydrophobic domains and only two relatively hydrophilic areas. Genetic approaches suggest a model describing the folding of the transmembrane protein within this lipidic environment (Figure 1.3 (a)). However, a molecular description of the three-dimensional structure would require the crystallization of the protein in its lipid environment. Furthermore,

the lipid of natural small HBsAg spheres had been reported to be similar to that of liver-derived high-density lipoprotein, which contains 60% phosphatidylcholine, 14% cholesteryl ester, 15% cholesterol, and 3% triglycerides. Lipids are apparently necessary for the formation of 20 nm spheres, because detergent-extracted small HBsAg proteins form large micelles after removal of the detergent. Thus, incorporation of HBsAg into lipid bilayers using liposome technology allowed immunization comparable to that induced by natural HBsAg particles.

Recombinant small HBsAg (*ayw* subtype) expressed in *Saccharomyces cerevisiae* yeast cells and the pRc/CMV HBS plasmid DNA which encodes for this antigen were applied in this study for investigating the effect of liposomes on the induction of immunity against HBsAg. Firstly, the antigenic structure of HBsAg will be illustrated and discussed. In the antigenicity of HBsAg, antibodies to the *a* determinants confer protection in adults to all the common subtypes of HBV. In all known serotypes of HBV, the *a* determinant epitopes are located within domains bordered by amino acids 120-147, a predicted double loop structure projecting from the surface of the HBV particle. The *a* determinants are predominantly located on the second of these two loops, between 139 and 147, and on the predicted loop regions are also located two subtype determinants, *d* or *y* and *w* or *r*. As the sequencing data shows, the *d* and *y* alleles correlate with the appearance of either lysine or arginine at position 122 respectively, and *w* and *r* correspond to the presence respectively of either lysine or arginine at position 160. This information will be applied to investigate the immunity induced by antigen and to design the peptide-based vaccines.

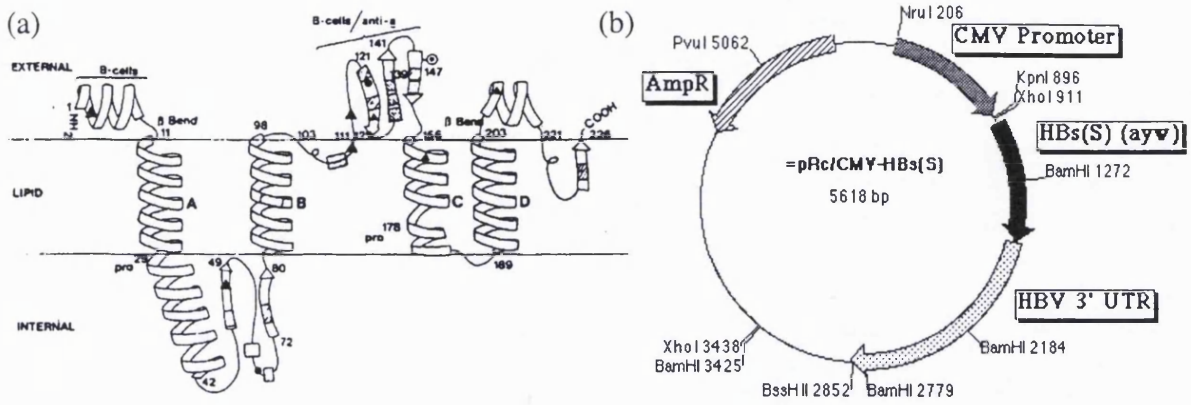

Figure 1.3: Schematic representation of HBsAg and the map of HBsAg-expressing plasmid pRc/CMV-HBS. The panel (a): Secondary structure of HBsAg in lipid bilayer predicted by computer modelling (Howard *et al.*, 1988). The panel (b):Map of plasmid pRc/CMV-HBs (S) (Davis *et al.*, 1993a).

In addition, plasmid pRc/CMV-HBS expressing sequences coding for the S (small) protein of hepatitis B virus was reconstructed by Dr. Robert Whalen to induce DNA immunization *in vivo* (Figure 1.3 (b)). It is reconstructed with the pRc/CMV vector, and encodes the HBsAg sequence within the genomic sequence of hepatitis B surface antigen (subtype *ayw*), which confers resistance to ampicillin (50-100 μg/ml) and uses the CMV promoter to construct a plasmid with 5,618 base pairs (Davis *et al.*, 1993a). This plasmid had been injected intramuscularly and induced the seropositive antibody response. This will aid the understanding of the structure-function relationships between the expressed antigen and the immune response to the antigen, and the formulation will be the new challenge in vaccine pharmaceutics.

1.3.1.3 Development of hepatitis B vaccines

The basis for development of inactivated hepatitis B vaccines stemmed from (1) the discovery of the Australia antigen (Blumberg *et al.*, 1969), (2) its subsequent identification as

HBsAg (Prince, 1968), and (3) the demonstration that heat-inactivated serum containing HBV and HBsAg was not infectious but was immunogenic and partially protective against subsequent exposure to HBV. The detection of anti-HBs in the serum of recipients of the heat-inactivated preparation indicated that the noninfectious HBsAg particle was the immunizing antigen needed for vaccine production (Krugman *et al.*, 1971).

The first licenced hepatitis B vaccines consist predominantly of purified 22-nm particles of the S antigen of HBsAg that is derived from the plasma of chronic carriers or from recombinant cells or organisms that produce HBsAg (Hilleman *et al.*, 1975). Some vaccines also include varying amount of pre-S_1 or pre-S_2 regions. Challenge studies in chimpanzees demonstrate protection by antibody response (Itoh *et al.*, 1986). However, the need for including pre-S1 or pre-S2 components in vaccines remains controversial. The methods used to purify the viral antigen in plasma-derived subunit vaccines and to inactivate any residual HBV or other infectious agents that might be present vary considerably, but all available vaccines have been shown to be safe and effective.

Recombinant technology for hepatitis B vaccine has involved insertion of segments of the HBV genome that encode HBsAg into plasmid, which in yeast or cultured mammalian cells then express HBsAg (Valenzuela *et al.*, 1982). Most recombinant vaccines have been composed of the S antigen, although some have also included the pre-S_2 region. The recombinant vaccines consists of the purified antigen adsorbed on an adjuvant, usually alum. The first recombinant hepatitis B vaccine has been available in the United States since July 1986.

Also, peptide vaccine had been developed with the progressing technique of peptide synthesis. A number of investigators have reported the synthetic peptides are poor immunogens. However, this is a very subjective characterization depending on adjuvant systems, species or strains immunized, form of the antigen and inclusion or exclusion of relevant T-cell epitopes. The existence of an effective recombinant subunit HBV vaccine raises the question of the necessity of a synthetic-based vaccine. Nevertheless, the knowledge gained from the analysis of synthetic peptide reagents in the HBV system has provided and will continue to provide insights relevant to the development of future generations of HBV vaccines and may serve as a model for the development of synthetic vaccines for diseases in which a recombinant subunit vaccine is not appropriate.

Recently, a novel approach to immunization is the induction of immune responses against an antigenic protein expressed *in vivo* from an introduced gene. DNA vaccines are attractive, because endogenous antigen synthesis induces CD8+ MHC I restricted cytotoxic T lymphocytes, such as are obtained with live viral vaccines but rarely with whole killed pathogen or subunit vaccines. Comparing with the HBsAg protein vaccines, the DNA is very stable, and the storage, transport and distribution of DNA-based vaccines would be facilitated. However, this can be followed by direct assessment of changed immunogenicity of the protein after direct injection of plasmid DNA. Furthermore, antigen synthesis over a sustained period may be advantageous for the immune system and as such might help overcome low responsiveness and eliminate or reduce the requirement for booster injections. Further studies about the optimization of immunization including the route of administration, dose and the adjuvant systems are being

processed and under development.

1.3.1.4 Application of immunological adjuvants for hepatitis B vaccines

Following the development of Hepatitis B vaccines, a lot of adjuvant systems had been applied in the formulations of these vaccines, especially for recombinant HBsAg protein, synthetic peptides and DNA vaccines. Most of the commercially available hepatitis B vaccines are based on recombinant HBsAg particles derived from yeast as antigens, and on alum as an adjuvant. The adjuvants had been applied in hepatitis B vaccines is demonstrated in Table 1.4.

Table1.4: Adjuvants for Hepatitis B vaccines.

Adjuvant	Major observations
Liposome (MLVs) (Manesis *et al.*, 1979)	Liposomes are likely to play a role in the preparation of vaccines against hepatitis B. Liposomal vaccines are mor efficacious than naked HBsAg in eliciting humoral responses.
Cationic liposomes (Gregoriadis *et al.*, 1997)	Cationic liposome-mediated DNA vaccination appears more effective than naked DNA in inducing both humoral and cell mediated immunity to HBsAg.
Microparticles (Lee *et al.*, 1997 and Singh *et al.*, 1997)	Novel double walled microparticles is a promising adjuvant for HBsAg in one dose vaccination.
Thymopentin (Zaruba *et al.*, 1983)	Thymopentin can enhance the humoral response to HBsAg.
Interleukin 2 (Jungers *et al.*, 1994)	Natural IL-2 at low dose overcome the non-responsiveness of patients with chronic uraemia to hepatitis B vaccines.
poludan ridostine amixine larifan myekonide (Barinskii *et al.*, 1994)	The use of these agents significantly enhanced specific antibody production to HBsAg and at least doubled cell-mediated immune response by 14 and 28 days postvaccination.
Interferon-α (Quiroga & Carreno, 1989)	Interferon-α may increase the seroconversion of hepatitis B vaccine non-responders.
GM-CSF (Tarr *et al.*, 1996)	A single injection of GM-CSF enhances antibody responses at HBsAg vaccination.
MF59 (Traquina *et al.*, 1996)	The dramatic immune response elicited by HBV/MF59 in baboons.
Alum (Manivel *et al*, 1993)	HBsAg adsorbed on Alum can enhance the antibody response.
Cytokine gene (Chow *et al.*, 1998)	Application of a cytokine gene in DNA vaccine formulation influence the differentiation of Th cells and the nature of immune response.
SBAS4 (Thoelen *et al.*, 1998)	The formulation promote rapid protection against hepatitis B infection.

Adjuvant	Major observations
Synthetic oligodeoxynucleotide (McCluskie & Davis 1998)	CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice.
BCG-CWS TDM MDP-Lys(L18) (Koike *et al*, 1998)	These agents are potential adjuvants to enhance the immunogenicity of recombinant human HBsAg to induce humoral and cellular responses.

1.3.2 Liposomes as immunological adjuvants for hepatitis B vaccines

As that mentioned above, liposomes had been demonstrated as immunological adjuvants in a variety of antigens. Considering the structure of hepatitis B viruses, the HBsAg is located on the envelop of the viral particles with lipid bilayers, so HBsAg reconstructed on liposomes may have advantages to induce the immune response *in vivo*.

1.3.2.1 Liposomes as immunological adjuvants for hepatitis B surface antigen

Since 1978, Gregoriadis had started to apply liposomes as an immunological adjuvant for HBsAg (Manesis *et al.*, 1979). In pervious works, HBsAg entrapped alone or together with MDP in MLVs or HBsAg mixed with MDP or adsorbed onto aluminium hydroxide produces a higher response than the free antigen. At the same time, it was found that administration of HBsAg in liposomes together with another adjuvant may be more effective in producing antibody responses than when the antigen is given in free or liposomal form.

Recently, HBsAg was reconstituted with different phospholipids and the results showed the antigenic activity depends on the physical state of the phospholipid moiety. The appropriate membrane fluidity is required for optimum conformation but, once this conformation is established, additional interactions imparted by various phospholipids give a difference in the

patterns of antigenicity. Of all the antigenic determinants, only those close to the lipid-protein interface would change upon direct interaction with the phospholipids, and the rest would depend on the correct protein conformation determined by the appropriate phospholipid composition.

Recombinant HBsAg had been encapsulated into MLVs and tested in humoral and CTL responses (Diminsky *et al.*, 1996). Comparison of vaccination using free HBsAg particles, particles adsorbed to alum, and particles encapsulated in liposomes demonstrated the liposomal vaccine was superior in eliciting humoral response with a low dose of antigens. Encapsulation in liposomes did not improve specific CTL response. The alum in the vaccine completely eliminates CTL response, though it improved the humoral response by increasing the linear range in the antigen dose-response curve. So far, these results just focus on the liposomes as immunological adjuvants for HBsAg in basic immunological studies. However, there are many questions unanswered in this issue, including the lipid compositions, vesicle sizes, the properties of HBsAg and the interaction between antigens and lipid bilayers. Therefore, in this study, the optimisation of liposomal formulations for HBsAg will be studied regarding these factors.

1.3.2.2 Liposomes as carriers for peptide vaccine against hepatitis B

The adjuvanticity of liposomes had been found for peptides vaccines. They exhibited the ability to generate immunological memory. It is likely that liposomal B peptides will behave as haptens producing an IgM response. However, it is conceivable that a liposome-entrapped B cell epitope could receive help from a co-entrapped T-cell epitope to produce an IgG response. Liposome-entrapped HBsAg pre-S_1 peptide was observed to do so when co-entrapped in the

same liposomes with a HBsAg S-peptide (Gregoriadis *et al.*, 1993). This result suggests that T and B epitopes would then interact with MHC class II molecules on APC and B cells respectively in the tri-molecular fashion proposed for peptide conjugates made of epitopes, to elicit immunological memory.

1.3.2.3 Liposomal applications in genetic vaccination for hepatitis B

Genetic immunization, one of the more realistic spin-offs of gene therapy, arose from the observation that intramuscularly injected plasmid DNA transfected cells whereupon production of the encoded protein induces humoral and cell-mediated immunity (Weiner and Kennedy, 1999). The immunity follows DNA uptake by muscle fibres, leading to the expression and extracellular release of the antigen. Potential disadvantages with naked DNA vaccination include uptake of DNA by only a minor fraction of muscle cells, exposure of DNA to nucleases in the interstitial fluid, the use of relatively large quantities of DNA and, often, the need to inject into regenerating muscle in order to enhance immunity. On the other hand, administration of antigen-encoding plasmid DNA via liposome could circumvent the need of muscle involvement and facilitate instead its uptake by antigen-presentation cells, for instance those infiltrating the site of injection or in the lymphatics and at the same time protecting the DNA from nuclease attack.

The previous works indicated the cationic liposome-entrapped DNA vaccines generate titres of anti-HBsAg IgG_1 antibody isotype in excess of 100-fold higher and increased levels of both IFN-γ and IL-4 when compared with naked DNA or DNA complexed with performed similar cationic liposomes (Gregoriadis *et al.*, 1997). Recent results demonstrated that plasmid

DNA act as a Th1 promoting adjuvant when mixed as such or co-entrapped in liposomes with a very low dose of antigen (Ulmer *et al.*, 1993). However, the adjuvant activity was lost when separate liposome entrapped formulations of both the antigen and the plasmid DNA were mixed, indicating a necessity for the antigen and the plasmid DNA to contact the same APC for optimal immune activation.

So far, the optimization of liposome as adjuvants for DNA vaccination is far from ideal. Considering the physical properties of DNA, the cationic substances are necessary for DNA condensation (Sagui and Darden, 1999). Cationic liposomes are suitable for this purpose. However, other materials, including calcium, calcium phosphate and cationic polymers, can interact with DNA and form the complex. Considering the mechanism of viral infection, the enveloped virus had envelope membranes, which are similar to the liposomal bilayers. These envelope membranes can protect and deliver the genetic materials into cells and induce pathogenic effects. This led to the ideas of designing the liposomes to entrap plasmid DNA and induce the immune responses.

1.4 SUMMARY

In summary, liposomes have the potential to develop as vaccine formulations against hepatitis B. The subunit protein (HBsAg) or the plasmid encoding HBsAg can be formulated in a liposomal form. The objective of this thesis is to optimize liposomal formulations for hepatitis B vaccines and to investigate the effect of these formulations on the induction of antibodies to HBsAg. The strategies adopted for the optimisation of liposomal adjuvanticity include tailoring

of the liposomal characteristics (e.g., lipid composition and vesicle size), choice of route of administration and approaches of antigen supply (i.e. direct or indirect) to the antigen presenting cells. The HBsAg (S region) and plasmid pRc/CMV HBS DNA encoding the HBsAg (S region) are entrapped into liposomes and these formulations are evaluated in terms of structural characteristics, stability upon storage and release kinetics of entrapped substances *in vitro*. The results and conclusions from this thesis justify the use of liposomes as vaccine adjuvants for hepatitis B protein and DNA vaccines.

Initially, the effect of lipid composition(PC or DSPC with equimolar cholesterol) on liposomes as hepatitis B vaccine adjuvants is investigated and the results are discussed in Chapter 3. Then, methods such as microfluidisation and entrapment in the presence of sucrose, are used to reduce the liposomal size to improve immune responses (Chapter 4). In addition, mannosylated HBsAg is prepared and entrapped into liposomes and their adjuvanticity on mannosylated HBsAg is studied. The results obtained are reported in Chapter 5. The cholera toxin B subunit (CTB) is conjugated with the HBsAg-containing DRVs which are then administered orally to study the systemic or mucosal immune responses generated against HBsAg (Chapter 6). Furthermore, an attempt is made to compare the DNA vaccination and protein vaccination using DNA encoding HBsAg and native HBsAg respectively. Calcium phosphate-DNA complexes entrapped in liposomes are introduced as a new adjuvant system for genetic immunisation in Chapter 7. Finally, General Discussion and Conclusions are presented in Chapter 8.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals

The following chemicals were purchased from Sigma Chemical Co., Poole, Dorset, UK: bicinchoninic acid solution, copper(II) sulfate, sodium dodecylsulfate, 2-mercaptoethanol, ammonium persulfate, N, N, N', N'-tetramethyl ethylenediamine, D(+)-mannose, bromphenol blue, citric acid, sucrose, protease (type I : crude from bovine pancrease), DL-dithiothreitol, heparin, bovine serum albumin (fraction V), L-lysine, L-cysteine, o-phenylenediamine, chloramine-T, SPDP(3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester), and SMCC((4-N-maleimidomethyl)-cyclohexane-1-carboxylic acid-N-hydroxysuccinimide ester).

Sodium Chloride, sodium hydroxide, sodium hydrogen carbonate, calcium chloride, tris (hydroxymethyl) methylamine, acrylamide, chloroform, methanol, sodium carbonate, and potassium chloride were brought from British Drug Houses (BDH), Lutterworth, Leicester, UK. Horse-radish peroxidase conjugated goat anti mouse IgG, IgG_1, IgG_{2a}, IgG_{2b}, foetal calf serum were supplied from Sera-Lab Ltd., Crawley Down, Sussex, UK. 96 well flat-bottomed microtiter plates (Immulon 1B) were purchased from Dynex Technologies, Inc., Chantilly, VA, USA. Carrier-free ^{125}I, ^{35}S-deoxyadenosine 5'-triphosphate(dATP), and ^{14}C-sucrose were got from Amersham International, Amersham, Bucks, UK.

2.1.2 Proteins and plasmid DNA

Hepatitis B surface antigen (HBsAg)(ayw subtype) was purchased from Genzyme diagnostics, Kingshill, Kent, UK. Mouse anti-HBsAg monoclonal antibody was obtained from

CHEMICON International, Inc., Temecula, CA, USA. Cholera toxin B subunit (CTB) was supplied by Sigma Chemical Co., Poole, Dorset, UK. Plasmid pRc/CMV HBS (5.6kb), expressing the sequence code for S (small) region of HBsAg (ayw subtype) was cloned by Dr. R. Whalen, Ecole de France, Paris.

2.1.3 Lipids

Egg phosphatidylcholine (PC) and distearoyl phosphatidylcholine (DSPC) were obtained from Lipid Products, Nutfield, Surrey, UK. Disteroyl phosphatidylethanolamine (DSPE) was supplied by Sygena Ltd., Eichenweg 1, CH-4410 Liestal, Switzerland. Dioleoyl phosphatidylethanolamine (DOPE) and 3((N-(N',N')-dimethylaminoethane)-carbamoyl)-cholesterol (DC-CHOL) were purchased from Sigma Chemical Co., Poole, Dorset, UK.

2.1.4 Buffers

Tris-Glycine running buffer: 3.02g of Tris base and 18.8 g of Glycine were dissolved into 900 ml of distilled water. Then, 50 ml of 10% (w/v) SDS solution was added into the mixture and adjusted volume to 1000 ml. Final concentrations will be 25 mM Tris; 250 mM Glycine; 0.1% SDS.

Borate buffer (pH 9.0): 50.0 ml of boric acid (0.2 M) mixed with 59.0 ml of sodium borate (0.2 M). Then, this mixture was diluted to a total volume of 200 ml with distilled water.

Coomassie blue staining solution: 500ml of methanol, 100ml of acetic acid and 2.5g of Coomassie blue R-250 were mixed. Then, distilled water was added to 1000 ml.

Destaining solution: 100ml of methanol was mixed with 50ml of acetic acid. Then, this mixture was adjusted to 1000 ml with distilled water.

Phosphate buffered saline (PBS, pH 7.4): 8.0 g of sodium chloride (NaCl), 0.2 g of monobasic potassium phosphate (KH_2PO_4), 1.15 g of dibasic sodium phosphate (Na_2HPO_4), 0.2 g of potassium chloride (KCl) and 0.2 g of sodium azide (NaN_3) were dissolved in 900 ml distilled water. Then, this solution was adjusted pH to 7.4 with 0.1N of sodium hydroxide or hydrochloric acid solution and made up to 1 litre.

Washing buffer: 1000ml of PBS added 0.25 ml of Tween 20 to mix up.

Coating buffer (pH 9.6): 1.59 g of sodium carbonate (Na_2CO_3), 2.93 g of sodium bicarbonate ($NaHCO_3$) and 0.20 g of sodium azide (NaN_3) were dissolved in 900 ml of distilled water. Then, this solution was adjusted pH to 9.6 with 0.1N hydrochloric acid solution and made up to 1 litre.

ELISA substrate buffer: 24.3ml of citric acid (0.1M), 25.3ml of di-sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) (0.2M), 50ml of distilled water, 40mg of o-phenylenediamine and 40µl of hydrogen peroxide (30%) were mixed to make up to 100ml and checked pH (pH should be pH 5.0 to 5.13).

Phosphate buffer: (1) Buffer stock A (0.1 M Phosphate): 6.9 g of di-sodium hydrogen orthophosphate (Na_2HPO_4) was dissolved and up to 500 ml with distilled water, (2) Buffer stock B (0.1 M Phosphate): 7.1 g of sodium phosphate (NaH_2PO_4) was dissolved in 500ml of distilled water. Buffer A and B were mixed to give a pH 7.4 solution to prepare 0.1 M of buffer (pH 7.5). 1 part of the 0.1 M buffer (pH 7.5)was diluted with 1 part distilled water and adjusted the pH to make 0.05 M buffer (pH 7.5).

Nick Translation Buffer (10X): 500 ml of Tris stock (1M),100 ml of $MgSO_4$ (1M), 10 ml of DTT (1M), 10 ml of BSA (BRL 50mg/ml sterile stock) and 380 ml autoclaved water were mixed and up to 1000ml. The buffer were frozen at -30°C.

HBS (HEPES buffered saline): 8.766 g of NaCl and 5.2 g of HEPES were dissolved in distilled water, then adjusted pH to 7.0 and made up to 1000ml with distilled water.

TE (Tris 10 mM, EDTA 1 mM, pH 7.4) buffer: 0.121 g of Tris was dissolved in 90 ml distilled water and adjusted pH to 7.4 with 6 N of hydrochloric acid solution. Then, 0.037 g of EDTA was added and disolved to make up to 100 ml with distilled water.

STE buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA): 0.121 g of Tris and 0.294 g of NaCl were dissolved in 90 ml of distilled water. The mixture was adjusted to pH 7.4 with 6 N of hydrochloric acid solution. Then, 0.037 g of EDTA was added and dissolved. Finally, distilled water was added up to 100 ml.

Tris buffer (20 mM, pH 6.8): 0.24 g of Tris base was dissolved in 60 ml of distilled water. Then, this solution was adjusted pH to 6.8 with 6N of hydrochloric acid solution and made up to 100 ml with distilled water.

2.1.5 Experimental Animals

Male Balb/c and T.O. mice (6-8 weeks old) were supplied from B&K Universal, Hull, UK. The mice was kept according to Home Office regulations in the School of Pharmacy, University of London and fed with standard mouse diet and water.

2.2 METHODS

2.2.1 SDS-PAGE of proteins

10 μg of HBsAg, mannosylated HBsAg, CTB or CTB-SPDP conjugate was applied into 15% polyacrylamide gel and running the electrophoresis in Tris-glycine buffer (pH 8.3) at 40mV. The gel was stained by staining buffer, fixed at 40% acetic acid for 1 hour and destained in destaining solution (10% acetic acid and 10% methanol). Finally, the gel was dried by air at 20°C for overnight.

2.2.2 Preparation of mannosylated HBsAg

The method used for the mannosylation of proteins was as described by Kataoka and Tavassoli (1984) and the mechanism of sugar coupling to the proteins is demonstrated in Figure 2.1. Firstly, 80 μl of thiophosgene was added into a solution of p-aminophenyl α-D-mannopyanoside in ethanol (10 ml, 80%(w/w)) and the concentration of the sugar was 0.185

μmole. The mixture was stirred for 1.5 hours at 20°C. At the end of this period, nitrogen gas was bubbling through the solution until most of the odours were removed. Then, the pH was adjusted to 6 with 2 M NaOH and the solution was completely dried by rotary evaporation. Finally, the sugar derivative was dissolved in distilled water (5 ml) and slowly added to 100μg of HBsAg in 0.01 M borate buffer (15 ml, pH 9.0). The molar ratio of sugar to protein is 412 in this experiment and the mixture was stirred at 20°C for 18 hours. The bound sugar was separated from the free sugar by extensive dialysis for overnight at 4°C against several changes of 1 litre of PBS, then the mixture was passed through a Sephadex G-25 column equilibrated with 0.1 M PBS (pH 7.4). The fractions (1 ml each) collected were analysed for their protein and sugar contents. The fraction corresponding to the protein peak was pooled. The concentration of protein in each fraction was estimated by the bicinchonic acid protein determination assay (BCA) with bovine serum albumin as the standard (2.2.2.1). At the same time, the concentration of mannose in collected fractions was measured by the phenol-sulfuric acid method with D(+)-mannose as the control (2.2.2.2).

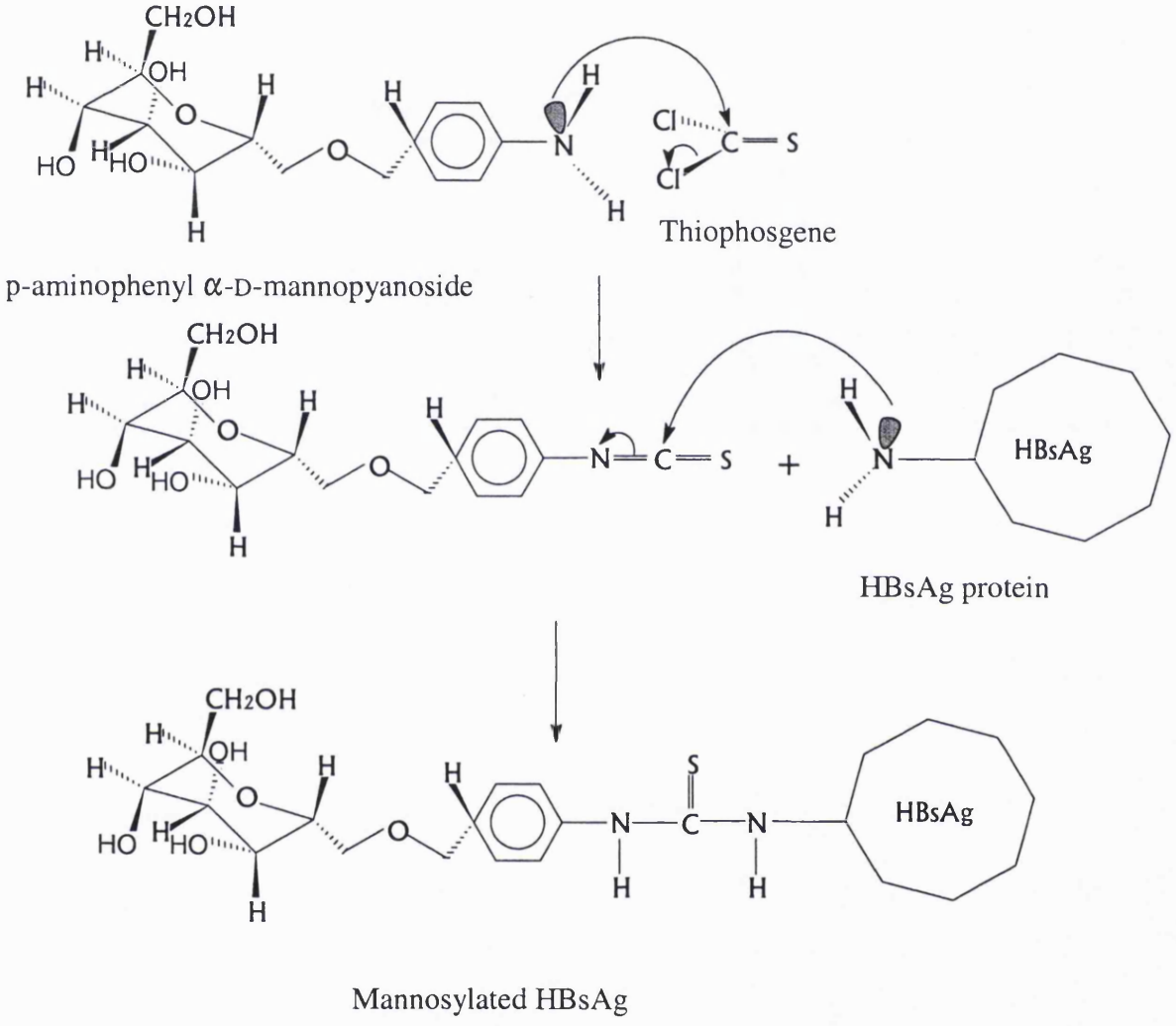

Figure 2.1: Scheme of HBsAg mannosylation.

2.2.2.1 Determination of mannose

Determination of mannose was carried out as described by Weissig *et al.* (1989). The standard solutions of mannose ranging between 25-125 μg /ml were prepared in double distilled water, then 200 μl of samples and these standards were analysed at various dilutions in pyrex glass tubes. 400 μl of 2% aqueous solution of phenol in distilled water was added to each tube and mixed. Then, 1 ml of sulfuric acid (6 N) was added into the mixture and the tubes were incubated at 20°C for 30 minutes to develop the colour. The OD value was quantified at 485 nm using the Wallac CompuSpec UV-visible spectrophotometer. The amount of sugar presented in the sample was determined by using the standard calibration curve.

2.2.2.2 Quantisation of protein content by bicinchoninic acid assay

Protein determination was carried out by bicinchoninic acid (BCA) method. 1 ml of bicinchoninic acid solution was mixed with 0.02 ml of copper(II) sulfate pentahydrate solution (4% w/v) and the mixture was added into 1ml of standards (10-100 μg/ml) and samples. Then, the solutions were kept at 37°C for 30 minutes and the absorbances were measured at 562 nm.

2.2.3 Radioiodination of proteins by chloramine-T method

Carrier free ^{125}I(2 μCi, 5 μl) was mixed with phosphate buffer (10 μl, 0.25 M, pH 7.5), and a solution of protein (100 μl, 0.5 mg/ml) was added to this mixture. To incorporate iodine onto tyrosine residues of the protein, chloramine-T (10 μl, 5 mg/ml in 0.05 M phosphate buffer, pH 7.5) was added and the mixture was incubated at 20°C for 10 minutes. In alkaline conditions, chloramine-T is slowly converted to hypochlorous acid which acts as an oxidation agent. When

the solution pH is less than 8.0, the oxidation results in iodine incorporation into tyrosine residues of proteins. The reaction was stopped by the addition of sodium metabisulphite (20 μl, 1.2 mg/ml in 0.05 M phosphate buffer, pH 7.5). The labelled protein was applied on a Sephadex G-25 column (10 cm^3) equilibrated with 0.05 M phosphate buffer (pH 7.5), and the fractions per ml were collected. 5μl from each fraction was transferred to gamma vials for counting in a 1275 Mini-gamma γ-counter (Wallac) and the protein amounts were measured by BCA method. The fractions corresponding to the maximum protein radioactivity peak were pooled and then dialyzed against four changes of 0.05 M phosphate buffer (pH 7.5) to remove the unbound ^{125}I. The amount of protein-bound radioactivity was assessed with trichloroacetic acid (TCA) precipitation. The 5 μl of labelled protein was mixed with 100μl of BSA (10%) and 1.25 ml of TCA (20%) was added to this mixture. After incubation at 4°C for 1 hour, the samples were centrifuged at 735g for 10 minutes. The supernatant and the pellet were transferred separately to gamma vials and their radioactivity was counted. While radiolabelled proteins give the ratio of counts in the pellet to that of the total radioactivity less than 90%, the dialysis equilibrium was reached.

2.2.4 Radiolabelling of plasmid DNA

The DNA labelling reaction in this study is based on the endonuclease activity of DNA polymerase I, which acts at the site of nicks in the plasmid and removes the nucleotides from 5' to 3' direction, while it is incubated in the nick-translation mixture. The radiolabelling of DNA was operated by the following steps: firstly, the ^{35}S-deoxyadenosine 5'-triphosphate (2μCi) was added into 2.5 μl of nick translation buffer (pH 7.5) composed of 0.5 M Tris-HCl (pH 7.5), 0.1

M magnesium chloride, 500 μg/ml BSA and 10mM dithiothreitol. Then, 1 μl each of deoxyguanosine, deoxythymidine and deoxycytidine triphosphates (dNTP's) (200mM) was added into the mixture. Finally, 5 μg of plasmid DNA and 2 units of E. coli DNA polymerase I was mixed with the mixture. The mixture was diluted to 50 μl with TE buffer (pH 8.0) supplemented with 0.9% NaCl (STE buffer) and applied to PROBEQUANT™ G-50 micro spin column, in an eppendrof tube which was centrifuged at 735g. Unincorporated dNTP's were retained within the column and the labelled plasmid DNA was eluted. The labelling efficiency of DNA was estimated by measuring ^{35}S radioactivity in the aliquots of the initial reaction mixture and the eluted radiolabelled plasmid on the scintillation counter 1400 (Wallac), then dividing the count in the eluted plasmid DNA by that in the initial reaction mixture.

2.2.5 Preparation of calcium phosphate-DNA Complexes

100 μg of plasmid DNA and tracer ^{35}S-labelled DNA was mixed with 10 μl of calcium chloride (2.5 M), and this mixture was transferred into 100 μl of hepes buffer saline (HBS) (pH 7.0). Then, the suspension of calcium phosphate-DNA complex in HBS was centrifuged at 735g for 15 minutes and washed with distilled water twice. Finally, the calcium phosphate precipitate was resuspended at 100 μl of distilled water for the entrapment into DRVs.

2.2.6 Preparation of liposomes

Liposomes can be prepared by different methods for various types. In this study, the methods applied to prepare the liposomes will be described by following steps.

2.2.6.1 Preparation of multi lamellar vesicles (MLVs)

The lipid mixture in chloroform was dried to thin film at 40°C by evaporation of the solvent at a low speed in a rotary evaporator (Buchi) connected to a running tap water pump. To remove residual organic solvent, the lipid film was left under a stream of nitrogen for 3 minutes. Rehydration and formation of MLVs were affected by the addition of 2 ml distilled water and shaking down on rotamixer.

2.2.6.2 Preparation of small unilamellar vesicles (SUVs)

MLVs prepared as described above were sonicated by an MSE sonicator (probe of 19 mm diameter) in an ice bath for 10 one-minute cycles alternating with 30 sec intervals to get SUVs. Then, SUVs produced were centrifuged at 735g for 10 minutes to spin down titanium particles shed from the probe of the sonicator.

2.2.6.3 Preparation of dehydration-rehydration vesicles (DRVs)

The preparation of DRVs was according to the method of Kirby and Gregoriadis (1984). SUVs prepared as above in the presence of distilled water were mixed with 1 ml of the substances to be encapsulated together with corresponding radiolabelled tracers when available, frozen at -20°C and freeze-dried overnight in Christ freeze-drier. The dried powder was rehydrated with 0.1 ml of distilled water and incubated above phase-transition temperature for 30 minutes. Then, 0.1 ml of sodium phosphate buffer (0.1 M, pH 7.4) containing 0.9% NaCl was added and the mixture incubated for another 30 minutes. After adding with 0.8 ml of PBS, the entrapped substances were separated by ultracentrifugation at 27300g for 20 minutes using a

Sorvall Combi Plus Du Pont ultracentrifuge. The liposome pellet was washed 2 more times with 5 ml of PBS and the final pellet was resuspended in adequate medium for further studies. The entrapment efficiency of protein is estimated by ^{125}I radioactivity and that of DNA is evaluated by ^{35}S radioactivity.

2.2.7 Microfluidisation of Liposomes

The DRVs prepared as the procedures described above were passed for 3, 6, 10 full cycles through a Microfluidizer® (model: 110 S, serial: 93124) provided by Microfluidics Corp. (Newton, MA, USA). The microfluidized DRVs were centrifuged for 30 minutes at 35000g and the pellets washed twice in PBS and resuspended in 1 ml of adequate medium for further studies. Solute retention values thus obtained correspond to the percentage of the original entrapment values in the preparations before microfluidisation.

2.2.8 Covalent conjugation of cholera toxin B(CTB) subunits to liposomal surface

In this study, the cholera toxin B was conjugated to the liposomal surface via a thioether bond and finished by the following steps (Harokopakis *et al.*, 1995) (Figure 2.2).

2.2.8.1 Synthesis of distearoyl phosphatidylethanolamine-4-N-maleimidomethylcyclo-hexane-1-carboxylate (DSPE-MCC)

DSPE-MCC was prepared by incubating 50 mg of DSPE with 35.8mg of SMCC in 3.6 ml of chloroform/methanol (9:1) containing freshly distilled triethanolamine (19μl), at room temperature for overnight (Figure 2.2 (a)). The water-soluble excess of SMCC and other reaction

byproducts were removed by diluting the solution with chloroform (5 ml) and extracted twice with 1 ml of NaCl (1%) aqueous solution. For further purification, the preparation was dried on a rotary evaporator, resuspend in 1 ml chloroform and passed through a silica column (12×0.8 cm). The purified reaction was analysed by thin layer chromatography and UV spectroscopy (Wallac) for each fraction.

2.2.8.2 Preparation of SPDP-CTB conjugates

The amine-reactive reagent, SPDP, was used to add thiol groups to the lysine residues of CTB (Figure 2.2 (b)). The thiol groups were necessary for reaction with the maleimide group of DSPE-MCC. In the initial step, CTB was dissolved in and dialyzed against Hepes buffer and subsequently incubated with SPDP at CTB: SPDP molar ratio (1: 50) in the dark at room temperature for 24 hours. The reaction was quenched with 10 µl of 20 mM L-lysine (20mM Tris buffer, pH 6.8) and the reaction products were reduced by adding 5 µl of 7.7 mg/ml dithiothreitol in distilled water. Then, the untreated substances and byproducts were removed by dialysis and the degree of substitution of CTB with pyridyldithiopropionate was estimated spectrophotometrically (Carlsson *et al.*, 1978). Finally, CTB and SPDP-CTB were applied in SDS-PAGE to confirm the alternation of CTB after conjugation with SPDP.

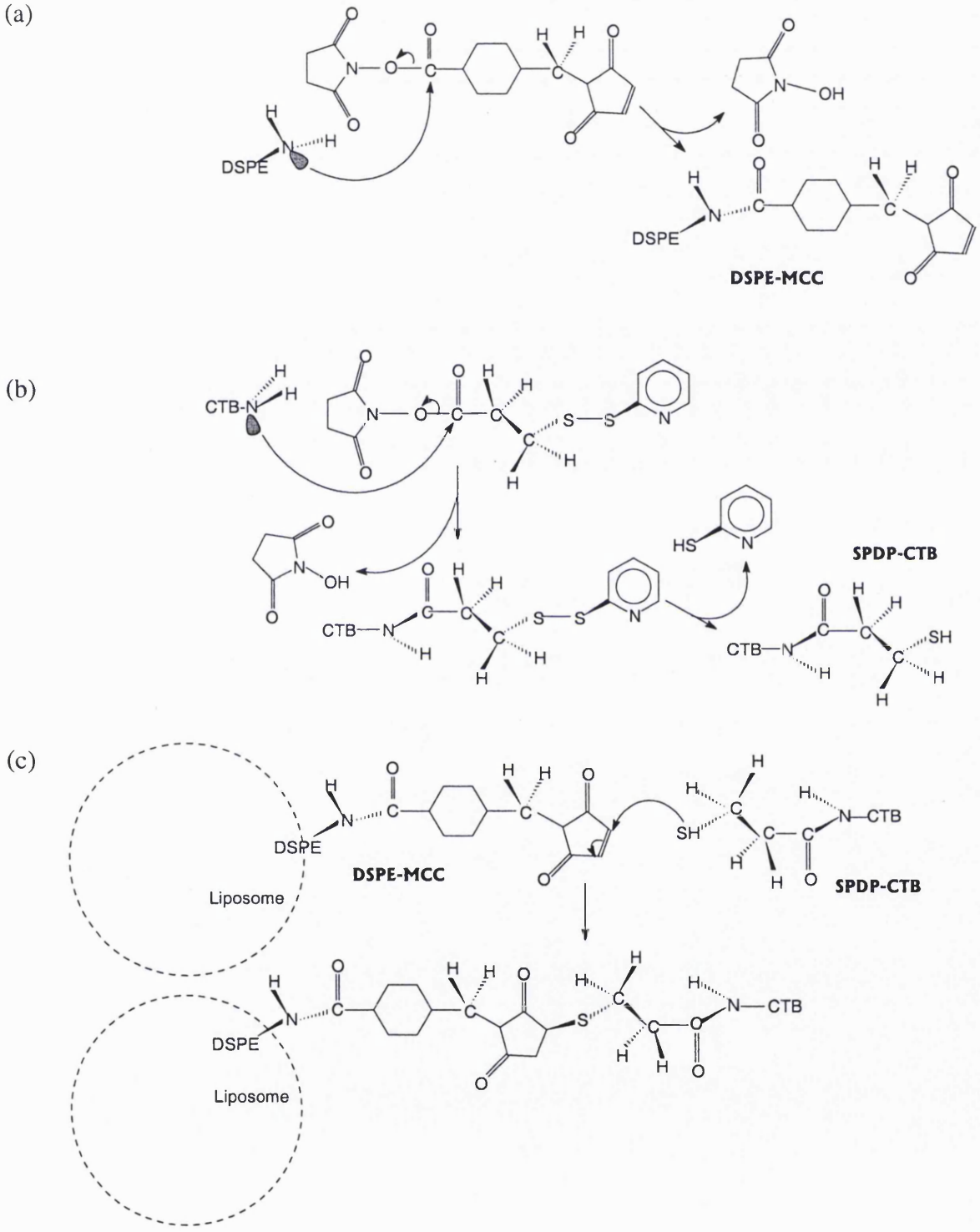

Figure 2.2: Chemical reactions involved in the conjugation of CTB to liposomes. (a) synthesis of DSPE-MCC, (b) preparation of CTB-SPDP, (c) CTB conjugation on liposomes.

2.2.8.3 Covalent coupling of CTB to the liposomal surface

Conjugation of CTB-SPDP to liposomes was carried out by incubating the reduced protein (approximately 1mg) with the dispersion of DSPE-MCC bearing DRVs at 4°C for overnight (Figure 2.2 (c)). The coupling reaction was stopped by adding 10μl of L-cysteine buffer (20mM L-cysteine, in 20mM Tris buffer, pH 6.8) to the reaction solution. The DRVs were separated from the unconjugated proteins and washed twice with PBS by centrifugation at 27300g for 30 minutes. The amount of CTB in washed DRVs pellet was determined by using ^{125}I radioactivity.

2.2.9 Measurement of particle size distribution

2.2.9.1 Sizing of vesicles by photon correlation spectroscopy

Particle size distributions were measured by photo correlation spectroscopy of samples diluted in water, using a Malvern Model 4700 apparatus (Malvern Instruments Ltd., Malvern, UK) equipped with a 25 mW helium/neon laser. Mean diameters and size distributions are obtained: the z-average mean diameter, polydispersity factor and cumulative percentage undersize data were recorded.

2.2.9.2 Sizing of vesicles and particles by laser diffraction

The volume mean diameter of vesicles and calcium phosphate-DNA complexes greater than 1 μm was measured by the laser diffraction method on a Malvern Mastersizer X at 20°C by diluting the suspensions to the adequate concentration in distilled water.

2.2.10 Measurement of particular zeta potential

In the laser Doppler spectroscopy, particle mobility in fixed electric field can be detected by Doppler shifts of laser light. The zeta potential, the electric potential in slipping surfaces of colloidal particles, can be calculated by electric mobility through the Helmholtz-Smoluchowski equation and be applied to evaluate the charges on the colloidal particles. In this study, the DRVs were resuspended in PBS with various pH and different concentration. The zeta potential was measured by microelectrophoresis on a Malvern Zetasizer 3000 at 25°C.

2.2.11 Morphological observation of liposomes

2.2.11.1 Transmission electron microscopy of liposomes

The DRVs were placed on a bare 700 mesh gold EM grids held by tweezers mounted on a spring-loaded plunger and stained with 1% aqueous phosphotungstic acid. After removing excess samples by bolting the grid with filter paper, the sample was taken a photograph at 100kV under a philips 201 transmission electron microscope (TEM).

2.2.11.2 Cryo-scanning electron microscopy of liposomes

The HBsAg-containing DRVs and CTB-conjugated HBsAg-containing DRVs were plunges frozen in Liquid Nitrogen Slush, transferred to the Philips XL20 scanning electron microscope (SEM) and surface moisture removed by sublimation at -40°C for 10 minutes. Then, the samples were transferred to cryo-work station, coated with gold at -180°C and taken a photograph under SEM.

2.2.12 Monitoring the retention of entrapped substances in liposomes

The retention of entrapped HBsAg and plasmid pRc/CMV HBS in DRVs was evaluated by the radioactivity of the entrapped substance. The DRVs entrapping HBsAg or calcium phosphate- plasmid pRc/CMV HBS DNA complexes were resuspended in PBS or 10% mouse muscular extract. Then, they were incubated at 4 or 37°C for different time courses. In addition, HBsAg-containing DRVs was incubated in PBS and 10% mouse gastrointestinal extract to evaluate the release of HBsAg from DRVs in GI tracts. The samples were taken out and centrifuged at 27300xg (for DRVs) or 35000xg (for smaller DRVs) for 30 minutes by using a Sorvall Combi Plus Du Pont ultracentrifuge. The retention ratio of protein is estimated by ^{125}I radioactivity and that of DNA is evaluated by ^{35}S radioactivity.

2.2.13 Immunisation protocols

2.2.13.1 Schedules for protein vaccination

Male Balb/C or T.O. mice (20-25 g) were used (in groups of five) in all immunisation experiments. Immunisation was carried out by intramuscular injection into the mouse leg using a 25-gauge needle and 0.1 ml of samples on days 0 and 29. The blood samples from the tail vein were collected on 28 days as the primary response and 10 days after the booster injection as the secondary response. In some cases where long term antibody response was monitored, blood samples were taken per month after the booster injection. Blood samples (50μl) in heparinized capillaries were transferred into eppendrof tubes containing PBS (450μl) and centrifuged at 1000 g for 10 minutes. The serum in supernatant was taken and stored at -20°C for further analysis.

2.2.13.2 Schedules for DNA vaccination

Male Balb/C mice (20-25 g) were used (in groups of five) in all immunisation experiments. In the DNA vaccination with multiple doses, immunisation was carried out by intramuscular injection into the mice leg using a 25-gauge needle and 0.1 ml of samples on days 0, 7, 14, 21 and 28. The blood samples from the tail vein were collected on days 7, 14, 21,28, 35 and per month after the forth booster injection. In the DNA vaccination with a single dose, immunisation was carried out by intramuscular injection into the mice leg on days 0 and the blood samples from the tail vein were collected per month after the injection. Blood samples were treated as in the method described in *2.2.13.1.*and stored at -20°C for further analysis.

2.2.13.3 Vaccination by oral administration

To apply the liposomal formulations for oral vaccination, male Balb/C mice (20-25 g) were used (in groups of five) in oral immunisation experiments. Immunisation was carried out by feeding the preparations into the mice using an oral dosing needle and 0.1 ml of samples on days 0 and 29. The blood samples from the tail vein and the faeces were collected on 28 days as the primary response and 10 days after the booster injection as the secondary response. Blood samples were treated as the method described in *2.2.13.1.* and the faecal samples were diluted with 10 fold volumes of PBS and centrifuged to take the supernatant. Then, these samples were stored at -20°C for further analysis.

2.2.14 Enzyme-linked immunosorbant assay (ELISA)

96 well flat-bottom microtiter plates were coated with HBsAg in 0.05M sodium carbonate buffer (0.318 g of Na_2CO_3 and 0.58 g of $NaHCO_3$ up to 200 ml of distilled water, pH 9.6). The 60μl of HBsAg (2μg/ml) was added into each well and incubated at 4°C for overnight. The unbound antigen was removed and the plates were washed three times with washing buffer (PBS containing 0.025% Tween-20) and dried by adsorbing liquid with tissue papers. To block the nonspecific binding of antibody, 60 μl of BSA in washing buffer (1%) was added into each well and the 60 μl of diluted sera were added into the first well of each column. Then, the serial dilution was operated by mixing and transferring 60μl mixture to the well in next row and discarded the final 60μl. The plates were incubated at 20°C for three hours and washed as above. After drying the plates, 50 μl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (total IgG, IgG_1, IgG_{2a}, IgG_{2b} subclasses and sIgA) were added at 1:2000 dilutions in washing buffer containing 1% BSA and 5% fetal calf serum to each well, and incubated at 20°C for three hours. Plates were washed and dried as above, and 200 μl of freshly prepared substrate solution (24.3 ml of citric acid (0.1 M), 25.3 ml of $Na_2HPO_4 \cdot 2H_2O$ (0.2 M), 50 ml of distilled water, 40 mg of o-phenylenediamine, and 40 μl of hydrogen peroxide (30%)) was added to the wells and incubation at 20°C for 30 minutes. Finally, the reaction was stopped by adding 25 μl of sulphuric acid (1.8 M) and the colour developed was measured at 492 nm by using a Titertek Multiskan® MCC/340 ELISA Reader (Flow Laboratories, Herts, UK). Pooled sera obtained prior to immunisation and a commercial anti-HBs antibody (CHEMICON, CA, USA) were employed as negative and positive controls respectively. The antibody responses were expressed in terms of serial dilutions and the values of end points were estimated as the

$\log_{10}$ of the reciprocal of serial (two-fold) serum dilution given an OD value of about 0.2. The means and standard deviation of the $\log_{10}$ serial dilution end points were calculated for each animal group.

2.2.15 Statistical analysis

The significance levels were evaluated by the p-value from one-tail Student's unpaired *t*-test or (U) critical value from Mann-Whitney test.

Chapter 3

Liposomes as Immunological Adjuvants for Hepatitis B Surface Antigen: Effect of Lipid Composition

3.1 INTRODUCTION

Most of the commercially available hepatitis B vaccines are based on recombinant hepatitis B surface antigen particles derived from yeasts as antigen and on alum as an adjuvant. However, alum as an immunological adjuvant is far from ideal, because it cannot induce cell-mediated immunity and is, therefore, unsuitable for a variety of antigens. It has been shown (Allison and Gregoriadis, 1974) that liposomes have immunological adjuvant properties. Earlier evidence (Manesis *et al.*, 1979) suggested that liposomes could play a role in vaccines against hepatitis B. Furthermore, the studies (Bakouche and Gerlier, 1986; Kinsky, 1978) on the effect of the bilayer fluidity of liposomes on the immune response to membrane-soluble antigens had shown that liposomes made of 'high melting' phospholipids (e.g., DSPC) with Tcs above 37°C, elicit strong antibody responses to such antigens. The reverse was observed (Gregoriadis *et al.*, 1987; Davis and Gregoriadis, 1987), however, when a water soluble antigen (tetanus toxoid) was used, with responses now being strong with liposomes made of 'low melting' phospholipids (with Tcs below 37°C) and nil or minimal with DSPC liposomes. The differential effects of DSPC on responses to the two types of antigens have been attributed to the direct transfer of membrane antigens from their liposome carrier to the plasma membranes of APC where they associate with MHC molecules, without being first processed.

Based on these studies, the HBsAg, a membrane protein on the envelope of hepatitis B viruses, was entrapped into liposomes composed of PC or DSPC with equimolar cholesterol (Chol) by the dehydration-rehydration method, yielding dehydration-rehydration vesicles (DRVs). The morphology, the size distribution and the zeta potentials of these vesicles were

studied. Furthermore, the stability of these vesicles was tested on storage and their antigen release profiles in different biological media were monitored. Finally, these formulations were injected by the intramuscular route and antibody responses in inbred and outbred mice were measured to evaluate the adjuvanticity of the liposomal formulations. Through this study, the characteristics of HBsAg-containing DRVs, composed of PC or DSPC and equimolar cholesterol, and the immune responses induced by these formulations were investigated.

3.2 CHARACTERISATION OF HEPATITIS B SURFACE ANTIGEN

To study liposomes as vaccine adjuvants for HBsAg, the HBsAg proteins had been characterized in terms of molecular weight by SDS-PAGE to confirm the integrity, and labelled with ^{125}I by Chloramine-T method to evaluate the entrapment efficiency in liposomes.

3.2.1 SDS-PAGE of HBsAg

The SDS-PAGE of recombinant HBsAg shows a sharp band near 24kDa (Figure 3.1.). It indicates that the antigen used is small HBsAg without glycosylation and sufficiently pure for immunization studies.

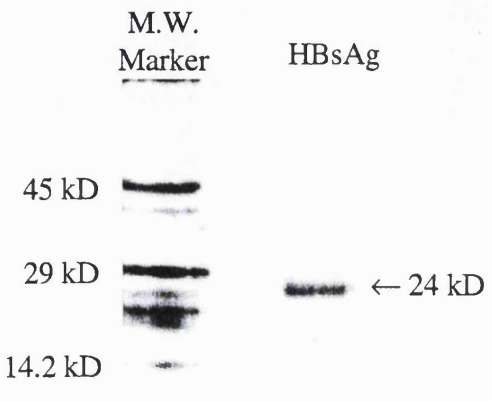

Figure 3.1: SDS-PAGE of HBsAg in 15% acryl amide gel. The molecular weight markers are α-lactalbumin (14.2kD), carbonic anhydrase (29kD) and chicken egg albumin (45kD). The band of HBsAg shows its molecular weight is around 24kD.

3.2.2 Radiolabelling of HBsAg

HBsAg was radiolabelled with ^{125}I by Chloramine-T method and passed through a G-25 column and fractions were collected. The radiolabelled HBsAg was eluted in the first peak and free $Na^{125}I$ in the following peak (Figure 3.2). The labelling efficiency of ^{125}I on HBsAg calculated from this result was 91.5%. This labelled HBsAg was used as a tracer in the following experiments to evaluate the amount of HBsAg in liposomes and decide the dose of HBsAg for immunization studies.

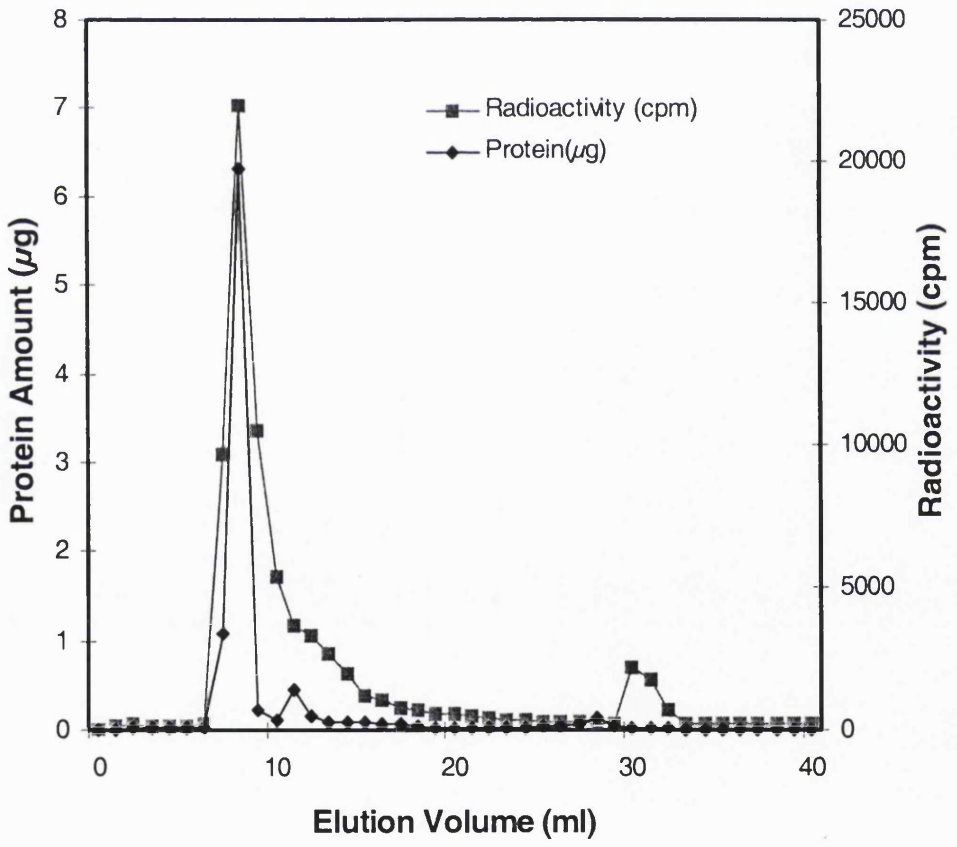

Figure3.2: Size exclusion chromatograms of ^{125} I-labelled HBsAg. ^{125}I-labelled HBsAg passed through a G-25 column and eluted with PBS. The fractions (one ml each) were collected and measured for ^{125}I radioactivity by a 1275 MINIGAMMA gamma counter and for protein amounts by BCA assay.

3.3 CHARACTERISATION OF HBsAg-CONTAINING DRVs

In order to investigate the effect of lipids on the immunization with HBsAg, this antigen was entrapped into liposomes composed of PC or DSPC with equimolar cholesterol (Chol) by the dehydration-rehydration method yielding dehydration-rehydration vesicles (DRVs). These DRVs were characterized by entrapment efficiency, vesicle size distribution, morphology and electrostatic properties. Furthermore, the stabilities of HBsAg-containing DRVs were carried out, which included antigen retention in DRVs on storage and the antigen release profiles.

3.3.1 Vesicle properties of HBsAg-containing DRVs

Vesicle properties play an important role as they influence the fate of liposomes *in vivo* and the immune response. To apply these formulations to the immunisation studies, the properties of these vesicles were firstly characterized and the results have been summarized in the following section.

3.3.1.1 Entrapment efficiency of HBsAg into DRVs

The entrapment efficiency of HBsAg will be used to decide the dose and the ratio of antigens to lipids in liposomes, which had been found to have an effect on the immunization results in the previous studies. Here, the entrapment efficiency of HBsAg into liposomes prepared by different procedures has been compared . The DRVs were prepared from precursor MLVs or SUVs to investigate the effect of sonication procedures, and the rehydration of freeze-dried lipid powder at 25°C or 60°C was investigated to show the effect of temperature control in this step. Furthermore, HBsAg was added in a rehydration step to avoid freezing damage in

the preparation of DRVs.

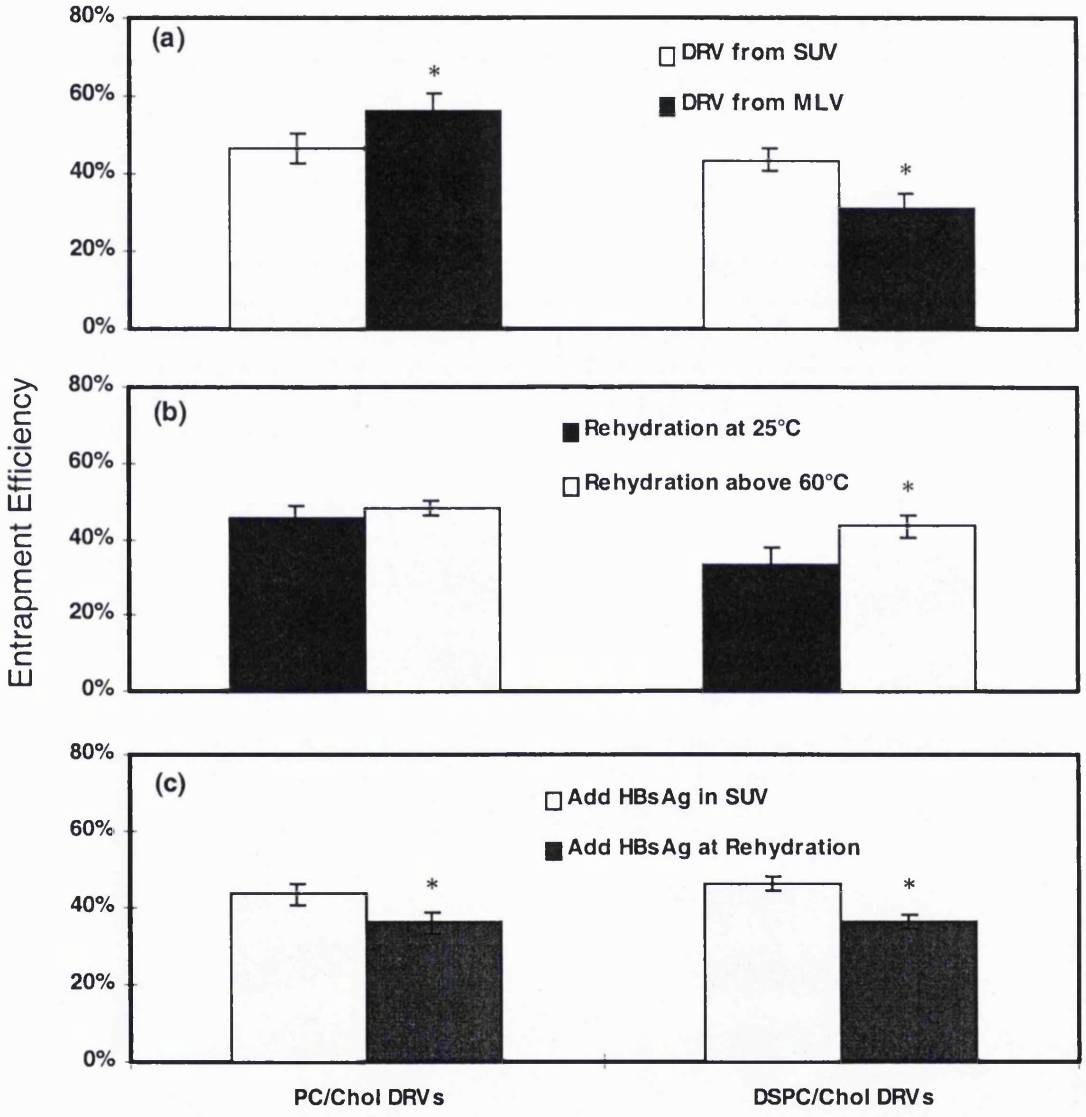

Figure 3.3: Comparison of the entrapment efficiency of HBsAg in DRVs with different preparation procedures. HBsAg (10μg) was entrapped into DRVs made of PC or DSPC with equimolar cholesterol (16:16μmoles). The entrapment amount of HBsAg in DRVs was evaluated by entrapping ^{125}I labelled HBsAg, and counting the radioactivity in a gramma counter. The entrapment efficiency was calculated by dividing the amount in the precipitated pellet after centrifugation with the total amount. Data shown are mean ± SD of three experiments. * Significantly different from the value of the control group, Mann-Whitney U test ($U \leq 0$ (critical value)).

The entrapment efficiency of HBsAg into DSPC/Chol DRVs was significantly reduced from 46% to 31% by removing the sonication step in the preparation procedure of DRVs (Figure 3.3 (a)). If the sample was rehydrated above the phase transition temperature, the entrapment efficiency of HBsAg into DSPC/Chol DRVs increased from 37% to 46% (Figure 3.3 (b)). Considering the instability of the protein in the freezing process (Chang *et al.*, 1996), the antigen was added in the rehydration step to avoid freezing damage during the lyophilization process, which reduced the entrapment efficiency of HBsAg from 45% to 36% in DRVs (Figure 3.3 (c)).

3.3.1.2 Loading capacity of HBsAg into DRVs

By entrapping varying amounts of HBsAg into DRVs, the loading capacity of HBsAg in DRVs was studied (Table3.1). The amount of HBsAg entrapped into these DRVs is directly proportional to the amount of HBsAg used. This indicates that the entrapped antigens did not achieve the saturation point. The loading amount of HBsAg in DSPC/Chol DRVs was comparatively higher than that in PC/Chol DRVs. When 40μg of HBsAg was used to entrap into DRVs, there was 20μg of protein entrapped in DSPC/Chol DRVs but only 14μg of protein in PC/Chol DRVs.

3.3.1.3 Vesicle size distribution of HBsAg-containing DRVs

The vesicle size distribution is another factor to influence the fate of liposomes *in vivo*. Although the liposomal size is not as important in terms of vesicle accumulation in the reticuloendothelial system (liver and spleen) for IM injection as it is for IV injection (Jackson, 1980), the size distribution is significant for cellular uptake in the local lymph nodes following

IM injection (Schwender *et al.*, 1984; Velinova *et al.*, 1996). When the applied amount of HBsAg in DRVs increased up to 40μg, the vesicle size became larger in PC/Chol DRV, but not in DSPC/Chol DRV (Table 3.1). This indicated that the vesicle size distribution of HBsAg-containing DRVs is controlled by the compositions of DRV and the lipid to antigen ratio.

Table 3.1: Characteristics of HBsAg-containing DRVs.

Formulations	Applied HBsAg (μg)	Entrapped HBsAg (μg)	Volume Mean Diameter (μm)	Span	Zeta Potential (mV)
PC/Chol (16:16μmoles) DRVs	5	2.55±0.12	3.72±0.32	1.28±0.14	-39.4±0.4
	10	4.58±0.25	3.74±0.35	1.21±0.20	-37.2±0.4
	20	7.75±0.36	3.94±0.37	1.18±0.23	-40.2±0.4
	40	14.04±0.47	4.52±0.32*	1.54±0.17	-38.8±0.4
DSPC/Chol(16:16μmoles) DRVs	5	2.45±0.12	2.92±0.18	2.17±0.42	-23.9±0.8
	10	4.39±0.26	2.74±0.17	1.64±0.08	-31.9±0.4
	20	11.29±0.46	2.89±0.21	1.41±0.25	-32.7±0.5
	40	19.63±0.51	3.09±0.18	1.71±0.11	-36.1±1.2

Values reported as mean ± SD of three experiments. *Significantly different from DRVs prepared in the presence of 5μg of HBsAg, Mann-Whitney U test ($U \leq 0$ (critical value)).

3.3.1.4 Morphology of HBsAg-containing DRVs

The photographs of HBsAg-containing DRVs under electron microscope show polydispersed vesicular structures of PC/Chol and DSPC/Chol DRVs. However, the concentric structures were observed only in the case of PC/Chol DRVs (Figure3.4). The polydispersed vesicular distribution (Table 3.1) may contribute to the sustained release of HBsAg from the DRVs and thus to prolonged immune responses.

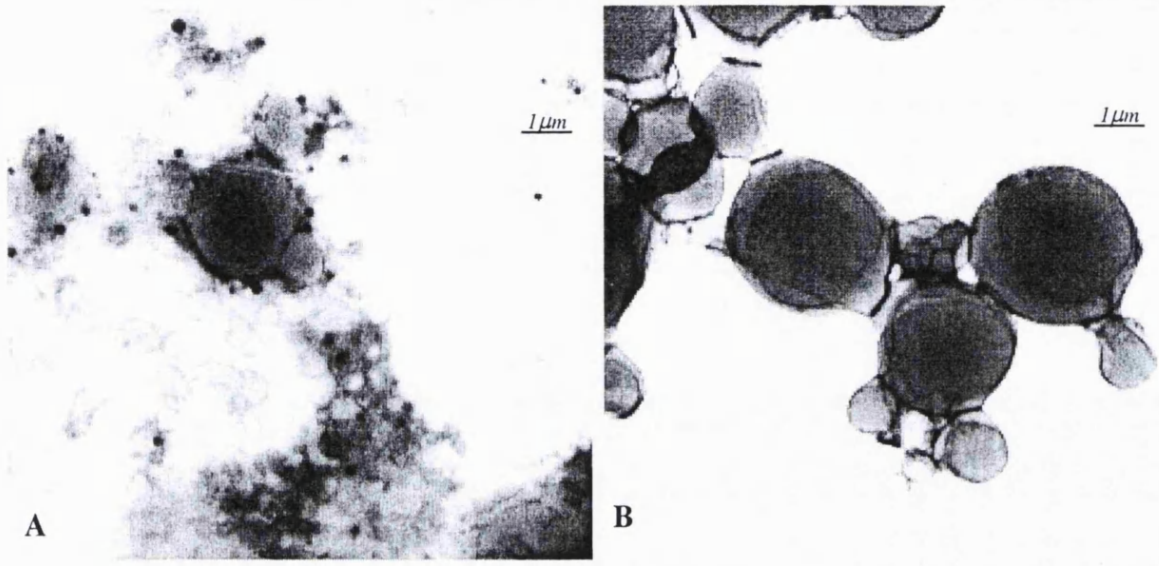

Figure 3.4: Photographs of HBsAg-containing DRVs under transmission electron microscopy. (A) HBsAg entrapped in PC/Chol DRVs, (B) HBsAg entrapped in DSPC/Chol DRVs. DRVs were examined in a Philips 201 transmission electron microscopy. The photos were taken at 100kV.

3.3.1.5 Electrostatic properties of HBsAg-containing DRVs

As mentioned above, the electrostatic properties play an important role in the stability of liposomes in dispersion. The zeta potentials of HBsAg-containing DRVs were measured in a medium varying in ionic strength and pH. The zeta potential approached a zero value with increasing ionic strength on a logarithmic scale at pH 7.4 (Figure 3.5a). Furthermore, the degree of flocculation in the dispersion of HBsAg-containing DRVs increased in PC/Chol DRVs but did not change in DSPC/Chol DRVs when the ionic strength increased (Figure 3.5b). However, the adsorption of counter ions on DSPC/Chol DRVs changed their zeta potentials but did not modify their degree of flocculation in the dispersion. This indicated that adsorbed ions on DSPC/Chol DRVs did not contribute to electrostatic repulsion in flocculation.

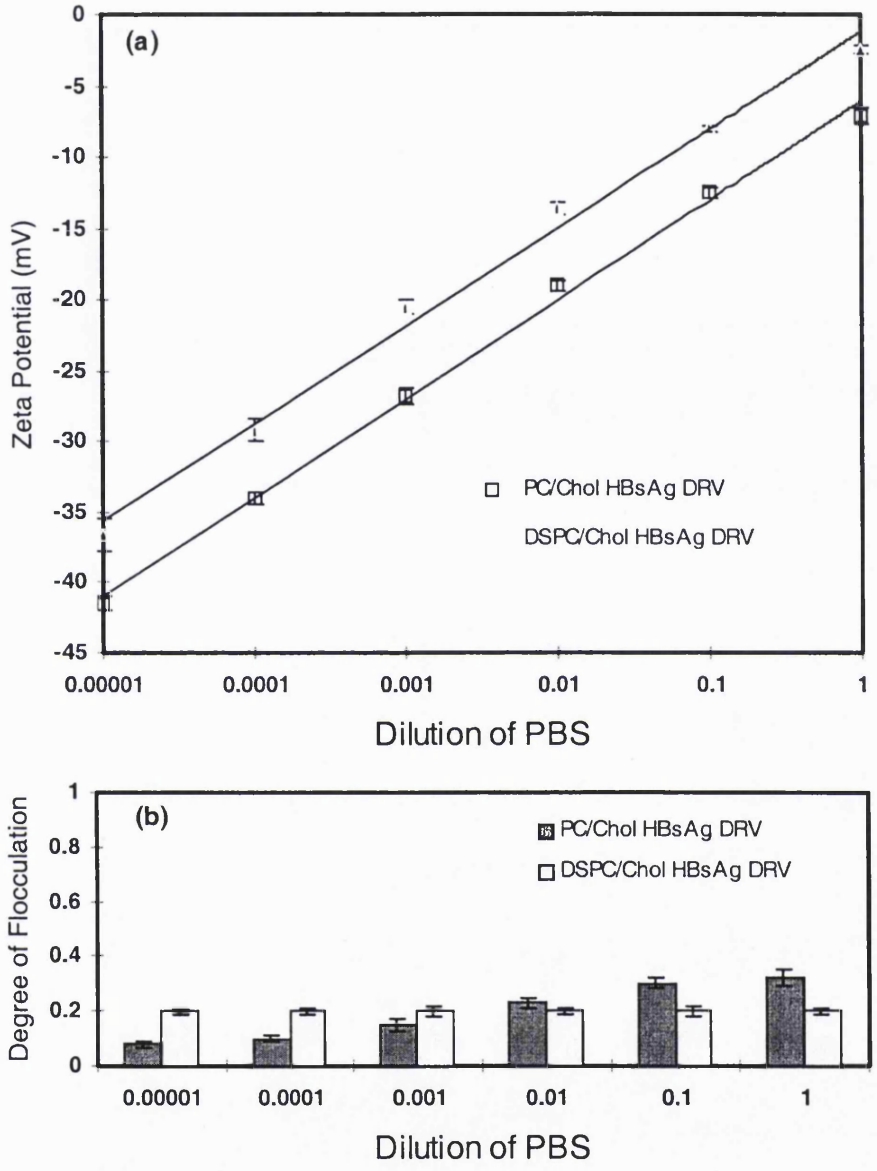

Figure 3.5: Effect of ionic strength on the zeta potential and the flocculation degree of HBsAg-containing DRVs in PBS. The HBsAg-containing DRVs (5μg of HBsAg in 16:16μmoles PC/Chol or DSPC/Chol) were resuspended in different dilutions of PBS and the zeta potentials of these vesicles were measured in a Zetasizer(a). The degree of flocculation (b) was evaluated with the sedimentation volume divided by the total volume. (All data shown are mean ± SD of five experiments).

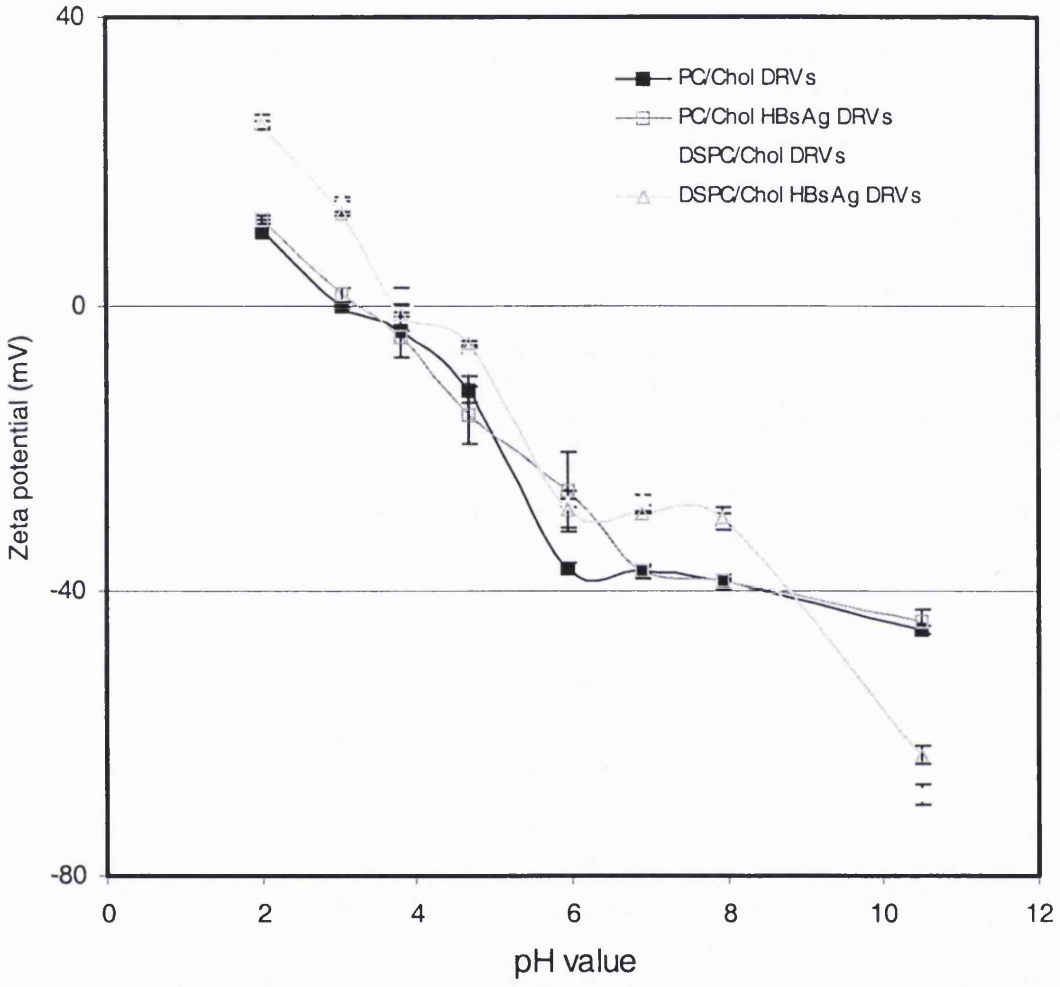

Figure 3.6: pH-zeta potential profiles of HBsAg-containing DRVs. The HBsAg-containing DRVs (5μg of HBsAg in PC/Chol or DSPC/Chol (16:16μmoles)) were resuspended in the 0.001 dilution of PBS with adjusting pH. Zeta potentials were measured in a Zetasizer (Data shown are mean ± SD of five experiments).

The zeta potentials of these liposomes are expected to change with pH. The isoelectric point of HBsAg-containing DRVs is around pH 3.2 in PC/Chol DRVs but 3.8 in DSPC/Chol. By comparison with the empty DRVs, the HBsAg did not change the zeta potential of DRVs significantly in DSPC/Chol case but induced a less negative zeta potential in PC/Chol DRVs

from -38 to -27 mV around pH 6 (Figure 3.6). The results indicated that the zeta potential of HBsAg-containing DRVs changing with pH is responsible for phospholipid composition but not for HBsAg in these formulations.

3.3.2 Stability of HBsAg-containing DRVs

The stabilities of HBsAg-containing DRVs, including the aggregation, HBsAg retention, and the release of HBsAg from DRVs, were characterized and discussed. Firstly, the aggregation phenomena were monitored at different time points. Then, the retention of HBsAg in DRVs was checked for several weeks. Finally, the release profiles of HBsAg were tested in various media, including the PBS, and a 10% muscular extract.

3.3.2.1 Retention of HBsAg from DRVs on storage

When the colloidal dispersions of HBsAg-containing DRVs were applied as vaccine formulations, the encapsulations of HBsAg inside the vesicles were the determining factor for immune responses. HBsAg-containing DRVs were stored at 4°C for 8 weeks and 65% of HBsAg was retained inside DRVs (Table 3.2). This study indicated that the HBsAg cannot stay for long in DRVs suspended in an aqueous phase. Considering the preparation procedures of HBsAg-containing DRVs, the freeze-dried powder of HBsAg-containing liposomes would be easier to apply in practical usage.

Furthermore, the sedimented HBsAg-containing DRVs didn't enlarge the vesicle size on storage, which shows the vesicle aggregation cannot induce the fusion between vesicles and vesicles in these formulations. However, the zeta potential changed with the storage time. This

phenomena may result from the leaking of HBsAg from the vesicles.

Table 3.2: Stability of HBsAg-containing DRVs on storage at 4°C.

Formulations	Storage Time	Retention (%)	Volume Mean Diameter (μm)	Zeta Potential (mV)
PC/Chol(16:16μmole) DRVs entrapped 5μg HBsAg	1 week	91.33±0.3	3.93±1.74	-37.0±0.9
	2 weeks	85.63±0.1	4.09±1.64	-38.0±0.5
	4 weeks	75.56±0.2	4.35±1.72	-39.4±0.2
	6 weeks	71.19±0.5	4.94±1.65	-41.5±0.4
	8 weeks	67.22±0.7	5.39±1.23	-42.9±4.9
DSPC/Chol(16:16μmole) DRVs entrapped 5μg HBsAg	1 week	92.03±0.3	2.76±1.75	-24.7±0.5
	2 weeks	87.55±0.1	2.87±1.76	-25.6±1.4
	4 weeks	79.96±0.1	3.01±1.81	-35.4±0.4
	6 weeks	75.29±0.3	3.50±1.78	-38.3±0.2
	8 weeks	69.78±0.3	3.53±1.97	-43.9±2.9

(Data illustrated are mean ± SD of three experiments).

3.3.2.2 Release studies of HBsAg from DRVs in vitro

In order to evaluate the possibility of applying these formulations *in vivo* using the intramuscular (IM) injection, preliminary studies were carried out to determine the release profiles of HBsAg from DRVs into the muscular tissue extract. In the case where HBsAg is released from DRVs in muscle tissue by IM injection, the characteristics of antigens delivered into the local lymph node and phagocytosized by macrophage are changed. This will affect the adjuvanticity of liposomes. Consequently, the release profiles of HBsAg from DRVs were carried out in both PBS and muscular tissue extract. Results indicated that the HBsAg is released from the PC/Chol DRVs but not DSPC/Chol DRVs in muscular tissue extract. Little HBsAg release was observed from the DRVs incubated in PBS (Figure 3.7). This meant that release of HBsAg

from DRVs may be due to the degradation of lipid bilayers. Furthermore, HBsAg release from PC/Chol DRVs only reached a level of saturation around 60%. This may be owing to the mode of HBsAg release from the liposomes. HBsAg, a membrane protein, would be incorporated in lipid bilayers when it is entrapped into DRVs. Since this antigen is inserted into lipid bilayers, it can not diffuse from DRVs. Hence, HBsAg can only be released from DRVs when lipid bilayers are degraded. Therefore, 100% saturation release was not achieved.

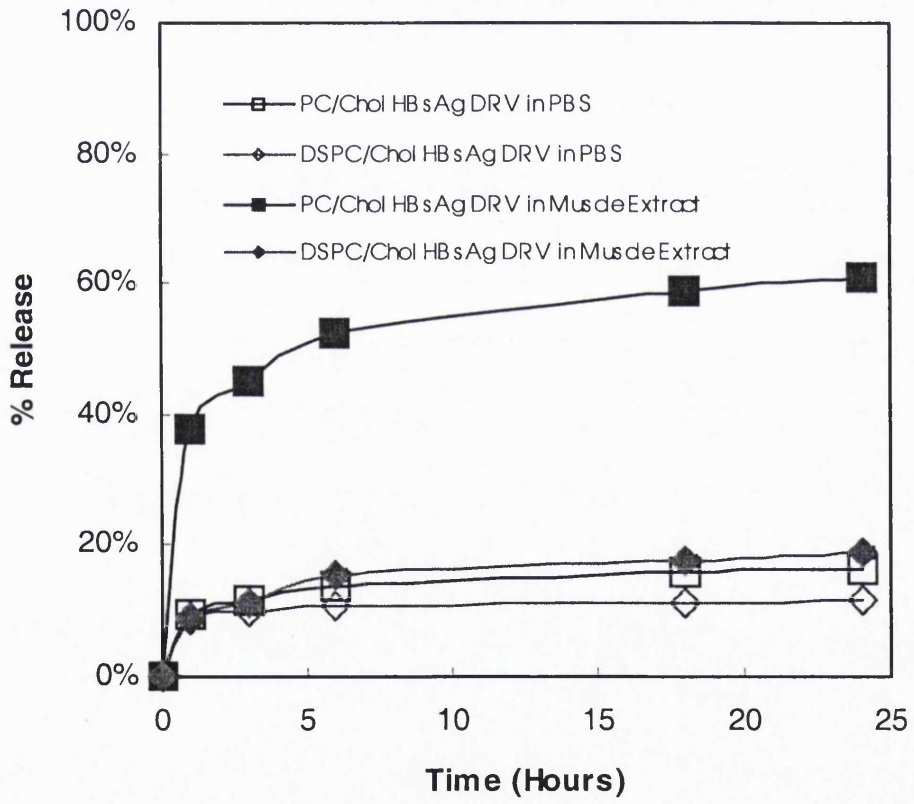

Figure 3.7: Release profiles of HBsAg from DRVs. The HBsAg-containing DRVs (5μg of HBsAg entrapped in PC or DSPC/Chol (16:16μmoles)) were resuspended in PBS or 10% muscular extract in PBS at 37°C and the samples were taken in different time courses (1, 3, 5, 18 and 24 hours) to test the ratio of HBsAg released from DRVs. (Data shown are mean ± SD of three experiments).

3.4 IMMUNE RESPONSES OF HBsAg-CONTAINING DRVs

The ability of these formulations to induce immune responses was investigated by injecting intramuscularly to mice. Firstly, the dose required in immunisation studies was decided by the experiments of dose-response curve. Then, T.O. and Balb/c mice were immunized with these formulations and the humoral responses in inbred and outbred animals were evaluated. Finally, the subclass IgG responses were tested to characterize the effect of liposomal adjuvanticity on the antibody responses of HBsAg.

3.4.1 Dose-response curve of HBsAg immunisation in Balb/C mice

In order to decide the optimal dose required for immunization studies, the ranging amounts (0.001, 0.005, 0.01, 0.1 and 1μg) of HBsAg were injected into the Balb/C mice and the antibody responses were tested by ELISA method (Figure 3.8). The primary response for total IgG was constant above 0.1μg , but the secondary response was observed to be increased with the dose up to 1μg. Compared to the primary response in the immunization, the secondary IgG titre was increased by 50 fold with1μg of HBsAg. The responses induced by 1μg of antigens may not achieve the saturation condition in immunized mice (Figure 3.8 (b)). This means that the immune responses induced by vaccination may not follow the reciprocal dose-response behaviour. Furthermore, the minimum dose required to trigger the antibody response for HBsAg is around 0.01μg, which is insufficient to induce the immunological memory. Based on these results, 0.1and 1μg of HBsAg were selected to formulate into DRVs for further studies.

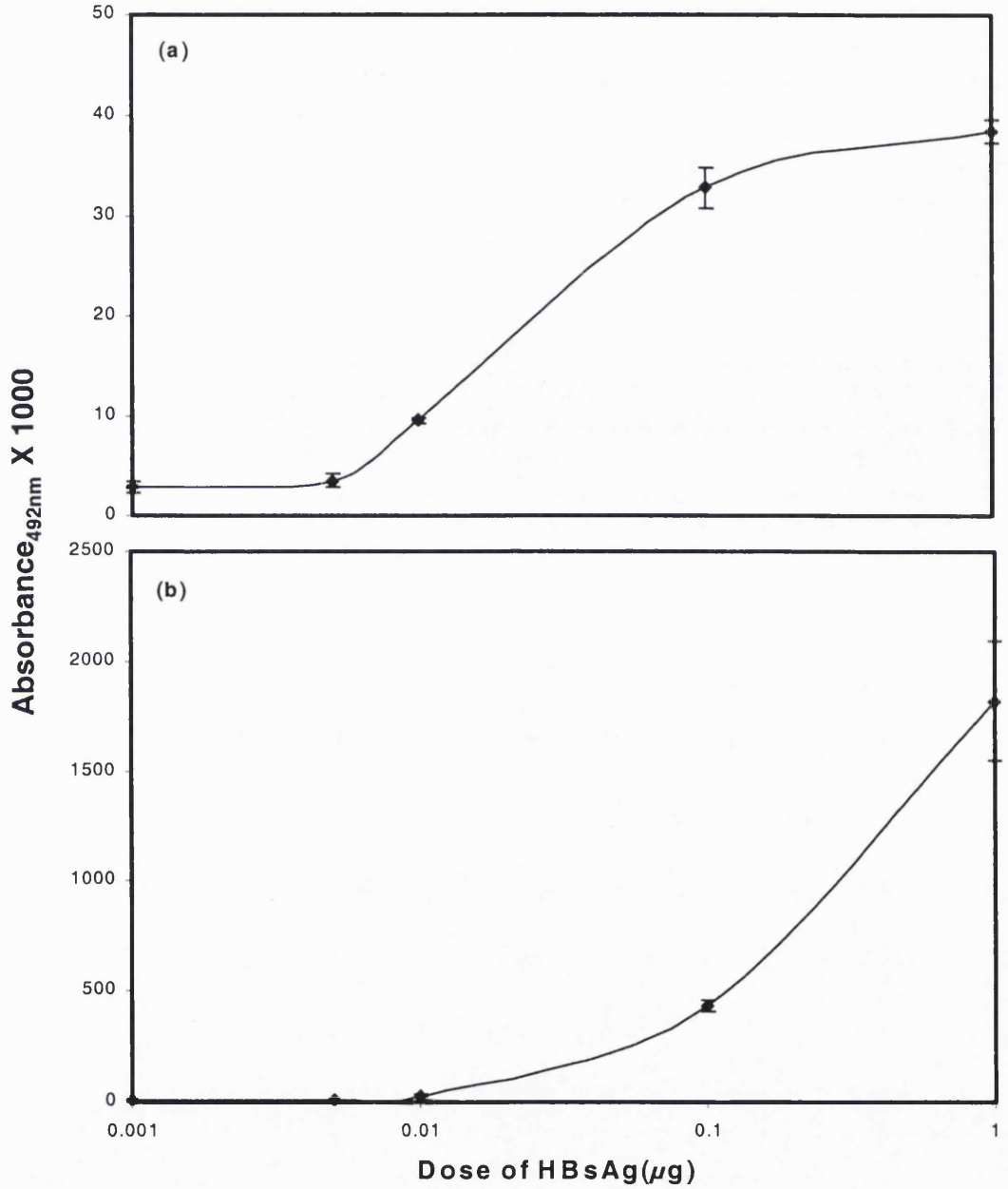

Figure 3.8: Dose-response curves of HBsAg in IM immunization. Balb/C mice were immunized with different doses of HBsAg and bled at 28 days after first immunization as the primary responses (a). At the same time, they were boosted by second doses and bled at 10 days after secondary immunization as the secondary responses (b). The total IgG responses in sera were assayed by ELISA and the values were 1000 folds of the optical density at 492nm by spectroscopical method (Data shown are mean ± SD, n=5).

3.4.2 Immune responses in different strains of mice

To compare the effect of liposomal adjuvanticity for HBsAg in inbred and outbred strains of mice, the HBsAg formulations were injected intramuscularly into T.O. and Balb/c mice. The significant augments in the primary total IgG responses were observed in the groups treated with DSPC/Chol DRVs entrapping HBsAg (1μg). Furthermore, the secondary antibody responses in Balb/c mice were up to the mean of serum IgG titre of 4.0 -5.2 (log_{10}) by immunisation with HBsAg or its liposomal formulations. However, IgG titres induced by these formulations were still kept on the mean of 3.2 (log_{10}) in T.O. mice. Better IgG titre levels were observed in mice treated with DSPC/Chol DRVs entrapping HBsAg than PC/Chol when the immunization dose was 0.1 or 1μg. PC/Chol DRVs increased the mean serum IgG titre from 4.1 to 4.8 (log_{10}) with 1μg of HBsAg (Figure 3.9).

3.4.3 Subclass IgG responses of HBsAg-containing DRVs

In order to evaluate the effect of liposomes on antibody responses induced by HBsAg in the immunological pathway, the subclass IgG responses elicited by these formulations in Balb/c mice were measured. In the PC/Chol case, the liposomal formulations can increase the serum IgG_1 titre to HBsAg from 3.5 to 4.5 (log_{10}) in the secondary response, but cannot change IgG_{2a} and IgG_{2b} titres. However, DSPC /Chol DRVs increased the titres significantly for IgG_1, IgG_{2a}, and IgG_{2b} subclasses. Liposomes increased the antibody production in IgG_1 dominantly but only slightly or not at all in IgG_{2a} and IgG_{2b} titres (Figure 3.10). These results indicated that the liposomal formulations generally improved the antibody responses in IgG_1, IgG_{2a} and IgG_{2b} subclasses. However, the enhancement in IgG_1 is more significant than that in IgG_{2a} or IgG_{2b}.

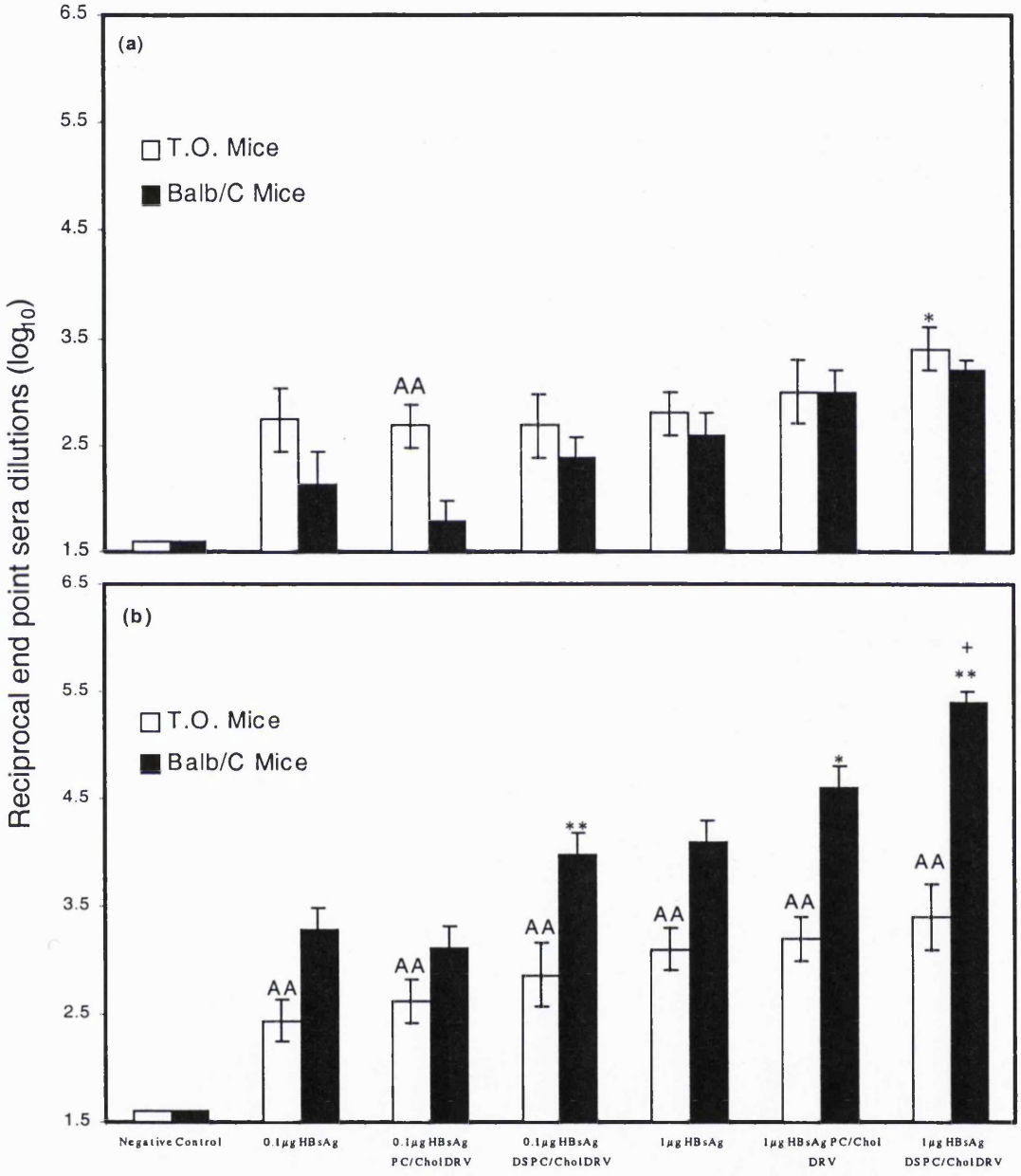

Figure 3.9: Total IgG responses of HBsAg with the liposomal formulations in T.O. and Balb/c mice. The sera bled at 28 days after the first IM immunization were the primary responses (a), and the sera bled at 10 days after the second IM immunization were the secondary responses (b). The IgG responses were assayed with ELISA method. The end point is the dilution with $O.D._{492}$ less than 0.2. Each bar represents mean ± SD (n=5). Significantly different from the groups immunised with free HBsAg:*$p<0.05$; **$p<0.005$. ^{AA} indicated data judged to be significantly different from IgG titres in Balb/c mice ($p<0.05$). $^{+}$Significantly different from the mice immunised with PC/Chol (HBsAg) DRVs, Student's unpaired *t*-test ($p<0.05$).

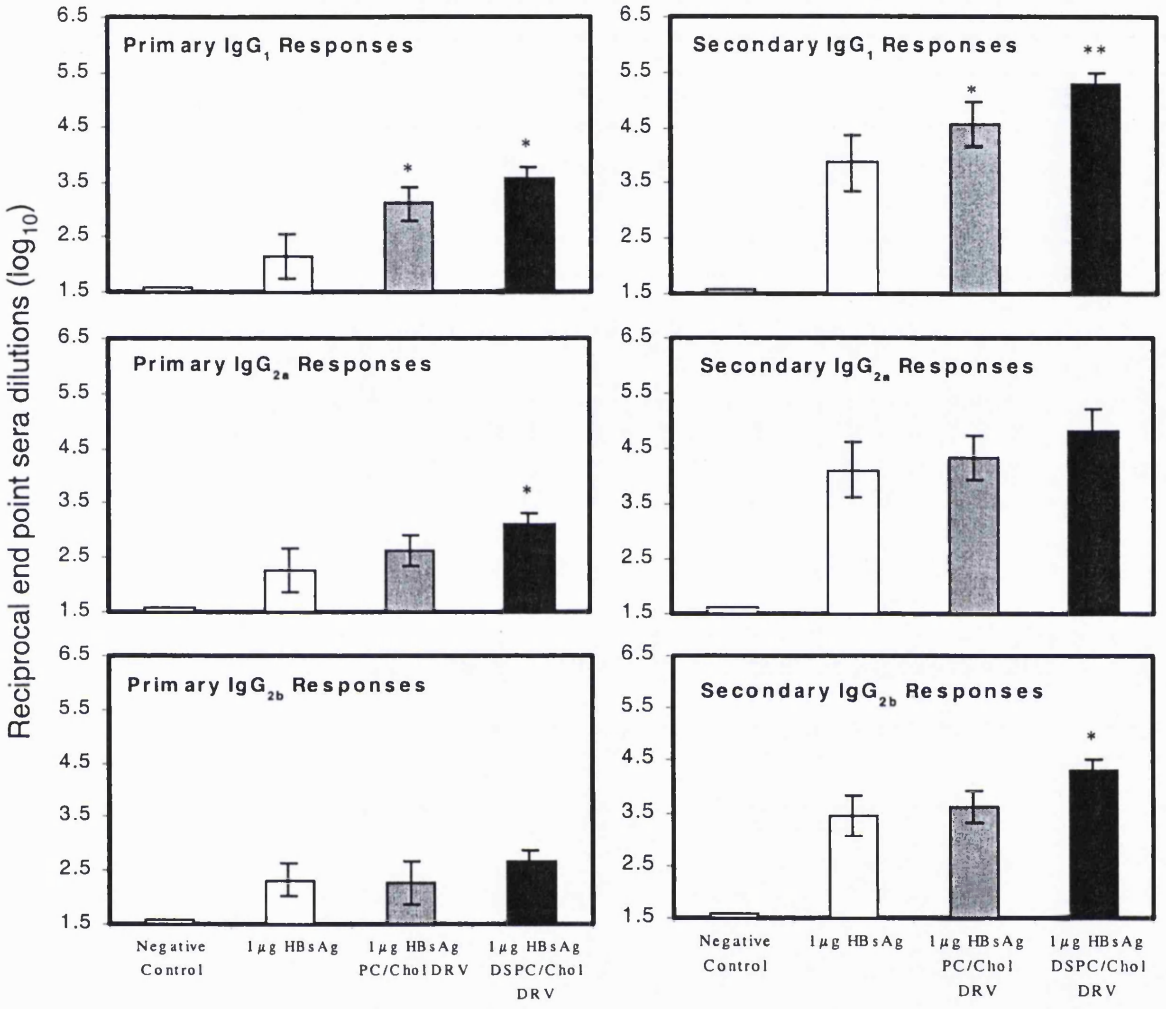

Figure 3.10: Subclass IgG responses of Balb/c mice for HBsAg with liposomal formulations. Balb/c mice were immunized with different formulations of HBsAg by intramuscular injection. The sera taken at 28 days after first injection were primary responses, and the sera bled at 10 days after the second injection were secondary responses. The subclass IgG (IgG_1, IgG_{2a}, IgG_{2b}) titres were assayed with ELISA method, and the $O.D._{492}$ of end point is less than 0.2. Each bar represents mean ± SD (n=5). Significantly different from the antibody titres in the mice immunised with 1μg of free HBsAg: *$p<0.05$, **$p<0.005$ (Student's unpaired *t*-test).

3.5 DISCUSSION AND CONCLUSIONS

Results indicated that HBsAg can be incorporated into DRVs composed of PC or DSPC with equimolar cholesterol and the entrapment efficiency of HBsAg into DRVs is controlled by the preparation procedures. The amount of HBsAg used and the phospholipid compositions affected the loading capacity of HBsAg into DRVs. The vesicle size distribution is controlled by the preparation procedures and the compositions of HBsAg-containing DRVs. Zeta potential is mediated by the compositions of DRVs and the condition in the suspended medium, including pH value and ionic strength. However, the stability of these DRVs upon storage at 4°C had shown the necessity for fresh preparation. Furthermore, the release profiles of HBsAg from DRVs and the immunisation results suggest liposomes, especially those made of DSPC/Chol, can protect entrapped HBsAg in muscle tissue *in situ* and may improve the delivery of HBsAg into macrophages for antigen presentation after intramuscular injection. Finally, in immunization studies using outbred T.O. and Balb/c mice, DSPC/Chol DRVs exhibited significantly greater immunological adjuvant activity (as judged from total IgG immune response) than PC/Chol DRVs. Results indicated that DSPC/Chol liposomes are a promising candidate as a vaccine formulation against hepatitis B. The conditions in this study were applicable to further studies for evaluating and studying the adjuvanticity of liposomal formulations for HBsAg.

During the preparation of DRVs, sonication made the lipid molecules disperse more homogeneously in the aqueous solution and increased the available surface area of liposomes for contacting with the HBsAg. This approach increased the entrapment efficiency of HBsAg in DSPC/Chol case but did not change that in PC/Chol DRVs. This might be due to the rigidity of

DSPC/Chol vesicles. Furthermore, the sizes of HBsAg-containing DRVs were observed to be larger without the sonication process. When the freeze-dried powder was rehydrated with the double distilled water above the transition temperature of phospholipid, the entrapment efficiency of HBsAg increased in DSPC/Chol DRVs. This indicates that the lipid bilayers are more flexible in temperatures above the phase transition and the HBsAg will be incorporated easily into the lipid bilayers (Epand, 1998). In order to protect the protein instability during the freezing process, the HBsAg was added to the freeze-dried powder at a rehydration step but the entrapment efficiency was found to decrease. This might be due to the limitation of the time course of protein interacting with the lipid in liposomal resealing. Generally, the characteristics of HBsAg-containing DRVs are controlled by the preparation procedures.

The physical properties of drug-carrier complexes play a critical role in determining the therapeutic efficiency and shelf-life of a pharmaceutical product. The characteristics of HBsAg-containing DRVs included the entrapment of HBsAg into DRVs, the morphology under electron microscopy, vesicle size distribution and the surface electrostatic property were characterized in this study. Before these formulations were applied in animal studies, the characterization of these formulations had been checked and kept the utility of these formulations in different manufactured batches for each immunisation.

The loading capacity of HBsAg in DRVs was characterized to evaluate the dose of HBsAg in these formulations. DSPC/Chol DRV can entrap more HBsAg than PC/Chol DRV, which may result from the interaction between lipid and HBsAg. DSPC, a saturated

phospholipid, can form the stable lipid bilayers to incorporate more membrane proteins (Gil *et al.*, 1998; May and Ben-Shaul, 1999), which may result in increasing amount of HBsAg entrapment in the DSPC/Chol DRVs.

Vesicle size distribution of HBsAg-containing DRVs is affected by the preparation procedures and the loading amount of HBsAg. If the DRVs were prepared in the absence of sonication, the vesicle size was observed to be larger. The difficulty of dispensing in rehydration of freeze-dried powder might produce large vesicles. Thus, vesicles from the freeze-dried powder of MLVs would reform from the fracture of MLVs and induce the larger vesicles in DRVs formation. It indicates the possibility to control the vesicle size of DRVs in this step. In the next chapter (Chapter 4), the vesicle size of HBsAg-containing DRVs was reduced and controlled to investigate the effect of vesicle size in liposomes as HBsAg vaccine adjuvants.

The electrostatic properties of colloidal dispersion play a decisive role in the stability of the colloid in the medium (Ortega-Vinnesa *et al.*, 1996). The zeta potential had been used to evaluate the electrostatic properties of colloidal particles. The zeta potential is controlled by the ionized groups on the colloidal surface and the ionic strength in the suspended medium. Its value is calculated by the Helmholtz-Smoluchowski equation. The zeta potential of HBsAg-containing DRVs in various dilutions of PBS exhibited the linear relationship between zeta potential and log scale of PBS dilution. It indicates that the surface charge is neutralized by the adsorption of counterions. However, the degree of flocculation in HBsAg-containing DRVs did not go up with increasing electrostatic repulsion. Furthermore, the zeta potential of HBsAg-containing DRVs

is changed with the adjusting pH. Results showed that the surface electrostatic properties of these DRVs are controlled by the phospholipid compositions dominantly, but are not changed by the protein ionization (Figure 3.6). In summary, the zeta potential is controlled by the condition of the disperse media, including pH and ionic strength and itself will influence the liposomal interaction upon storage. Thus, PBS (pH7.4) with 0.001 dilution was selected as micro-electrophoresis media in further studies.

The stability of HBsAg-containing DRVs in this study was characterized by monitoring their characteristics at various time points. In addition, the release profiles of HBsAg from DRVs in mice muscular extracts were investigated at 37°C to evaluate the possibility of HBsAg delivery to local lymph nodes by liposomes. By monitoring the characteristics of HBsAg-containing DRVs on storage, the stability of HBsAg-containing DRVs in aqueous medium was determined and the availability of colloidal dispersion dosage form was evaluated. The results showed that 30% HBsAg leaked from DRVs after 2 months and vesicle properties, such as vesicle size and zeta potential, had a little change. From these results, it has been shown that the freshly prepared liposomal formulation is essential for pharmaceutical application and the freeze-dried powder is advisable for clinical application.

The ability of HBsAg to be delivered to the lymph node *in vivo* is the decisive factor in improving the immune responses. Release profiles of HBsAg from the HBsAg-containing DRVs in PBS and muscular extract indicate that the HBsAg was released from PC/Chol DRV, but not from DSPC/Chol DRV. It means that DSPC/Chol DRVs may be able to deliver the HBsAg

through the muscle tissue and prevent it from the degradation *in vivo*. This can increase the efficiency of delivering antigens to the antigen-presentation cells in the local lymph node. This was subsequently confirmed by the antigen distribution monitoring after IM injection and the antibody responses in the immunization studies. Generally, HBsAg can be incorporated into the lipid bilayers of liposomal formulations and these formulations protect HBsAg in the delivery process to local lymph nodes. Consequently, liposomes increase the efficiency of HBsAg used by the antigen-presentation cell and enhance the antibody responses induced by this antigen.

The immune response to HBsAg-containing DRVs was carried out with the antibody titres. The mice were immunized intramuscularly by different formulations with various doses. In the first step, the different doses of HBsAg were injected by IM routes to investigate the dose-response curve during immunization. Then, the optimal doses of HBsAg were applied in the liposomal formulations and injected intramuscularly to carry out antibody responses. Finally, the subclass IgG responses were assayed and the immune responses regulated by liposomal formulation were investigated.

The pharmacological effect of vaccination is important in vaccine formulations. Based on the traditional methodology in biopharmaceutics, the dose-response curve was constructed, and the concentration of therapeutic agents in the target organ was quantified. Unfortunately, the biological activities in the immune response are too complicated to be summarized as the relationship between antigen dose and antibody response. Therefore, this complication will make the study in vaccine adjuvants difficult to be compared with each other. However, in order to

optimize the liposomes as adjuvants for HBsAg, the relationship between the doses of HBsAg and the antibody responses elicited by this antigen had been evaluated in this study. From the dose-response curve in these results, the antibody response was triggered while the dose up to 0.01μg and curve was shifted to the high dose in the secondary responses, indicating that more antigens are required to saturate the immune system for the secondary responses. However, considering the time course of antibody production, this may change the response curve and make the dose-response different in the primary and secondary responses. In summary, these data provide information about the dose range necessary to study the adjuvant effect of liposomes for HBsAg.

The immune responses elicited in T.O. and Balb/c mice immunised with these formulations were compared in the different preparations at the varying doses in order to carry out the effect on the outbred and inbred strain of mice. In T.O. mice, the primary responses are similar to the secondary responses and the antibody titres are low. By comparison with the responses in T.O. mice, the immune responses induced by these formulations in Balb/C mice show strong responses in the secondary responses. It indicated that liposomes present their adjuvanticity in the Balb/c mice more significantly than T.O. mice. Results show that the Balb/c mice are more sensitive than T.O. mice in the immunization with HBsAg and the immunological memory in Balb/c mice are better than T.O. mice.

The IgG subclass responses to HBsAg immunization in Balb/C mice indicated that the liposomal formulations increase IgG_1, IgG_{2a}, and IgG_{2b} levels unequally. Thus, liposomes increased the titres of IgG_1 but not those of IgG_2 as significantly, indicating a change of immunological pathways towards the Th2 type (Harnett and Harnett, 1999). However, liposomes did not inhibit the antibody titres in each subclass and the total IgG responses induced by DSPC/Chol DRVs were higher than those elicited by PC/Chol DRVs. Previous studies (Gregoriadis *et al.*, 1987 and Kinsky, 1978) suggested that the immunogenicity of liposomal antigens is a function of bilayer fluidity. The completely opposite conclusions reached here may reflect the nature of the antigens used. Membrane antigens may pass into the membrane of the antigen-presenting cells without being first processed. On the other hand, soluble proteins must, by their nature, be taken into such cells and processed before they can be exposed on cellular surfaces. The presence of DSPC in the liposomal bilayer may influence the microenvironment around each HBsAg molecule in a way that enhances its binding to immune cells. Furthermore, injection of DSPC-containing liposomes, characterized by a relatively high Tc, resulted in a marked popliteal lymph node (PLN) reaction. This could have increased the numbers of CD4+ cells, CD8+ cells and subsets bearing specific markers of early activation (Desjardins *et al.*, 1995). Therefore, DSPC DRVs may act to improve HBsAg binding to immune cells and promote the activation of lymphocytes to modulate IgG subclass responses.

Based on the results in this chapter, HBsAg incorporated DSPC/Chol DRVs were selected to study the optimisation of liposomes as vaccine adjuvants for HBsAg in advance. The first approach was to reduce the vesicle size for improving the cellular uptake *in vivo* and the results are summarized in Chapter 4. To apply this formulation by oral route, the vesicles were conjugated with CTB subunits and the data is presented in Chapter 6. The optimal conditions obtained in this chapter, including preparation and immunisation were applied in the following studies in an attempt to improve the liposomal formulation for HBsAg.

Chapter 4

Liposomes as Immunological Adjuvants for Hepatitis B Surface Antigen: Effect of Vesicle Size

4.1 INTRODUCTION

In chapter 3, the DRVs composed of PC or DSPC with equimolar cholesterol were studied for their adjuvanticity for HBsAg. The vesicle sizes for these formulations are around 2-5μm. Prevoius study reported that there was an inverse correction between liposome sizes and their uptake by macrophage (Allen *et al.*, 1991). Thus, the uptake of these vesicles by antigen-presentation cells may be not easy. The vesicle size of liposomes can be controlled or reduced by various methods, such as sonication and microfluidisation. The microfluidisation technique allowed to reduce the vesicle size and was used to produce liposomes with various sizes in this study. These vesicle were applied to study the effect of vesicle size on liposomes as immunological adjuvants for HBsAg. Another approach for size reduction consisted of adding the sucrose during DRV preparation. The resulting size upon rehydration was found to be small. In the same way, the animals were immunised with liposomes prepared in the presence of sucrose to carry out their adjuvantity.

4.1.1 Reduction of liposomal size

The liposomes prepared by dehydration-rehydration method and reverse-phase evaporation are large with diameters approaching the micrometer size range. Intramuscularly injected large liposomes (about 400nm diameter) are unable to reach the regional lymph nodes efficiently (Tumer et al., 1983) and to deliver vaccines and other agents to these sites (Gregoriadis, 1988). Such tasks, however, can be carried out by smaller vesicles of about 200nm or less in diameter. Unfortunately, these tends to exhibit low entrapment yield when prepared by conventional techniques (Mayhew et al., 1984). Previous works (Chapter 3) had shown that

DRVs can entrap HBsAg up to high amount. Subsequently, microfluidisation of these DRVs reduced their size down to 100nm in diameter. These microfluidized liposomes still retained 10-100% of the originally entrapped solutes. Experimental investigations on lyophilized and freeze-thawed liposomes have shown that sugars can suppress vesicle fusion and phase separation of lipids (Strauss *et al.*, 1986; Crowe *et al.*, 1987). Hence, this was exploited to generate DRVs exhibiting smaller size. In summary, these two methods, microfluidisation and sucrose addition, were used to prepare DSPC/Chol HBsAg-containing DRVs with small size. The effect of vesicle size on the immunisation with these formulations was studied.

4.1.2 Microfluidisation

Microfluidisation is a method to prepare the liposomes, fine emulsions and dispersions in the formulations (Vuillemard, 1991). The working principle of this technique is the dynamic interaction of two liquids at very high velocity flowing in microchannels. Studies have shown that this technique allowed working with viscous liquids to achieve a size lower than 1μm. It is reproducible and can be easily scaled up. It was already applied as a method of size reduction of DRVs in previous studies (Gregoriadis *et al.*, 1990; Skalko *et al.*, 1996). Microfluidisation distorts large and flexible vesicles by extrusion through a capillary, thereby increasing the number of vesicles of similar size which 'bud off' from simple parent vesicles. This method may reduce the size of HBsAg-containing DRVs and increase the adjuvanticity of liposomes for HBsAg.

4.1.3 Effect of sucrose on liposomal preparation

Some carbohydrates are capable of protecting membranes and vesicles from dehydration damage and of maintaining the integrity and barrier function of membranes. The protective effects of sugars have been related to their ability to form hydrogen bonds between the polar region of the membrane lipids and the carbohydrate hydroxy groups (Crowe *et al.*, 1984). The interactions result in membrane fluidization of dehydrated lipids, which without sugars would undergo phase transitions and phase separations. Thus, damage of membranes consisting of fluid lipids can be prevented due to pseudohydration. In contrast, phase transition of rigid lipids which are in the gel phase is not affected (Crowe *et al.*, 1988). There is only sparse knowledge of both the specific mechanisms of pseudohydration generation and the reasons for the different efficiency of various carbohydrates as cryoprotective agents. In the preparation procedure of DRVs, the SUVs and the substance to be entrapped were frozen in the deep-freezer. The sucrose added into the mixtures of SUVs and the substance to be entrapped allowed cryoprotection by inhibiting membrane fusion and generating smaller DRVs. This method is suitable for liposomal preparation for vaccine formulations. The cryoprotective agent is used for both the antigen protection and the vesicle size reduction. Results are presented in the following section and compared with the results obtained from microfluidisation.

4.2 MICROFLUIDISATION OF HBsAg-CONTAINING DRVs

HBsAg-containing DRVs were transfered into the microfluidizer and passed through the interaction chamber for different number of cycles by controlling the time course of operation. The microfluidized liposomes were separated from the free antigen by ultracentrifugation and

resuspended in PBS (pH 7.4) with 0.001 dilution in characterisation and stability studies.

4.2.1 Characterisation of microfluidized HBsAg-containing DRVs

Microfluidized HBsAg-containing DRVs showed that the reduction vesicle sizes and a decrease in encapsulation efficiency were due to leakage of HBsAg. The characteristics in terms of vesicle size distribution, antigen entrapment and the morphology of these microfluidized vesicles were determined.

4.2.1.1 Vesicle size distribution of microfluidized HBsAg-containing DRVs

The vesicle size distributions of HBsAg-containing DRVs microfluidized in water, measured by photon correlation spectroscopy, are presented in Figure 4.1. The results were in good agreement with the findings obtained from other author (Talsma *et al.*, 1989). Smaller vesicle size was achieved for formulations processed in water. Considerable reduction in the size was observed after the initial 3 cycles of microfluidisation. Mean volume diameter distribution data revealed the narrowing of the sizes distribution was due to increasing number of small vesicles obtained after processing for the first few cycles. However, the vesicle size distribution exhibits the gradual downward drift (Figure 4.1). This result is different from previous studies showing a transfer of sizes from one size band to another (Gregoriadis *et al.*, 1990). It shows clear evidence of the marked narrowing of the polydispersity index of the vesicles.

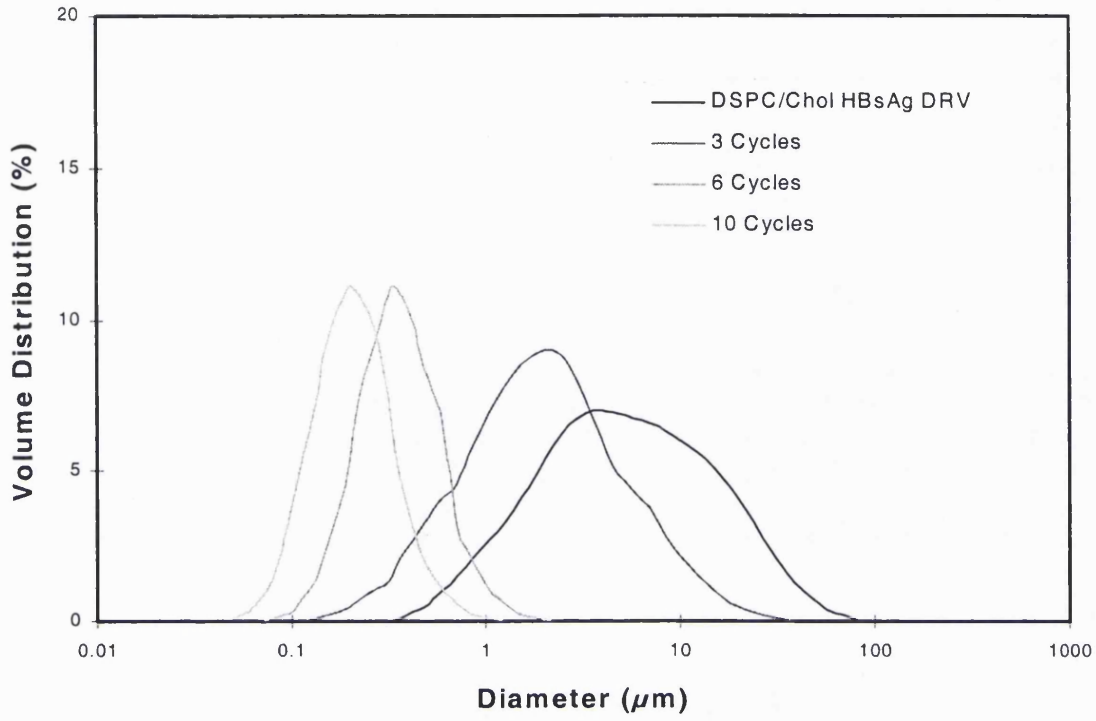

Figure 4.1: Particle size distribution of HBsAg-containing DRVs after microfluidisation. The size distribution of HBsAg-containing DRVs prepared in the presence of water as a function of the number of cycles of microfluidisation shows the progression towards a distribution with a mean size of approx 100nm after 10 cycles.

4.2.1.2 Retention of HBsAg in DRVs through microfluidisation

As shown in previous studies (Kirby and Gregoriadis, 1984; Gregoriadis *et al.*, 1987), entrapment of solutes in DRVs was efficient, ranging from 19.2 to 66.1% of the starting material and dependent on the amount of phospholipid used. Retention values (quoted as a percentage of original entrapment values) by HBsAg-containing DRVs microfluidized for up to 10 cycles in the water are shown in Figure 4.2. These values are similar to those obtained for toxoid and which remained virtually unchanged (at around 75%) after microfluidisation (Skalko *et al.*, 1996). It is conceivable that during the microfluidisation, a process entailing breakage of the integrity of the vesicle membrane and consequent leakage of entrapped solutes occurs, large

molecules such as proteins leak at slower rates than smaller molecules. The presence of unentrapped solute during microfluidisation diminishes solute leakage perhaps by reducing the osmotic rupture of the vesicles and initial concentration gradients across the membrane.

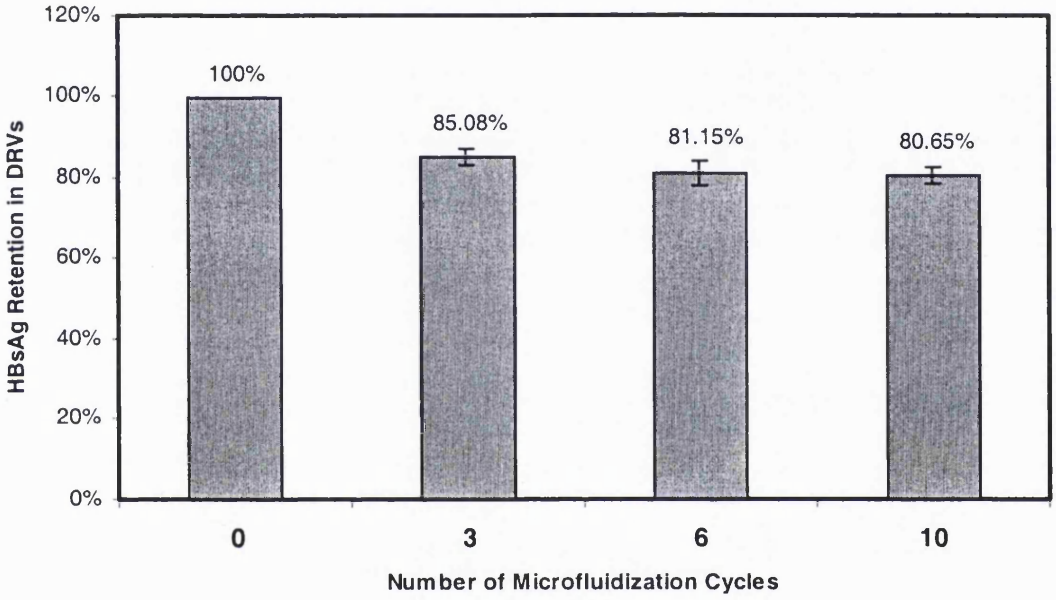

Figure 4.2: Retention of HBsAg in DRVs after microfluidisation. ^{125}I-labelled HBsAg - containing DRVs composed of equimolar DSPC and cholesterol were microfluidized in the presence of water for up to 10 cycles (Data shown are mean ± SD of three experiments).

4.2.1.3 Morphology of microfluidized HBsAg-containing DRVs

The morphology of the microfluidized HBsAg-containing DRVs were examined under TEM. The vesicle shape remained unchanged but pronounced size reduction was observed after 3 cycles of microfluidisation. This is in good agreement with PCS measurements. In constrast, microfluidisation for up to 10 cycles damaged the structure of the vesicle as shown by Figure 4.3. This indicated that the microfluidisation for a long period may make the vesicle be unable to reseal and produce a kind of lipid particles exhibiting difference from usual vesicle structures.

Indeed, the HBsAg inserts itself into the lipid bilayers and its retention within the liposomes is not affected by microfluidisation. This shows that the microfluidisation can be used as a method of liposomal size reduction for membrane proteins-containing DRVs.

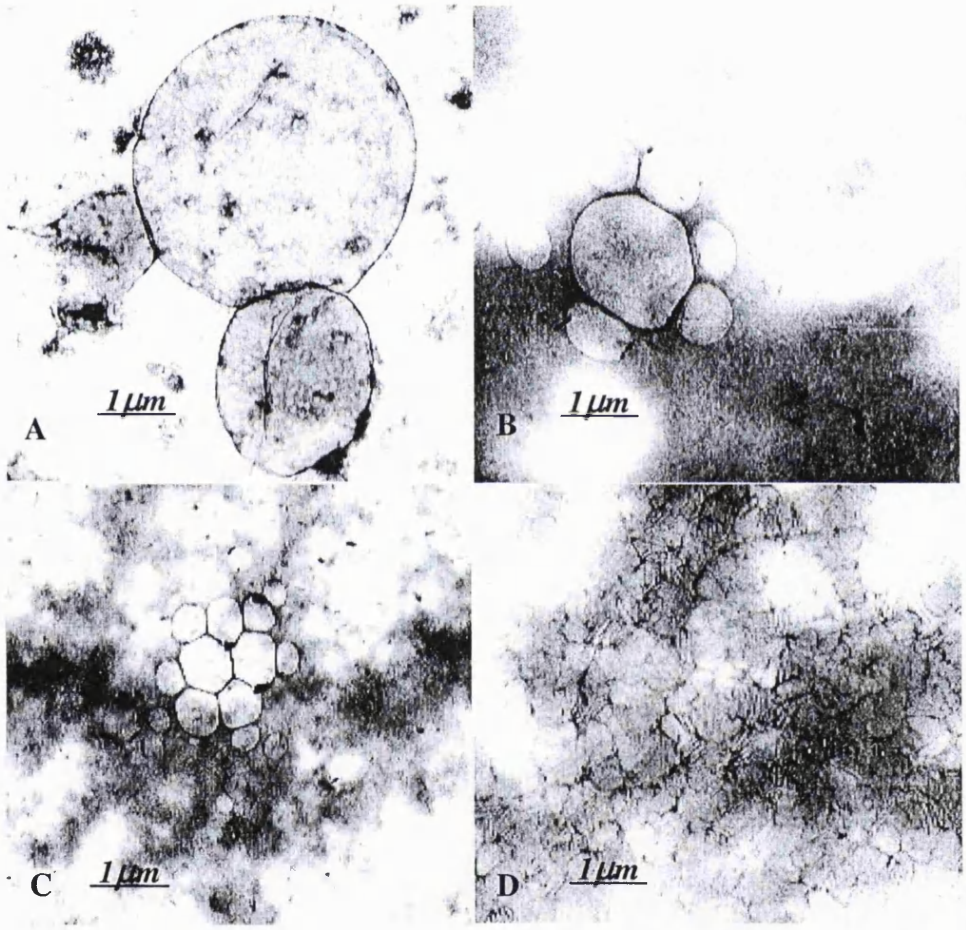

Figure 4.3: Photographs of microfluidized HBsAg-containing DRVs under transmission electron microscopy. (A) DSPC/Chol HBsAg-containing DRVs. (B) Microfluidized DSPC/Chol HBsAg-containing DRVs for 3 cycles. (C) Microfluidized DSPC/Chol HBsAg-containing DRVs for 6 cycles. (D)Microfluidized DSPC/Chol HBsAg-containing DRVs for 10 cycles. DRVs were examined in a Philips 201 transmission electron microscope and the photos were taken at 100 kV.

4.2.2 Stability of microfluidized HBsAg-containing DRVs

4.2.2.1 Retention of HBsAg in microfluidized DRVs on storage

In order to evaluate the stability of microfluidized HBsAg-containing DRVs at 4°C, the retention of HBsAg, sizes and the zeta potentials were measured and compared at different storage time (Table 4.1). Microfluidisation led to leakage of HBsAg easily from liposomes. Liposomal size and the zeta potentials remained unchanged upon storage. Microfluidisation generated smaller liposomes. The increase of the total surface area of these liposomes caused the leakage of HBsAg from liposomes. Reduction of the size of liposomes resulted in slower sedimentation rate and less aggregation consequently. Due to the leakage from the microfluidized HBsAg-containing DRVs, these formulations must be processed just before needed. This limited the application of this technique since it is cumbersome and generates leaky liposomes exhibiting problems upon storage.

Table 4.1: Stability of Microfluidized HBsAg-containing DRVs during the storage at 4°C.

Formulation	Storage Time	Retention (%)	Volume Mean or Z-Average Diameter (μm)	Zeta Potential (mV)
DSPC/Chol HBsAg DRVs	1 week	91.88±0.08	2.64±0.45	-27.6±0.3
	2 weeks	88.12±0.04	3.08±0.33	-31.3±0.1
	4 weeks	79.62±0.10	3.21±0.24	-35.9±1.0
	6 weeks	75.76±0.19	3.40±0.53	-37.9±2.9
	8 weeks	70.00±0.27	3.49±0.49	-42.3±0.8

Formulation	Storage Time	Retention (%)	Volume Mean or Z-Average Diameter (μm)	Zeta Potential (mV)
Microfluidized DSPC/Chol HBsAg DRVs (3 cycles)	1 week	92.58±0.33	0.57±0.08	-36.1±1.0
	2 weeks	82.67±0.67	0.65±0.01	-38.5±0.4
	4 weeks	75.83±0.70	0.67±0.01	-35.4±3.5
	6 weeks	57.19±0.17	0.67±0.05	-30.4±3.0
	8 weeks	46.06±0.69	0.69±0.03	-34.4±0.9
Microfluidized DSPC/Chol HBsAg DRVs (6 cycles)	1 week	89.64±0.05	0.28±0.01	-39.5±0.2
	2 weeks	81.33±0.11	0.31±0.01	-39.3±0.6
	4 weeks	72.60±0.51	0.31±0.01	-37.9±2.5
	6 weeks	63.37±0.42	0.32±0.04	-35.9±0.5
	8 weeks	55.69±0.31	0.35±0.09	-32.4±2.5
Microfluidized DSPC/Chol HBsAg DRVs (10 cycles)	1 week	90.05±0.11	0.22±0.02	–40.1±0.6
	2 weeks	83.80±0.25	0.25±0.03	-47.8±1.0
	4 weeks	75.73±0.58	0.27±0.03	-40.4±2.4
	6 weeks	66.64±0.77	0.28±0.01	-37.9±0.4
	8 weeks	59.74±0.68	0.29±0.05	-33.0±0.7

(Data shown are mean ± SD of three experiments)

4.2.2.2 Release profiles of HBsAg from microfluidized HBsAg-containing DRVs at 37°C

In order to confirm the hypothesis that the smaller size induce faster release due to larger surface area and reduced lamellarity as shown in Figure 4.3, the release profiles of HBsAg from these microfluidized HBsAg-containing DRVs were determined in PBS and muscle extract (Figure 4.4). The results were found to be in line with the hypothesis.

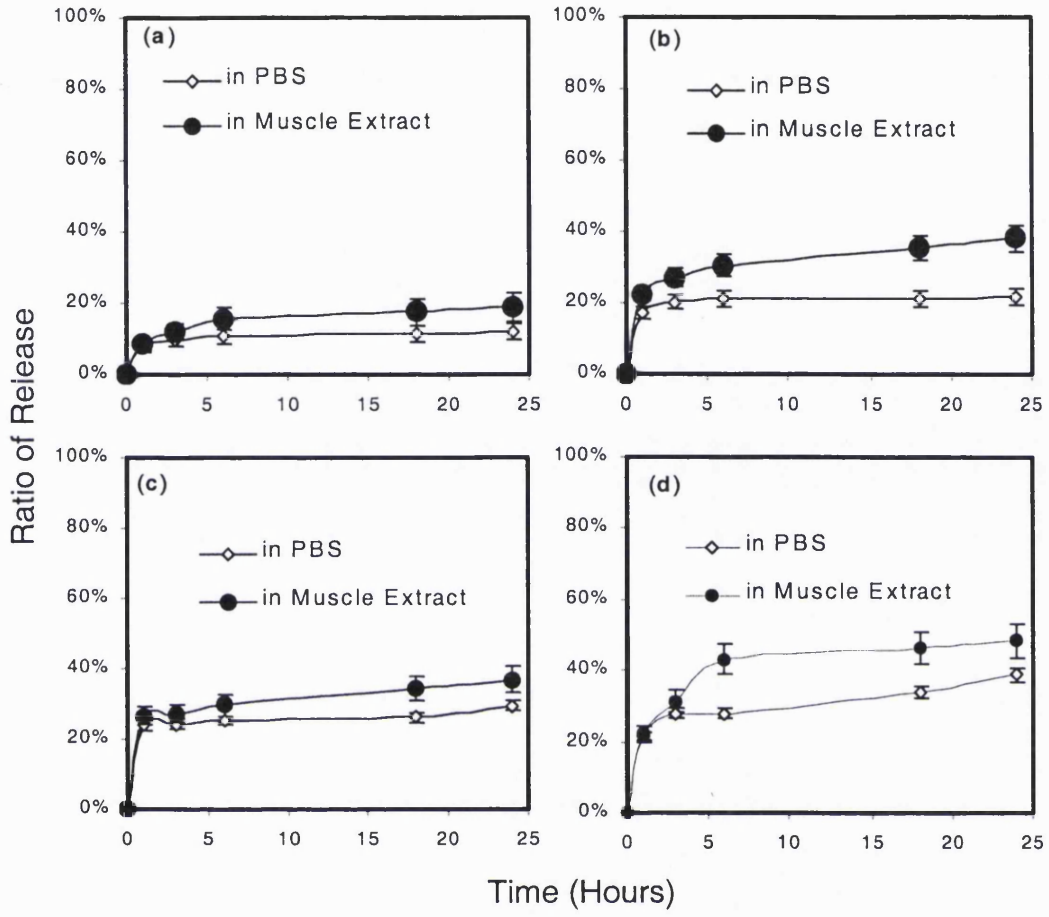

Figure 4.4: Release profiles of HBsAg from HBsAg-containing DRVs or microfluidized DRVs. DSPC/Chol (16:16 μmole) DRVs (a) or microfluidized DRVs ((b) 3 cycles; (c) 6 cycles; (d) 10 cycles) containing 5 μg HBsAg were resuspended in PBS or 10% muscle extract in PBS at 37°C. The samples were taken at different time courses (2, 4, 6, 18, and 24 hours) and centrifuged with the ultra-centrifuge to evaluate the released amount present in the supernatant by measuring the radioactivity. (Data shown are mean ± SD of three experiments).

The release profiles of HBsAg from the microfluidized HBsAg-containing DRVs indicated that HBsAg release from the liposomes was increased with size reduction. In addition, the release profiles of HBsAg from DRVs suggested that the released amount of HBsAg from DRV in muscle extract was higher than that in the presence of PBS. However, the difference between the HBsAg release from DRVs incubated in PBS and in muscle extract is not significant during the initial stage of incubation. This might be due to the properties of DSPC/Chol liposomes, which can resist the enzyme attack *in vivo* (Gregoriadis, 1988). Furthermore, the released amount of HBsAg from microfluidized DRVs in PBS increased when these DRVs were microfluidized for additional number of cycles. A possible explanation could be that the HBsAg released from these vesicles after desorption from the surface of these vesicles. In vaccine formulation, the liposomal sizes should be reduced to increase their uptake by antigen-presentation cells. In contrast, smaller size may reduce the protective effect of liposomes leading to premature leakage of the antigen.

4.3 HBsAg-CONTAINING DRVs PREPARED IN THE PRESENCE OF SUCROSE

Using the second approach, the liposomes size could be kept small when the sucrose was added during the lyophilisation step of DRV preparation. Vesicle size reduction of HBsAg-containing DRVs was achieved with sucrose co-entrapment. The investigation of the sucrose effect on the characteristics of these DRVs, including the entrapment efficiency, morphology and the stability tests, were conducted in the following section.

4.3.1 Characterisation of HBsAg-containing DRVs with sucrose co-entrapment

4.3.1.1 Effect of sucrose on the vesicle size distribution of HBsAg-containing DRVs

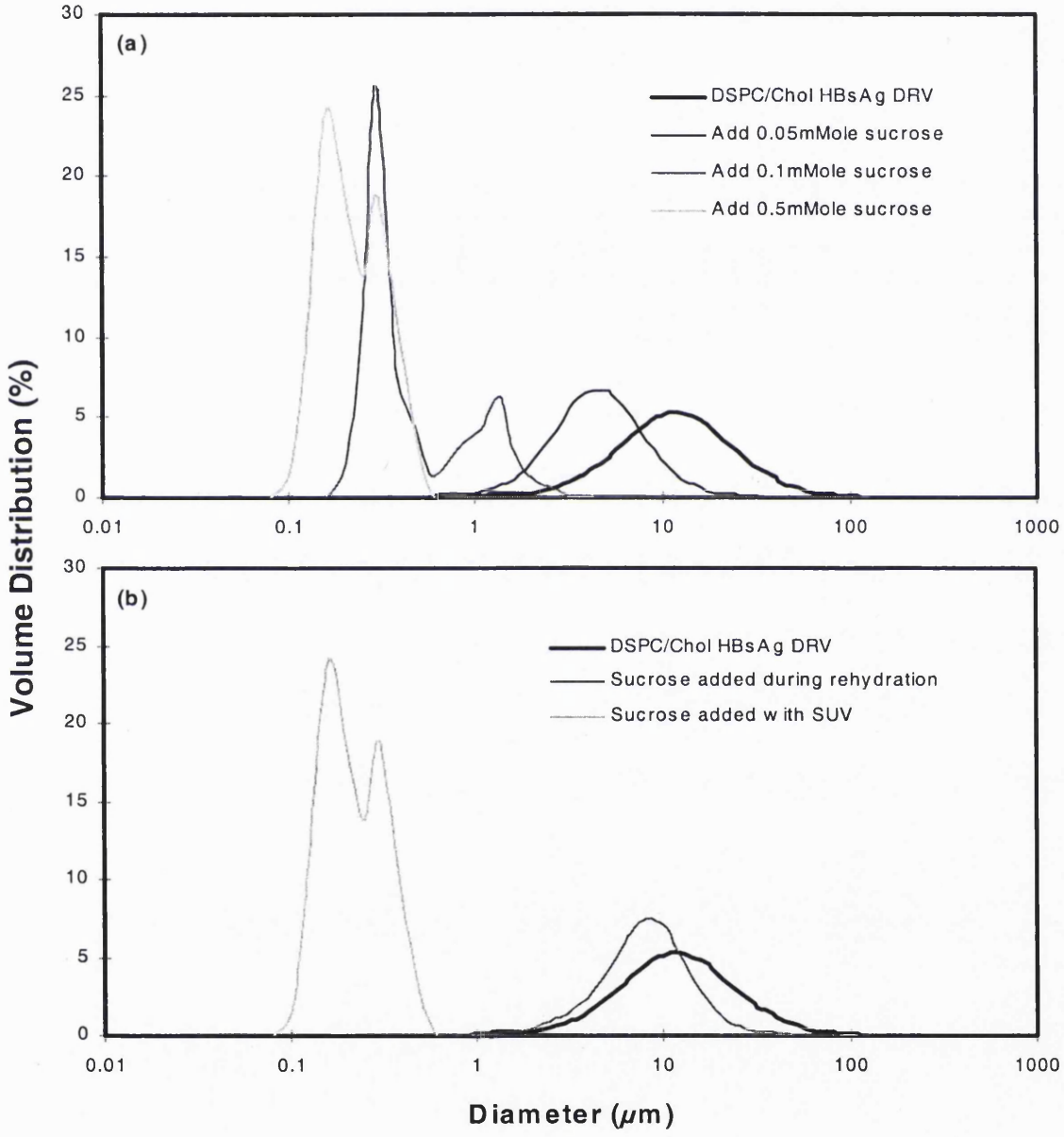

Figure 4.5: Effect of sucrose on the vesicle size distribution of HBsAg-containing DRVs. 5 μg of HBsAg was entrapped into the DSPC/Chol (16:16 μmole) DRVs with different amount of sucrose (a), and the vesicle size distribution is determined by using a Mastersizer and PCS. The vesicle size distribution of HBsAg-containing DRVs with addition of 0.5mMole sucrose in SUV or rehydration step (b). The condition of DRV preparation in (b) is as same as that in (a).

The vesicle size distribution of HBsAg-containing DRVs prepared by using sucrose co-entrapment was assessed function of the sucrose concentration and compared to the classical DRV method (Figure 4.5). The vesicle size distribution of HBsAg-containing DRVs prepared in the presence of sucrose indicated the vesicle sizes of these formulations depended on the molar ratio of sucrose to lipid. When the molar ratio of sucrose to lipid was increased to16, the vesicle size of HBsAg-containing DRVs was found to be the original SUV size. This showed that the high concentration of sucrose can inhibit the fusion of lipid bilayers during the rehydration step leading to smaller size. This observation was confirmed in the control experiments in which the same amount of sucrose was added during the rehydration step in DRV preparation, which generated vesicles in the micrometer range. Thus, the vesicle size of HBsAg-containing DRVs can be controlled by the addition of sucrose in the early stage of DRV preparation. The vesicle size of these DRVs prepared in the presence of sucrose depended on the amount of sucrose added to the SUV dispersion.

4.3.1.2 Entrapment efficiency of HBsAg into DRVs prepared in the presence of sucrose

The entrapment efficiency of HBsAg into the DRVs was not significantly affected by sucrose co-entrapment (Figure 4.6). This might result from the fact that HBsAg is located in the inter bilayer but not in the aqueous space of DRVs. The vesicle size reduction did not limit the HBsAg incorporation. This study indicates that sucrose co-entrapment is a better alternative method to microfluidisation for the preparation of small HBsAg-containing DRVs. Furthermore, sucrose acted as a cryoprotective agent during lyophilisation of labile HBsAg.

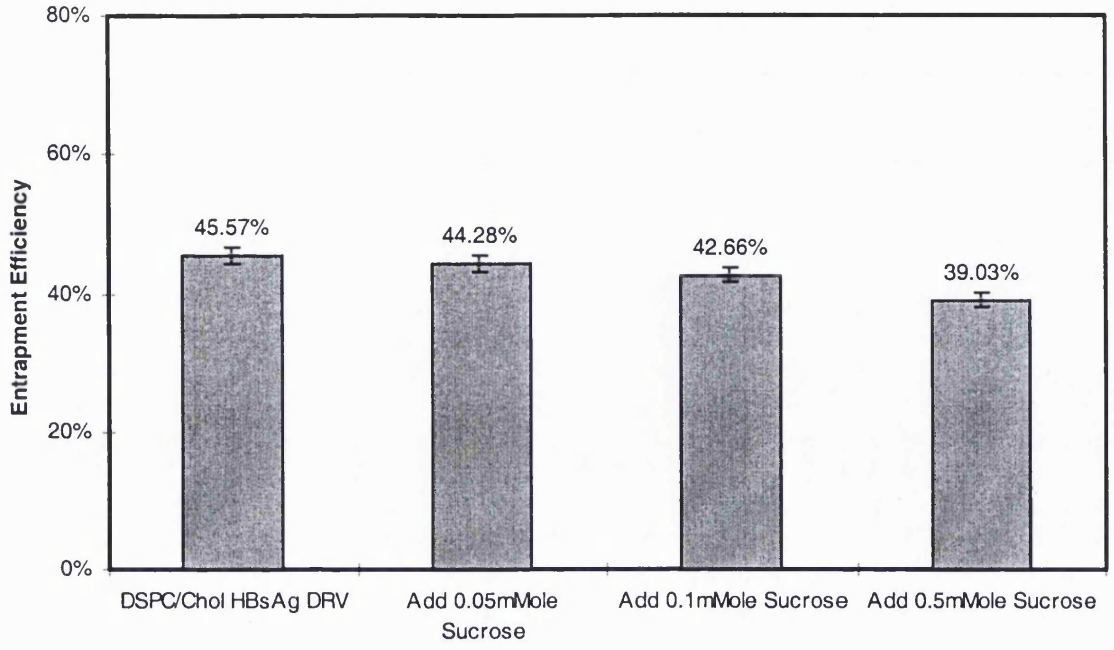

Figure 4.6: Entrapment efficiency of HBsAg in HBsAg-containing DRVs prepared in the presence of sucrose. 5 μg of HBsAg was applied into DSPC/Chol (16:16μmoles) DRVs or DRVs with various amount of sucrose. The entrapment efficiencies of HBsAg into DRVs were evaluated by monitoring free and encapsulated radiolabelled HBsAg (Data shown are mean ± SD of three experiments).

4.3.1.3 Entrapment efficiency of sucrose in HBsAg-containing DRVs prepared in the presence of sucrose

The formulations of HBsAg-containing DRVs with sucrose addition entrapped sucrose entrapped considerable amount of sucrose. In order to evaluate the effect of sucrose in these formulations, the entrapment efficiency of ^{14}C-radiolabelled sucrose was measured (Figure 4.7). The entrapment amount of sucrose in HBsAg-containing DRVs exhibited linear relationship when low amount was used. The entrapment of sucrose in these liposomes was saturated when high amount (0.5 mMole) was added originally. The co-entrapped sucrose allowed the generation of smaller vesicle at high molar ratio of sucrose to lipid. Sucrose presented in high amount lead

to hypertonic formulations. The requirements for isotonicity and smaller vesicle size had to be met for the application *in vivo*. In order to evaluate the effect of the encapsulated sucrose (as shown in Figure 4.7) on the liposomal adjuvanticity for HBsAg, equal amount of sucrose was added externally to the DRV formulations. These formulations were used to immunize animals and compared to the immune responses in mice treated with DRVs entrapping HBsAg and sucrose. The results are discussed in section 4.4.2.

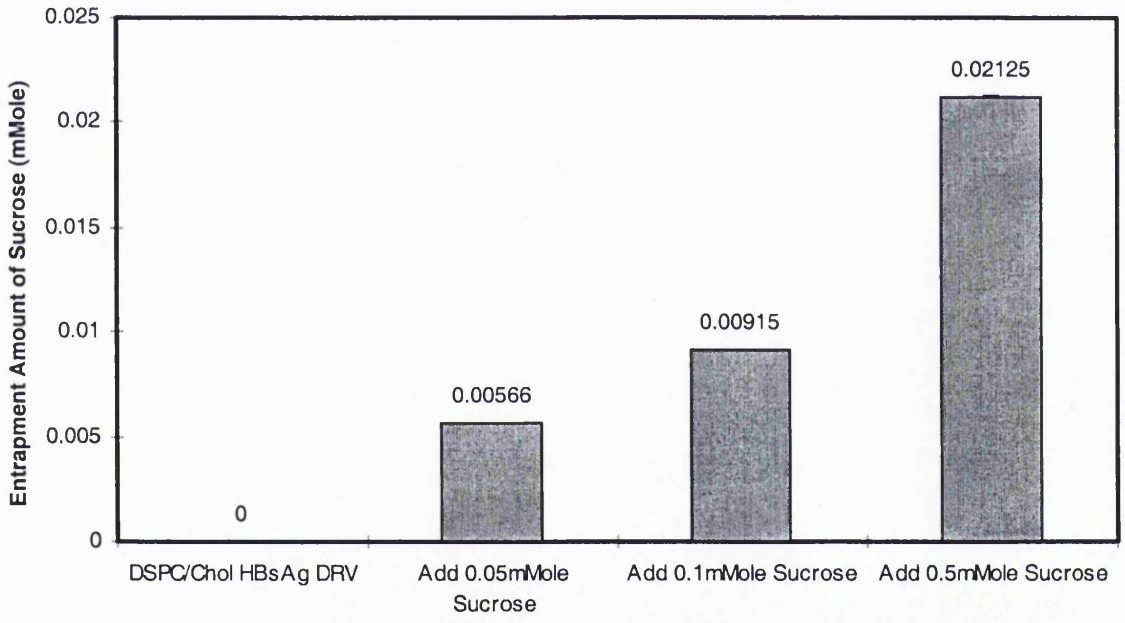

Figure 4.7: Entrapment of sucrose in HBsAg-containing DRVs prepared in the presence of sucrose. Various amount of sucrose and HBsAg was added into the SUV for encapsulation. Entrapment of sucrose in HBsAg-containing DRVs was evaluated with the entrapment amount of ^{14}C-labelled sucrose by radioactivity (Data shown are mean±SD of three experiments).

4.3.1.4 Morphology of HBsAg-containing DRVs prepared in the presence of sucrose

Photographs of HBsAg-containing DRVs prepared in the presence of sucrose under transmission electron microscopy exhibited that vesicle sizes reduced with increasing amount

of sucrose used (Figure 4.8). However, these vesicles showed a different morphology from microfluidized HBsAg-containing DRVs (Figure 4.3). This is probably due to the presence of sucrose and to the preparation method.

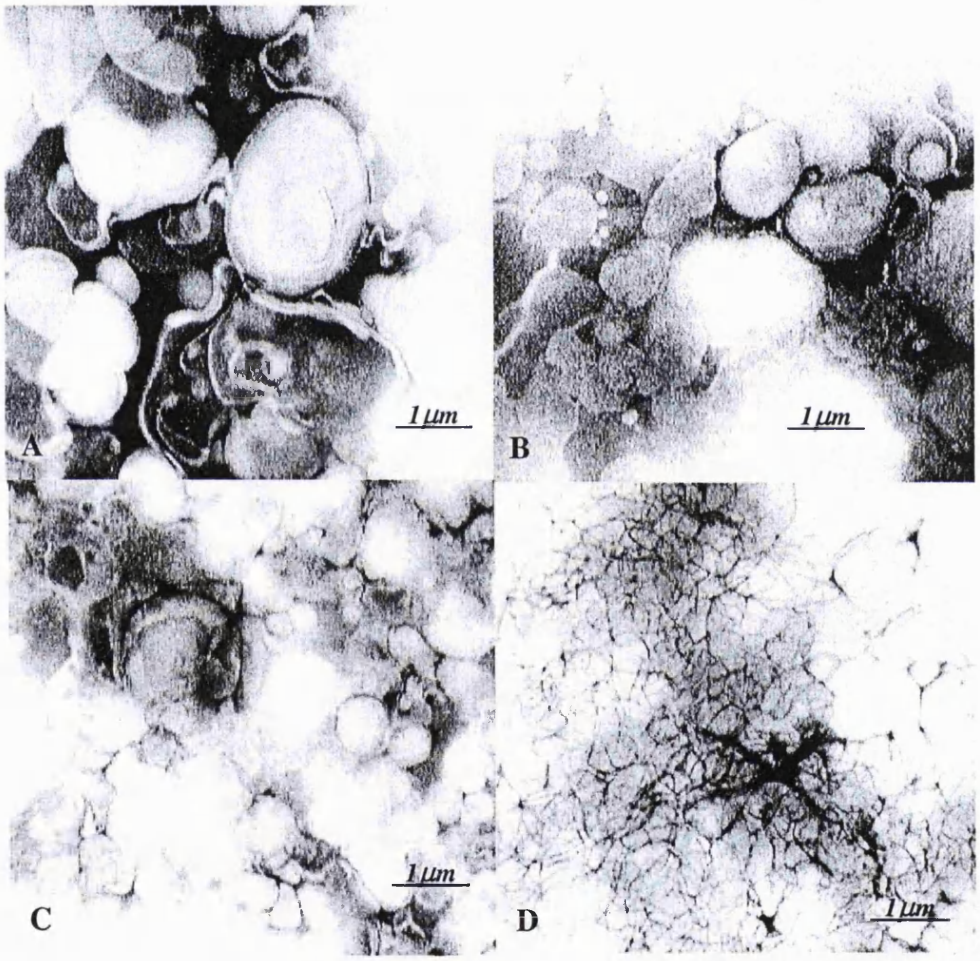

Figure 4.8: Photographs of HBsAg-containing DRVs prepared in the presence of sucrose under transmission electron microscopy. (A) DSPC/Chol (16:16 μmoles) HBsAg-containing DRVs. (B) DSPC/Chol (16:16 μmoles) DRVs entrapping HBsAg and 0.006mMole of sucrose. (C) DSPC/Chol (16:16 μmoles) DRVs entrapping HBsAg and 0.009mMole of sucrose. (D) DSPC/Chol (16:16 μmoles) HBsAg-containing DRVs and 0.021mMole of sucrose. DRVs were examined in a Philips 201 transmission electron microscope and the photos were taken at 100kV.

4.3.2 Stability of HBsAg-containing DRVs prepared in the presence of sucrose

Entrapment of HBsAg into DRVs prepared in the presence of sucrose reduced the vesicle size (Figure 4.5) and changed the vesicle morphology (Figure 4.8). Freeze-drying liposomes without cryoprotectant is known to result in their fusion and, in the case of SUV, to lead to larger vesicles on rehydration (Schenk *et al.*, 1989). The presence of sucrose during freezing and dehydration interferes with fusion. Sucrose acts by influencing the crystallization behaviour of intraliposomal ice through its formation into glass as well as through the interaction between phospholipid and sucrose molecules (Crowe *et al.*, 1988). Previous studies (Zhang *et al.*, 1997) suggested the leak-in pattern of the sample freeze-dried and rehydrated without sugar was very similar to that of the sugar-containing liposomal dispersions. However, another report (Viera *et al.*, 1993) suggested that intercalation of sugar molecules between the phospholipid head groups was responsible for the increased bilayer permeability which lasted at least 24 hours after rehydration. In the following section, the effect of freeze-drying in the presence of sucrose on the retention of HBsAg in DRVs on storage and the release profiles of HBsAg from DRVs *in vitro* was investigated.

4.3.2.1 HBsAg retention in DRVs prepared in the presence of sucrose upon storage

The stability of HBsAg-containing DRVs prepared in the presence of sucrose was monitored at the different time courses upon storage and compared to the formulations prepared in the absence of sucrose. The data suggested that the HBsAg leaked out much more easily from the liposomal formulations entrapping sucrose (Table 4.2). However, both formulations showed the same stability after one week of storage. An alternative solution consists of storing the freeze-

dried formulations in the powder form. This will bring the issues about the stability of these freeze-dried powders as well as the isotonicity of the rehydrated formulations for intramuscular injection. Advantageously, the rehydrated formulations prepared in the presence of sucrose does not require any additional processing for size reduction. In summary, these results suggest that freeze-dried formulations containing sucrose may consist a suitable solution to the stability problems of liposomal dispersion.

Table 4.2: Stability of HBsAg-containing DRVs prepared in the presence of sucrose on storage at 4°C (Values shown are mean ± SD of three experiments).

Formulation	Storage Time	Retention (%)	Z-average size (μm)	Zeta Potential (mV)
DSPC/Chol HBsAg DRVs	1 week	91.88±0.08	2.64±0.45	-27.6±0.3
	2 weeks	88.12±0.04	3.08±0.33	-31.3±0.1
	4 weeks	79.62±0.10	3.21±0.24	-35.9±1.0
	6 weeks	75.76±0.19	3.40±0.53	-37.9±2.9
	8 weeks	70.00±0.27	3.49±0.49	-42.3±0.8
DSPC/Chol + 0.5 mMole of Sucrose HBsAg DRVs	1 week	92.08±0.50	0.130±0.007	-17.3±0.3
	2 weeks	48.10±0.55	0.144±0.016	-21.9±0.5
	4 weeks	22.59±2.13	0.150±0.016	-30.8±0.5
	6 weeks	15.47±1.20	0.154±0.009	-35.9±0.5
	8 weeks	9.58±1.01	0.180±0.045	-37.9±0.7

4.3.2.2 HBsAg release profiles from DRVs prepared in the presence of sucrose at 37°C

The release profiles of HBsAg from DRVs suggested that vesicle size is an important factor. The released amount from liposomes prepared in the presence of sucrose was up to 50% when they were incubated in muscle extract (Figure 4.9). The release profiles of HBsAg from the DRVs prepared in the presence of sucrose are similar to those from vesicle with the same size but prepared by using microfluidisation.

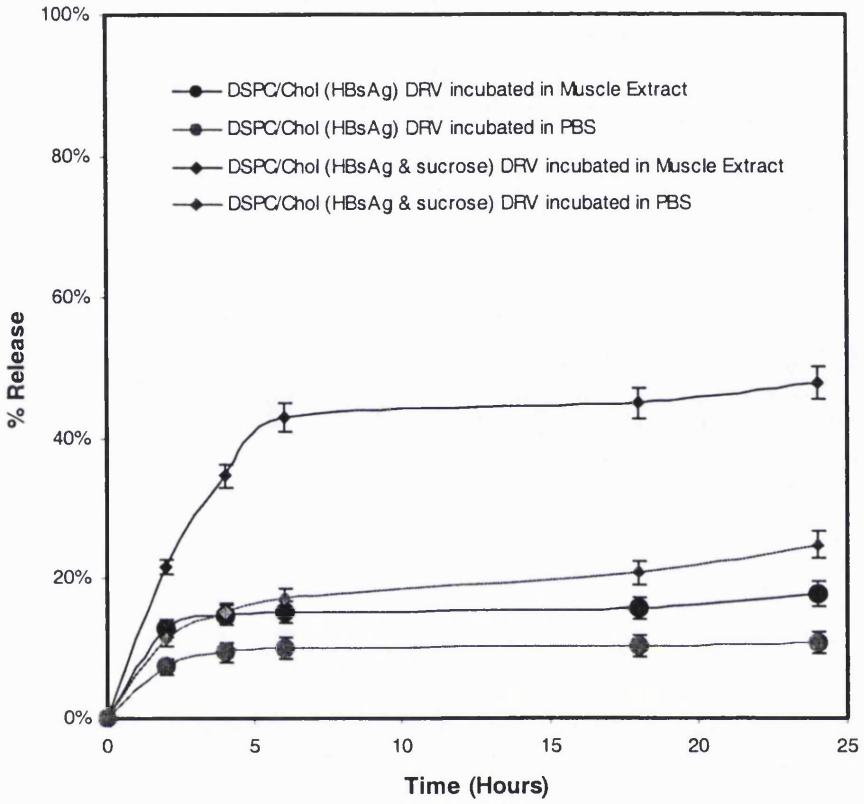

Figure 4.9: Release profiles of HBsAg from DRVs prepared in presence or absence of sucrose. DSPC/Chol (16:16 μmole) DRVs containing 5 μg of HBsAg with or without 0.021mMole of sucrose were resuspended in PBS or 10% muscle extract in PBS. Aliquots were taken in different time courses (2, 4, 6, 18, and 24 hours) and centrifuged with the ultracentrifuge to evaluate the released ratio in the supernatant (Data shown are mean ± SD of three experiments).

4.4 EFFECT OF VESICLE SIZE ON IMMUNE RESPONSES OF HBsAg-CONTAINING DRVs

The vesicle size was reported to be a factor which contributes to the control of the adjuvanticity of liposomes for OVA (Brewer *et al.*, 1998). These liposomes modulated the Th1 and Th2-type antibody responses. In this study, the vesicle size of HBsAg-containing DRVs was reduced by using the microfluidisation or by adding sucrose during the preparation procedure of DRVs. Results obtained from the mice immunised with these formulations are summarized

below.

4.4.1 Immune responses of microfluidized HBsAg-containing DRVs

The responses of total IgG, IgG_1, IgG_{2a} and IgG_{2b} subclasses in the mice immunised with these microfluidized HBsAg-containing DRVs were measured and compared to those obtained from the formulations exhibiting different sizes. The results summarized here confirmed the hypothesis that vesicle size affect the regulation of Th1/Th2 pathways.

4.4.1.1 Total IgG responses

The total IgG responses in Balb/c mice immunized with these formulations showed that DSPC/Chol DRVs exhibited adjuvanticity for HBsAg. However, the mice treated with microfluidized HBsAg-containing DRVs did not induce significantly higher titres than original DRVs in the primary or secondary responses (Figure 4.10). The secondary antibody responses also showed that the mean of $\log_{10}$ values of serum IgG titre induced by microfluidized HBsAg-containing DRVs decreased from 5.5 to 4.5. These results are contrary to the hypothesis that the liposomes with smaller size will improve the immune responses by increasing the uptake of liposomes by macrophage. This may be explained by the decrease in the protective effect of microfluidized DRVs compared to the original DRVs. The release profiles *in vitro* supported this hypothesis (Figure 4.4). Therefore, microfluidized HBsAg-containing DRVs cannot induce stronger immune responses than original DRVs. In conclusion, the microfluidized DRVs may be unsuitable as formulations for HBsAg vaccination.

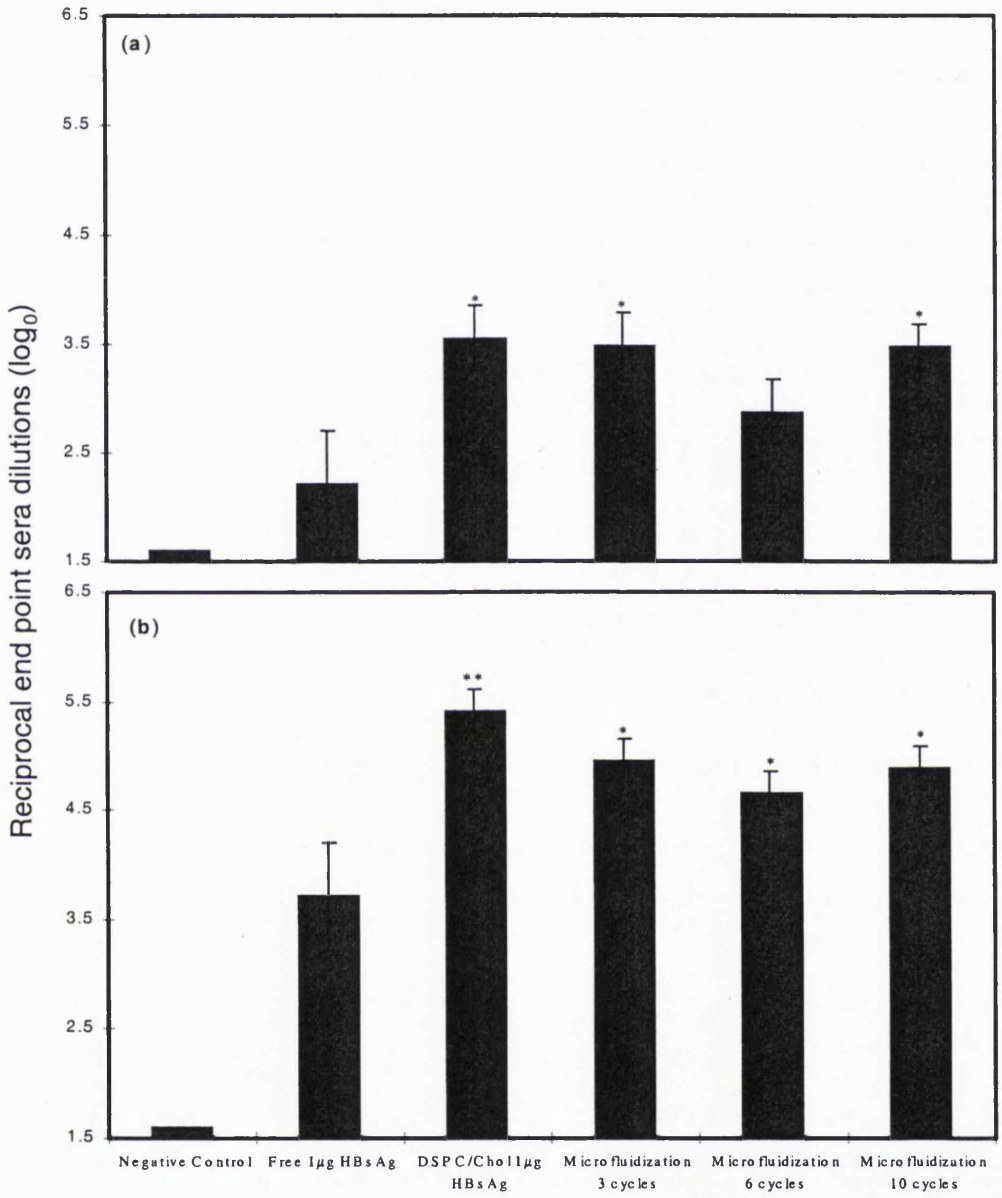

Figure 4.10: Comparison of total IgG responses for the microfluidized DSPC/Chol DRVs as HBsAg adjuvants. Balb/C mice in groups of 5 were injected intramuscularly on days 0 and 28 with 1μg of HBsAg entrapped (or retained) in DSPC/Chol (16:16μmoles) DRVs or microfluidized DRVs (3, 6 and 10 cycles' microfluidisation). The sera taken at 28 days after the first IM injection were tested for primary responses (a) and those bled at 10 days after second IM immunisation at 28 days were tested for secondary responses (b). Sera was taken and tested as the previous description. Data represented are mean ± SD for 5 individually tested animals per group. Significantly different from the group immunised with free HBsAg: **$p<0.005$; *$p<0.05$ (Student's unpaired *t*-test).

4.4.1.2 Subclass IgG responses

For the subclass IgG responses (Figure 4.11 and 4.12), the microfluidized HBsAg-containing DRVs could not induce higher responses than original DRVs in the immunised mice. In the primary responses, the mean of log values of serum IgG_{2a} titres was decreased from 3.1 to 2.3 by treating the mice with microfluidized DRVs. However, these results disagreed with the observation in the recent studies (Brewer *et al.*, 1998), which reported that the lipid vesicle size determines the Th1 or Th2 responses to entrapped antigens. Furthermore, the microfluidized HBsAg-containing DRVs induced higher IgG_1 and IgG_{2b} titres, but not IgG_{2a} responses, which is similar to the results from obtained from DRVs without microfluidisation. Generally, the result could not illustrate the effect of vesicle size on Th1 or Th2 dominance and showed that DSPC/Chol DRVs prefer Th2 pathway to induce higher IgG_1 response.

By comparison to the subclass IgG responses induced by the microfluidized HBsAg-containing DRVs, the immune responses did not decrease with size reduction. Only the HBsAg-containing DRVs processed for 6 cycles of microfluidisation reduced the ability to enhance some subclass IgG responses. However, the size of microfluidized HBsAg-containing DRVs with 6 cycles is close to the ones with 10 cycles, but their immune responses were found to be different. This might be due to the modification in the properties of vesicles, such as the vesicle structure and the interaction between antigen and lipid. In summary, the subclass IgG responses did not relate to the vesicle size of microfluidized HBsAg-containing DRVs and microfluidisation could not enhance immune responses of HBsAg-containing DRVs.

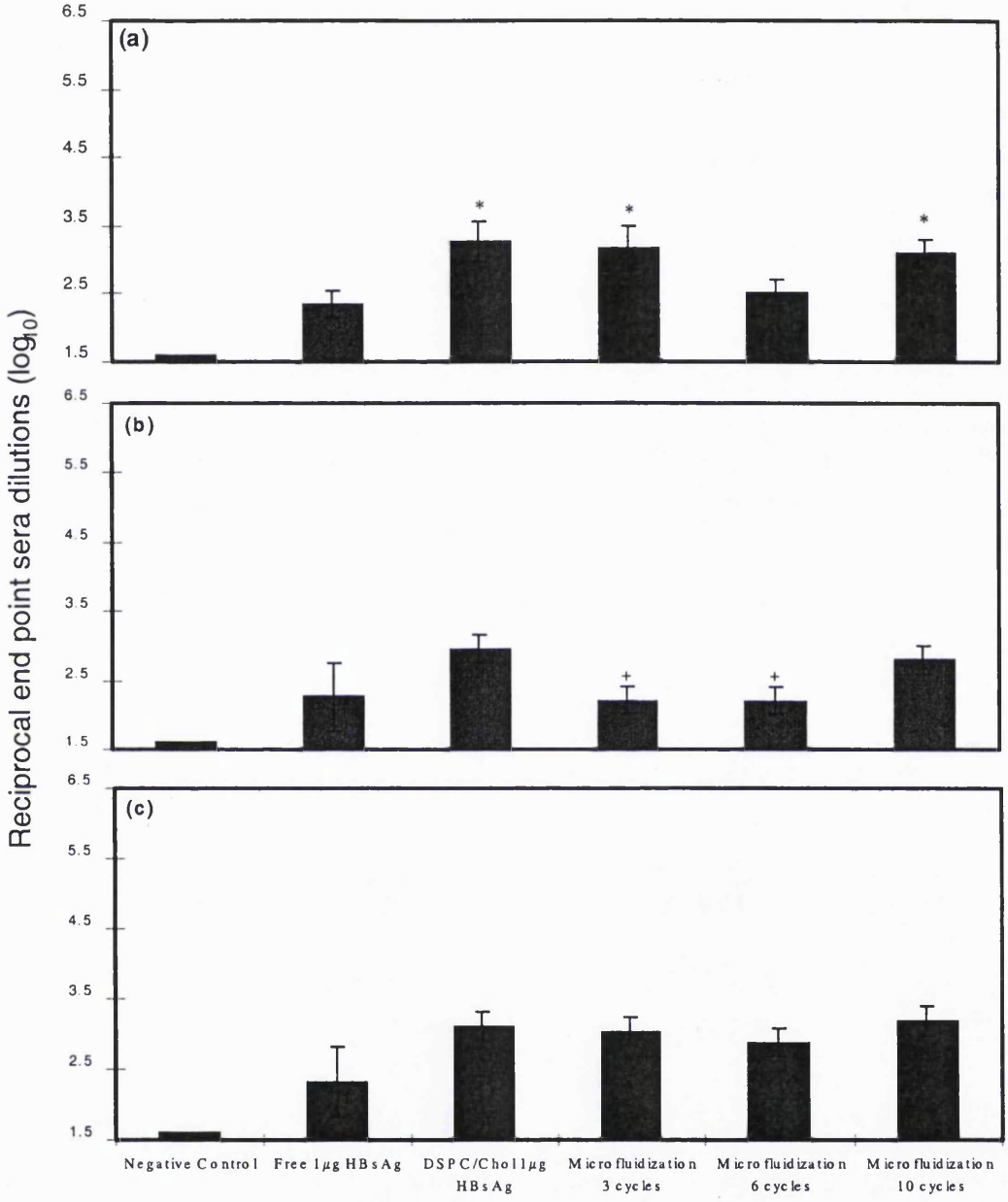

Figure 4.11: Comparison of the primary subclass IgG responses obtained by using microfluidized DSPC/Chol DRVs as HBsAg adjuvants. Experiments were done with the same procedures in Figure 4.10 and the sera taken on the 28 days after first immunisation were assayed for primary responses. The subclass IgG responses ((a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b}) were measured by ELISA method. Data shown are mean ± SD for 5 individually tested animals per group. *indicates data judged to be significantly different from the mice treated with free HBsAg ($p<0.05$). +denotes values for the group treated with microfluidized DRVs were sigificantly lower ($p<0.05$) than those for the group immunised with DSPC/Chol (HBsAg) DRVs (Student's unpaired *t*-test).

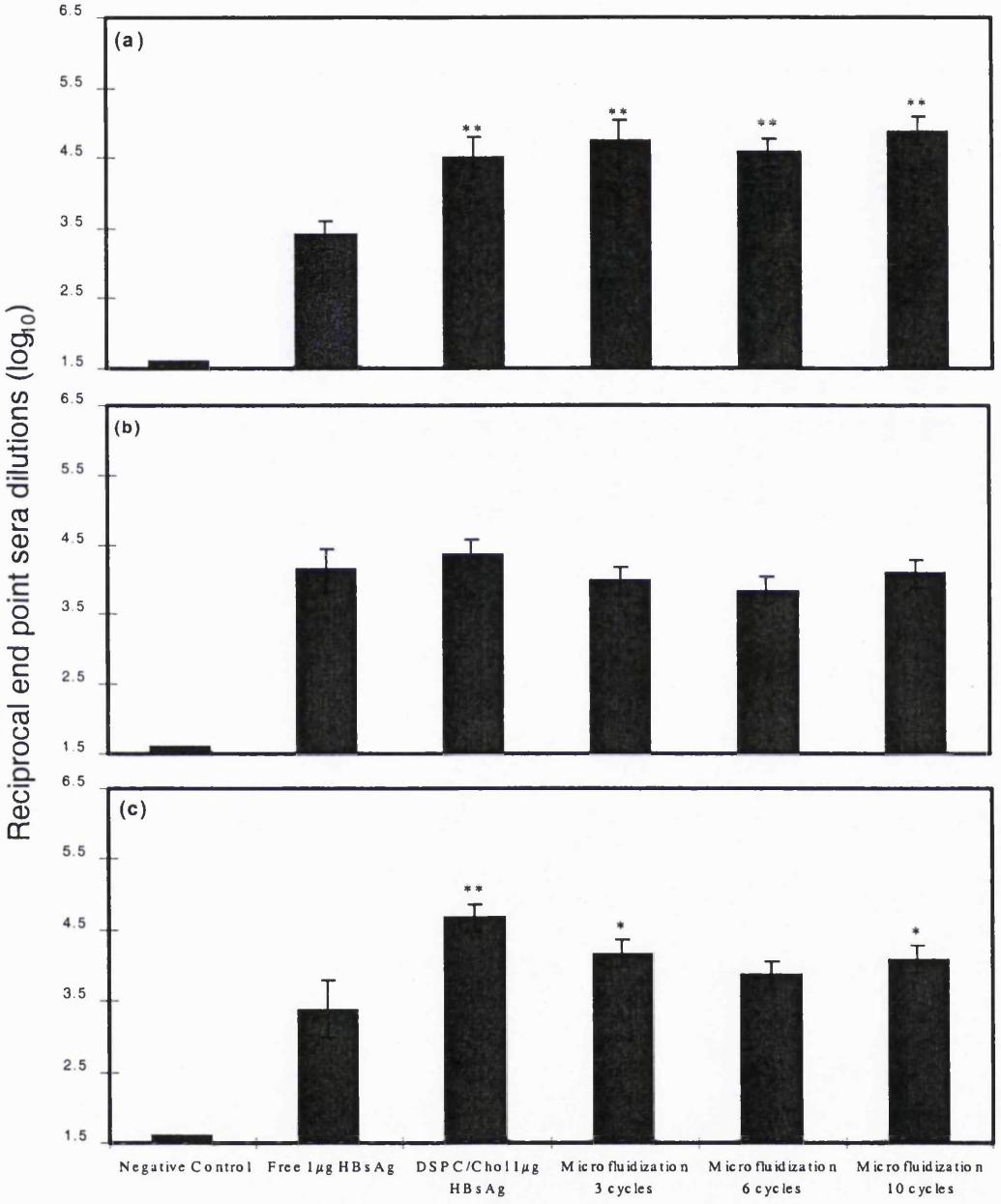

Figure 4.12: Comparison of the secondary subclass IgG responses obtained by using microfluidized DSPC/Chol DRVs as HBsAg adjuvants. Experiments were done with the same procedures in figure 4.10 and the sera taken on the 10 days after secondary immunisation were measured for secondary responses. The subclass IgG responses ((a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b}) were measured by ELISA method. Data shown are mean ± SD for 5 individually tested animals per group. Significantly different from the group immunised with free HBsAg: **$p<0.005$; *$p<0.05$ (Student's unpaired *t*-test).

4.4.2 Effect of sucrose on immune responses of HBsAg-containing DRVs

The immunisation results corresponding to microfluidized HBsAg-containing DRVs suggested that the size reduction could not improve IgG responses and did not agree with the hypothesis of increased uptake of smaller vesicles by antigen presentation cells. To confirm the size effect on the adjuvanticity of liposomal formulations, 0.011 mMole of sucrose was co-entrapped into HBsAg-containing DRVs (1μg) and injected intramuscularly to mice to study its effect. At the same time, HBsAg-containing DRVs in which sucrose was added externally were intramuscularly injected to Balb/C mice as the control group to evaluate the antibody responses.

4.4.2.1 Total IgG responses

The total IgG responses in Balb/C mice immunised with DRVs entrapping HBsAg and sucrose showed significant enhancement in anti-HBsAg titres(Figure 4.13). By treating mice with DRVs entrapping sucrose and HBsAg, the mean of $\log_{10}$ values of serum IgG titres was increased from 3.0 to 4.2 in the primary response and the level was enhanced from 4.7 to 6.2 in the secondary responses. The enhancement in antibody responses will allow to reduce the dose used for immunisation. However, the increasing effect on antibody response was not observed for the preformed DRVs which sucrose was added externally. Thus, the mice immunised with DRVs entrapping HBsAg and sucrose can increase the IgG titres. This may result from the alternation in membrane property of HBsAg-containing DRVs prepared in the presence of sucrose. Results indicated that HBsAg-containing DRVs prepared in the presence of sucrose may be promising formulation for the development of hepatitis B vaccines.

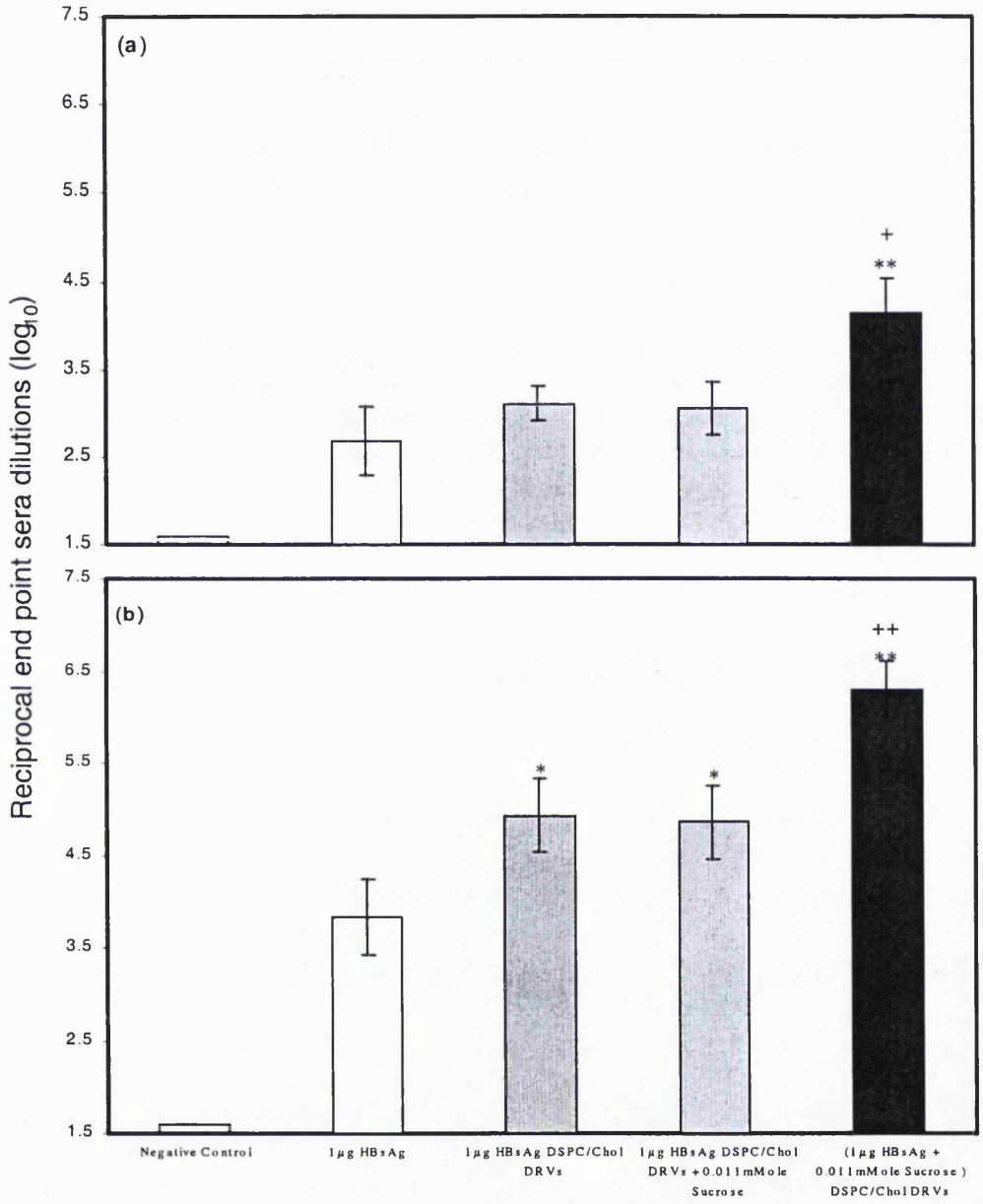

Figure 4.13: Total IgG responses in Balb/C mice immunized with HBsAg-containing DRVs prepared in the presence of 0.011 mMole of sucrose. The sera taken at 28 days after the first IM injection were used to evaluate the primary responses (a) and those taken at 10 days after the second IM immunisation at 28 days were for assaying the secondary responses (b). The total IgG titres were measured by ELISA. The end point is the dilution required for OD_{492} to reach a value of about 0.2. Each bar represents mean ± SD (n=5). *p<0.05, **p<0.005 compared with mice immunised with free HBsAg. Significantly different from the group immunised with HBsAg-containing DSPC/Chol DRVs: +p<0.05; ++p<0.005 (Student's unpaired *t*-test).

4.4.2.2 Subclass IgG responses

The effect of sucrose on the subclass IgG responses in mice immunised with HBsAg-containing DRVs showed that the IgG_1 and IgG_{2a} responses induced by DRVs entrapping HBsAg and sucrose were higher than those obtained from the mice vaccinated with HBsAg-containing DRVs (Figure 4.14 and 4.15). It showed that immunisation with DRVs entrapping HBsAg and sucrose can enhance both Th1 and Th2 pathway in antibody responses of mice. The subclass responses in animals treated with HBsAg-containing DRVs prepared in the presence of sucrose showed that only IgG_1 response increased in the primary responses but both IgG_1 and IgG_{2b} enhanced in the secondary responses.

The subclass IgG titres in mice could be enhanced by immunisation with DRVs prepared in the presence of sucrose but not with microfluidized DRVs. This observation showed that the effect of vesicle size on immune responses of HBsAg-containing DRVs also depended on other parameters. Probably, the structure of the lipid bilayers and the size reduction methods employed could provide an explanation to this phenomena. The integrity of HBsAg in the microfluidized DRVs was checked with protein gel electrophoresis. The microfluidisation didn't alter the molecular weight of HBsAg. Furthermore, the animals treated with HBsAg-containing DRVs mixed with sucrose did not show the argument in antibody responses. These results indicated that the vesicle size may be not the real factor responsible of increasing subclass IgG titres for HBsAg. Since DRVs entrapping HBsAg and sucrose could enhance the subclass IgG responses, they could be tested in lower doses in further studies.

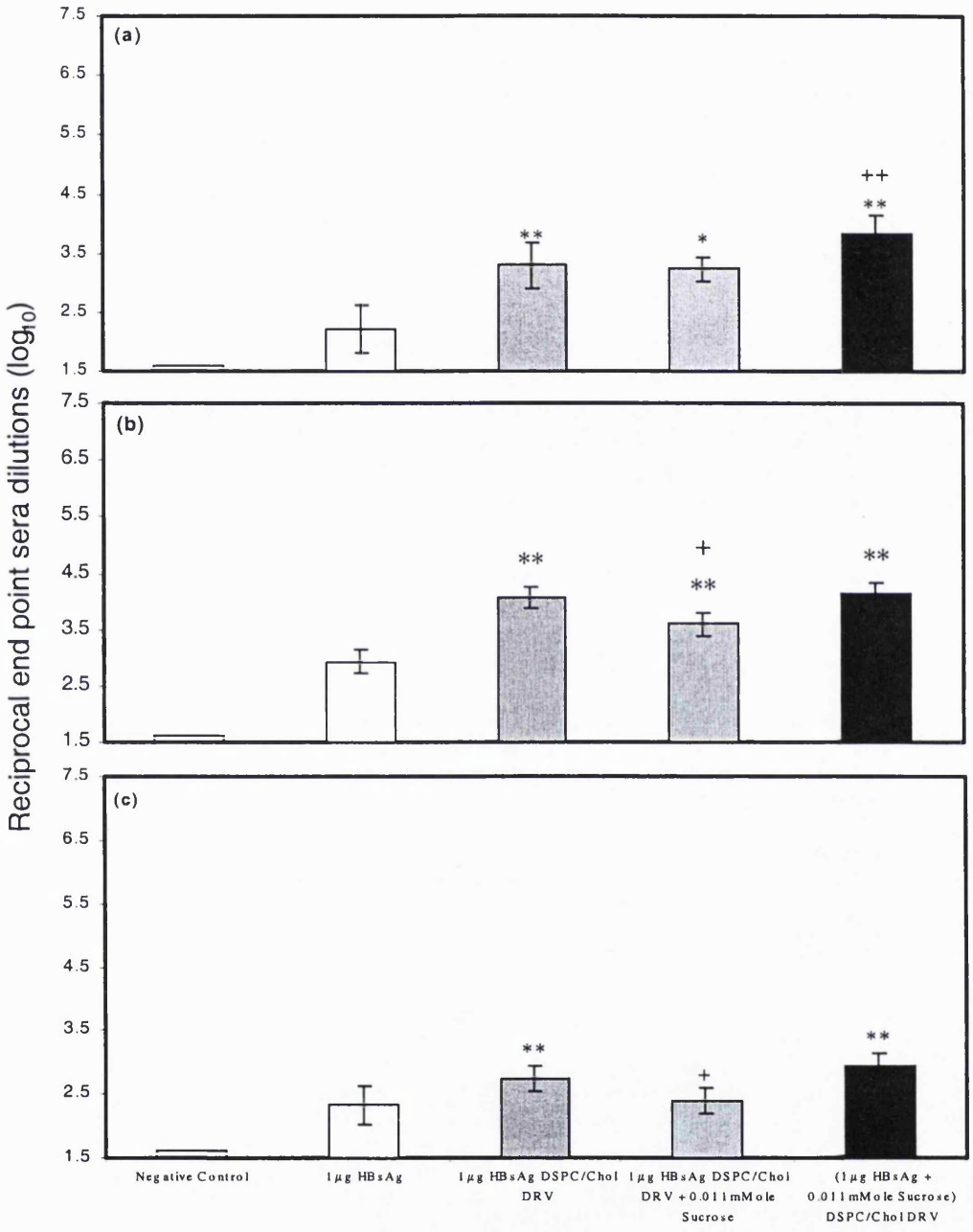

Figure 4.14: Primary subclass IgG (IgG_1, IgG_{2a}, IgG_{2b}) responses in Balb/c mice immunized with HBsAg-containing DRVs prepared in the presence of sucrose. The subclass IgG titres ((a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b}) were assayed with ELISA. The end point is the dilution required for OD_{492} to reach a value of about 0.2. Bars are mean value ± SD (n=5). *$p<0.05$, **$p<0.005$ compared to mice immunised with HBsAg. Significantly different from the groups treated with DSPC/Chol (HBsAg) DRVs: +$p<0.05$; ++$p<0.005$ (Student's unpaired *t*-test).

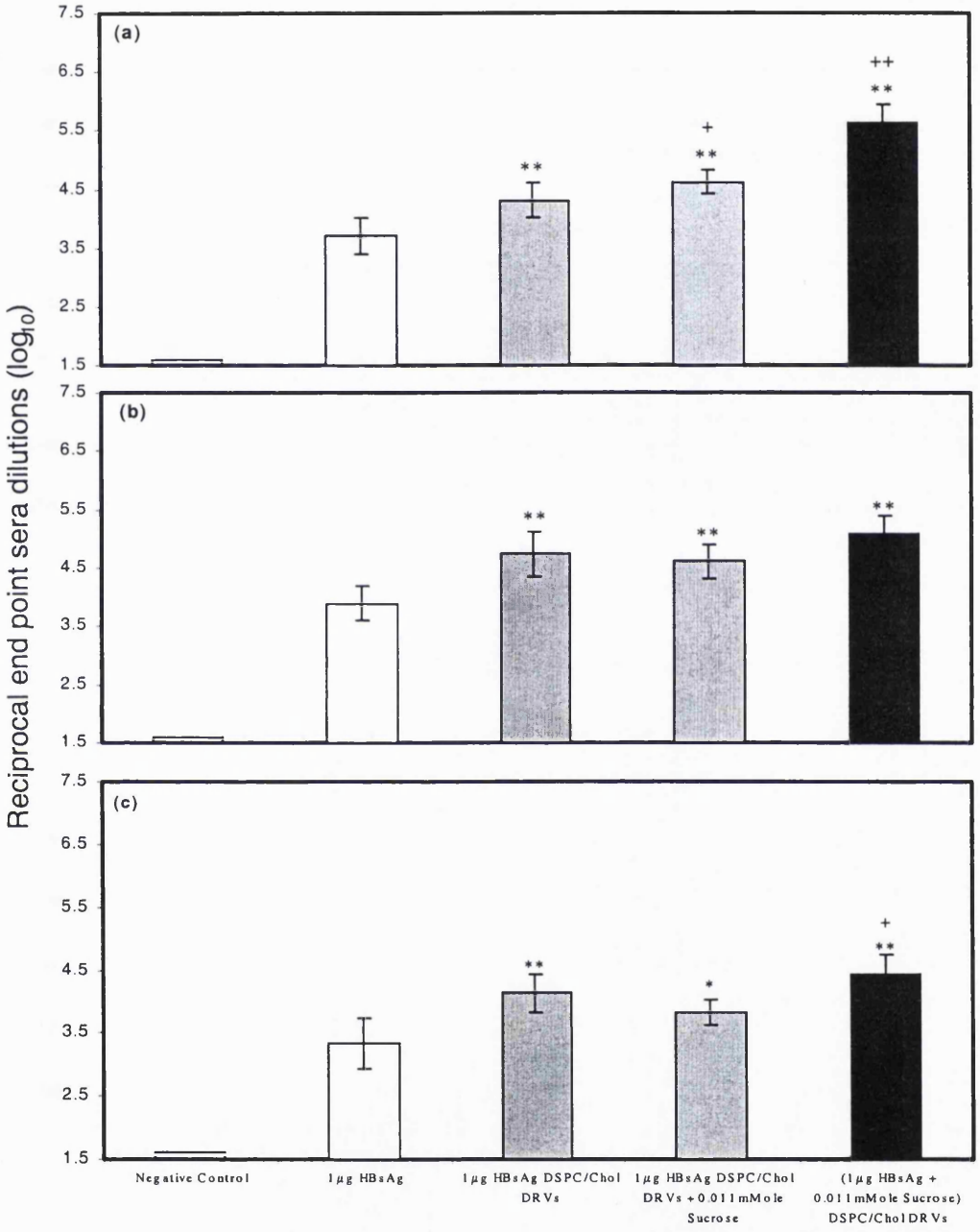

Figure 4.15: Secondary subclass IgG (IgG_1, IgG_{2a}, IgG_{2b}) responses in Balb/c mice immunized with HBsAg-containing DRVs prepared in the presence of sucrose. The subclass IgG titres ((a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b}) were assayed with ELISA. The end point is the dilution required for OD_{492} to reach a value of about 0.2. Each bar represents mean ± SD (n=5). *p<0.05, **p<0.005 compared to the mice immunised with HBsAg. Significantly different from the groups treated with DSPC/Chol (HBsAg) DRVs: +p<0.05; ++p<0.005 (Student's unpaired *t*-test).

4.5 DISCUSSION AND CONCLUSIONS

In this study, the methods used to reduce the vesicle size, such as microfluidisation and sucrose co-entrapment methods, had been employed successfully to reduce the size of HBsAg-containing DRVs. Their characteristics and immune responses induced by these formulations in mice were evaluated. However, these liposomes prepared by these two methods generated completely different immune responses. These results challenged the hypothesis that "the smaller vesicle size will increase the uptake of antigens by antigen-presentation cells and enhance the immune responses". The methods used for vesicle size reduction led to liposomes exhibiting different properties and inducing various immune responses. The details will be discussed below.

The vesicle size of liposomes could be reduced by using these two approaches. The first way is to break the large vesicles to smaller ones by applying mechanical force. The second one is to maintain the small size by inhibiting the fusion and/or aggregation of lipid bilayers. Microfluidisation was the method using mechanical force to shear the vesicle for size reduction. The sucrose, a cryoprotecant, was added in the preparation of DRVs to protect the lipid bilayers during the dehydration-rehydration process. The vesicle size of the HBsAg-containing DRVs can be controlled to the size range below 200nm by using one of these two methods. These liposomes were used to study the effect of vesicle size on immune responses induced by HBsAg-containing DRVs. The size reduction of HBsAg-containing DRVs could be confirmed by examination under transmission electron microscopy. The morphology of liposomes generated by these two methods is different from each other.

The stability of these HBsAg-containing DRVs exhibiting small vesicle size had been evaluated on storage. The data indicated that the retention of HBsAg in microfluidized HBsAg-containing DRVs is similar to that of HBsAg-containing DRVs. However, leakage of HBsAg from DRVs entrapping HBsAg and sucrose were much faster than those prepared in absence of sucrose. A possible solution to maintain the stability of HBsAg-containing DRVs prepared in the presence of sucrose could consist of storing the sample with sealed freeze-dried powder and adjusting the isotonicity of the rehydrated dispersion.

The release profiles of HBsAg from DRVs with smaller size showed that the released ratio obtained from the HBsAg-containing DRVs with small size is higher than that from control groups. This might be owing to the decreased lamellarity of small HBsAg-containing DRVs. Weaker protection made the enzyme in the muscle extract destabilize the lipid bilayers more easily. This could constitute a limitation for the use of liposomes as carriers to deliver antigens to antigen-presentation cells. It may illustrate the contradiction between the protection effect of liposomes for antigens *in vivo* and the cellular uptake of these formulations. Thus, the uptake of these liposomes by antigen-presentation cells needed to be quantified to evaluate the effect of vesicle size on antigen delivery into cells in further studies.

In the animals treated with microfluidized HBsAg-containing DRVs, the immune responses could not be improved by size reduction. Improving the uptake of liposomes by antigen-presentation cells and protecting them from the enzyme destabilizing during the delivery in muscular tissue are two necessary conditions for optimal use of these formulations. However,

the HBsAg-containing DRVs prepared in the presence of sucrose showed enhanced immune responses but less protection when incubated in muscular tissues extract. This indicated that the uptake of antigens by antigen-presentation cells is more important than the integrity of liposomal antigens during the delivery process. These results suggested that DRVs entrapping HBsAg and sucrose were a promising candidate for the development of hepatitis B vaccines.

The vesicle size reduction using microfluidisation or sucrose co-entrapment did not affect the subclass IgG responses in the immunised mice. On one hand, the production of the IgG_{2a} subclass of antibody by murine B cells is associated with Th1-type cytokine production. On the other hand, IgG_1 antibody, is associated with Th2-type cells (Snapper & Mond, 1993). Previous results (Brewer *et al.*, 1998) using OVA as model protein indicated that when the vesicle size was reduced, its adjuvant effect switched from inducing a mixed Th1/Th2-type response characterized by B cell IgG_1 and IgG_{2a} production to the production of a Th2-dominated response in the absence of IgG_{2a}. However, the results in this study showed that the microfluidized liposomes cannot alter the IgG_1 and IgG_{2a} titres. Moreover, HBsAg-containing DRVs prepared in the presence of sucrose can enhance IgG_1 but not IgG_{2a} independently of the vesicle size. The property of lipid bilayers in the DRVs entrapped sucrose may be able to explain these results. Therefore, the vesicle size may not determine the Th1 or Th2 responses to the entrapped antigen. The data obtained in this study showed that the Th2-dominance is still maintained when these liposomal formulations are used, irrespective of the vesicle size.

In conclusion, the studies described in this chapter indicated that the vesicle size effect on the adjuvanticity of liposomes for HBsAg vaccines depends on the methodology used for vesicle size reduction. The DRVs entrapping HBsAg and sucrose may be a promising formulation for HBsAg vaccination. The freeze-dried powder form could constitute a solution to improve the stability of this liposomal preparation. The minimum dose required for HBsAg in DRVs prepared in the presence of sucrose needs to be determined in further study.

Chapter 5

Liposomes as Immunological Adjuvants for Hepatitis B Surface Antigen: Effect of HBsAg Mannosylation

5.1 INTRODUCTION

Hepatitis B surface antigen (HBsAg), a type of membrane protein, is located on the lipid bilayers of hepatitis B virus envelopes (Howard and Allison, 1995). When this antigen was entrapped into the liposomes, it was proposed that it is incorporated into the lipid bilayers. Previous studies had shown that detergent soluble HBsAg can induce cellular immunity (Schirmbeck *et al.*, 1994). Liposomes can deliver water-soluble antigens to induce cellular immunity *in vivo* (Huang *et al.*, 1992). Therefore, the HBsAg can be modified to be more water-soluble. Then this modified HBsAg can be entrapped into the liposomes which can fuse with the cell membrane. Consequently, it may be delivered by the exocytosis and processed through the MHC I pathway to induce cellular immunity (Barnaba *et al.*, 1990). When CD4+ CTL are activated, they preferentially eliminate activated MHC II-positive cells, i.e. macrophages, B or T cells (Hahn *et al.*, 1995). This elimination of APCs will reduce the antibody response induced by antigens. In order to confirm this idea, the modified HBsAg was entrapped into the DRVs which were used to immunize the mice and study their immune responses.

Conjugation of carbohydrates to HBsAg had been concerned to make this protein be more hydrophilic. In addition, the macrophage plays a key role in the processes of immune response and contains a lot of mannose receptors on its surface. Consequently, by conjugating with mannose, HBsAg will improve its water-solubility and may target to the macrophage *in vivo*. However, HBsAg particles derived from yeast are leaky vesicles or lipoprotein-like structures (Diminsky *et al.*, 1997). The detergents, such as SDS, can unfold this protein and expose all the amino groups to conjugate with the activated mannose residues. Thus, HBsAg was conjugated

with mannose by the method described by Kataoka and Tavassoli (1984) and SDS was added as an unfolding agent in mannosylation of HBsAg. Mannosylation of HBsAg may change the interaction among amino acid residues in its peptide chain. Thus, the characteristics of mannosylated HBsAg were proposed to be different from those of native HBsAg.

The mannosylated HBsAg was entrapped into DRVs made of different lipid compositions. These DRVs were prepared in the presence of sucrose to reduce the vesicle size. Due to the modification of HBsAg, the protein may not be incorporated in the lipid bilayers of DRVs. This change encouraged the use of liposomes to be the delivery carriers for the peptide chain. However, antibody responses were induced through antigen processing and presentation by the MHC II pathway. Thus, the amino acid sequence is more important than the epitope conformation for recognition (Jenson, 1993). Furthermore, liposomes can be designed as the carriers to deliver the antigen molecules into the antigen-presentation cells (APCs) by endocytosis to process MHC II presentation or by fusion to process MHC I responses (Buiting *et al.*, 1992). Here, liposomes of conventional (PC/Chol or DSPC/Chol) and fusogenic (DSPC/DOPE) compositions were used to entrap HBsAg or mannosylated HBsAg. The procedures for the preparation of these formulations, their preparation and the antibody responses induced by them in immunised mice were studied.

5.2 CHARACTERISATION OF MANNOSYLATED HBsAg

In order to confirm the mannosylation of HBsAg, the reaction products were passed through column chromatography and the protein or mannose content was measured by the

radioactivity and BCA assay or phenol-sulfuric acid method respectively. Furthermore, the molecular weight of mannosylated HBsAg was determined by using the native PAGE.

5.2.1 Preparation of mannosylated HBsAg

The mannosylation of HBsAg was prepared by using the method described by Kataoka and Tavassoli (1984). To compare the mannosylation of HBsAg in the folding and unfolding states, sodium dodecylsulfate (SDS) was applied to unfold the HBsAg in mannosylation. Then, the resulting product was purified by column chromatography and characterized by native gel electrophoresis. The mannosylated HBsAg was radiolabelled with ^{125}I by using chloramine-T method. An amount of ^{125}I-labelled HBsAg was used as tracer in the following experiments.

5.2.1.1 Purification of mannosylated HBsAg

The mannosylated HBsAg was purified by passing through a G-25 column chromatography. The protein content in each fraction was measured by using both BCA assay and radioactivity. The mannose was quantified by phenol-sulphuric acid method. The mannosylation of HBsAg led to a reduction of the molecular weight of HBsAg (Figure 5.1). This could be confirmed by the native gel electrophoresis (Figure 5.3). In the gel chromatography, the mannosylated HBsAg exhibited a longer elution time than the native HBsAg by the elution with PBS (Figure 5.2). However, the mannosylated HBsAg produced from reactions conducted in the presence or absence of SDS showed similar elution pattern. This purified products were used in further studies.

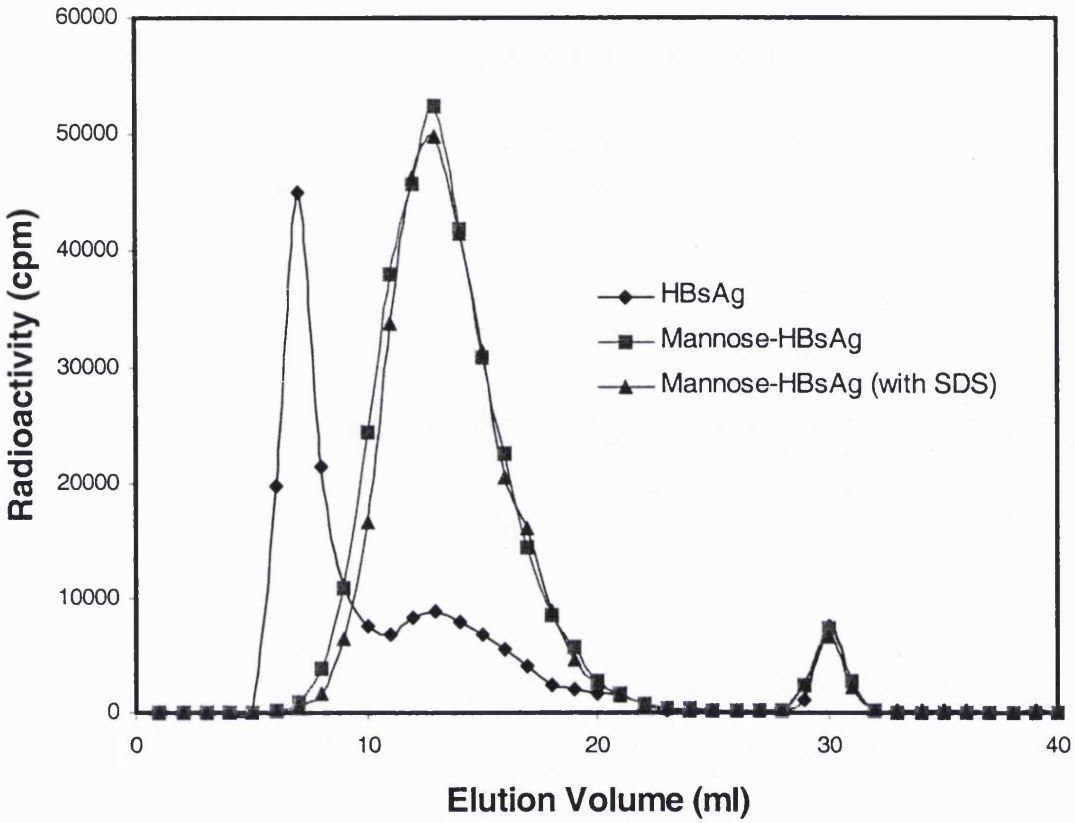

Figure 5.1: Size exclusion chromatograms of mannosylated HBsAg. 1μg of ^{125}I-labelled HBsAg or mannosylated HBsAg passed through a G-25 column and eluted with PBS. The fractions (one ml each) were collected by fraction collector and measured for ^{125}I radioactivity by 1275 MINIGAMMA gamma counter.

5.2.1.2 Effect of SDS on mannosylation of HBsAg

SDS was used in this experiment as unfolding agents to expose more amino groups of the polypeptide chain for mannosylation. The results indicated that HBsAg could be conjugated with more mannose in the presence of SDS during the reaction of mannosylation. In addition, SDS could improve mannose conjugation on low molecular weight HBsAg as judged with the results from size exclusion chromatography (Figure 5.2).

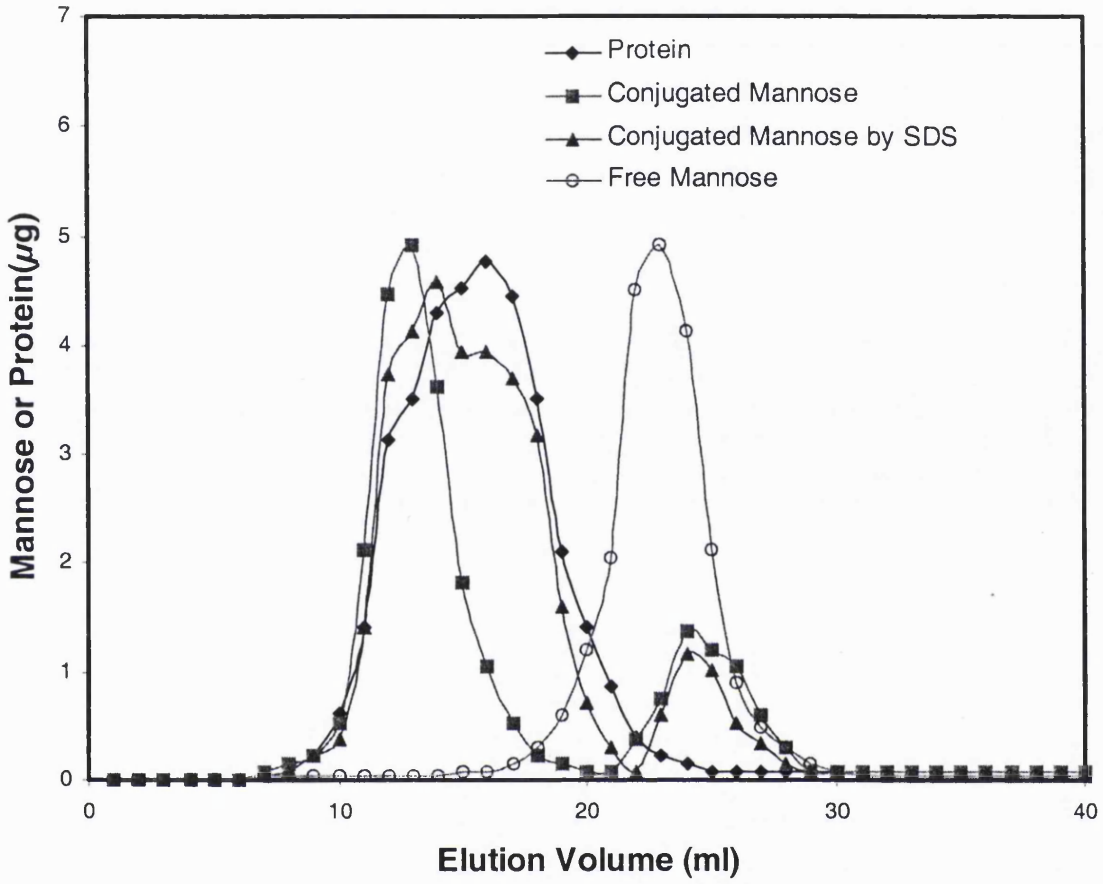

Figure 5.2: Size exclusion chromatograms of mannosylated HBsAg. 50μg of HBsAg was mannosylated with 0.1μmoles of mannose at room temperature overnight. Then, the mixtures were passed through a G-25 column and eluted with PBS. The fractions (one ml each) were collected by fraction collector. The amount of mannose and protein in the factions was determined by the method of Weissig *et al.* (1989) and by the BCA method.

There are 13-15 amino groups on the side chain of amino acids in the amino acid sequence of HBsAg. By considering the conformation of HBsAg, there are 5-7 amino groups exposed on the protein surface. The amount of conjugated mannose on HBsAg mannosylation in the absence of SDS was one half of the total amount of conjugated mannose in the product obtained from the reaction in the presence of SDS (Figure 5.2). However, SDS increased the mannose conjugation on HBsAg and might alter the HBsAg conformation at the same time. To

improve the mannosylation of HBsAg, the reaction was conducted in the presence of SDS. The resulting product was used in this study.

5.2.1.3 Native-PAGE of mannosylated HBsAg

To characterize the mannosylation of HBsAg, native HBsAg and mannosylated HBsAg were applied in the native poly-acylamide gel electrophoresis (PAGE). The gel was running with 40 mV for 2 hours and stained with coomassie blue G, and distained with the distaining solution. The gel (Figure 5.3) showed the molecular weight of mannosylated HBsAg is around 24 kD. This corresponds to the molecular weight of small HBsAg monomer. However, native HBsAg stayed on the top of the separation gel in native PAGE (Figure 5.3) but exhibited a band near 24kD in SDS-PAGE (Figure 3.1). Gel electrophoresis indicated that mannosylation made the HBsAg from entity to monomers.

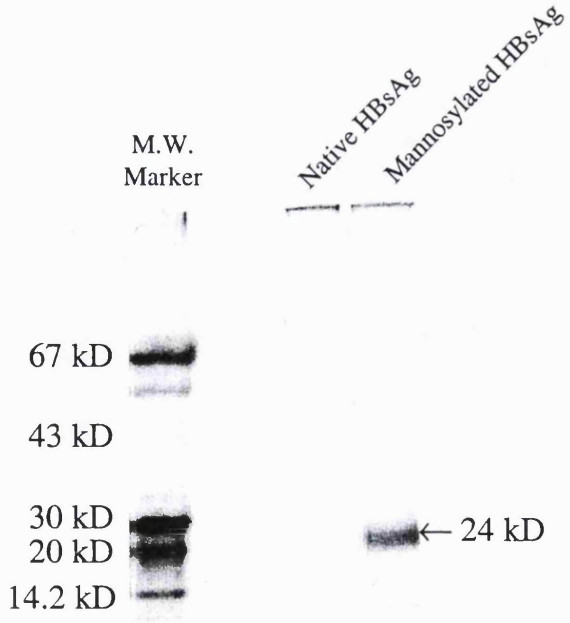

Figure 5.3: Native PAGE (10% acrylamide gel) of HBsAg and mannosylated HBsAg. The bands in gel showed that the native HBsAg is entity and mannosylated HBsAg is monomer (around 24 kD).

5.2.2 Radiolabelling of mannosylated HBsAg

Mannosylated HBsAg was labelled with ^{125}I by using chloramine-T method. The product was passed through a G-25 column chromatography to separate the free isotope from the labelled protein. The fraction corresponding to the ^{125}I-labelled mannosylated HBsAg was eluting in the first peak and the free $Na^{125}I$ was in the following peak (Figure 5.4). The ^{125}I labelling efficiency of mannosylated HBsAg was 92.8%. This labelled protein was used as tracer for further studies.

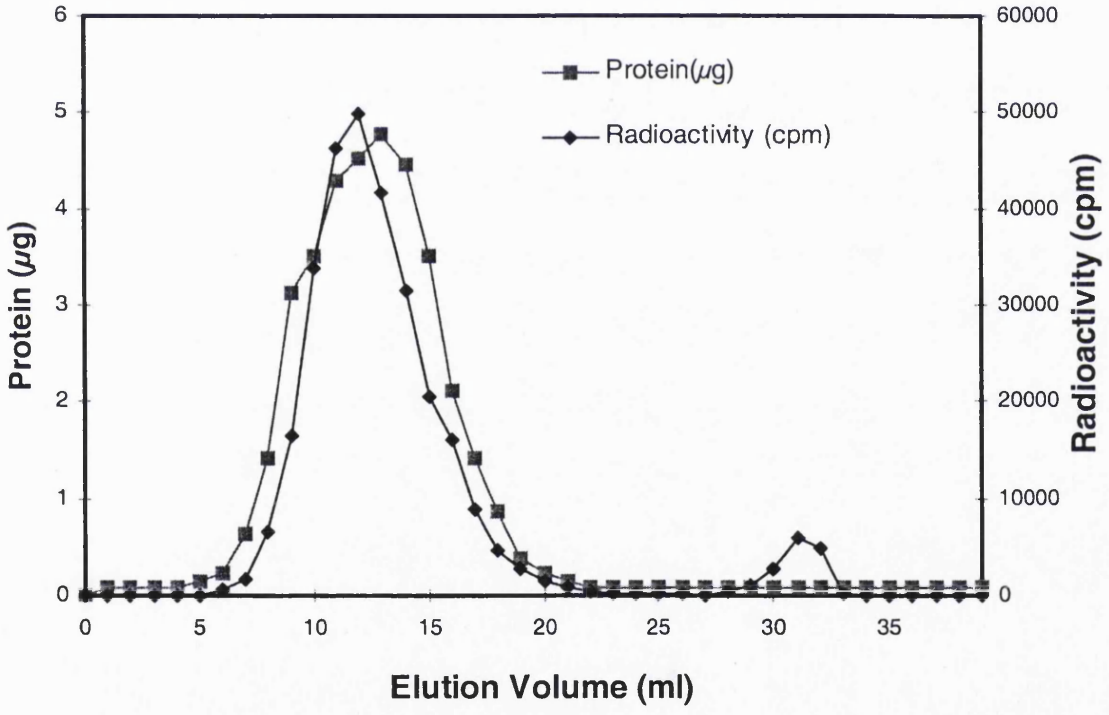

Figure 5.4: Size exclusion chromatograms of ^{125}I-labelled mannosylated HBsAg. 50 μg of mannosylated HBsAg was passed through a G-25 column and eluted with PBS (0.15M, pH 7.4). The fractions (one ml each) were collected by fraction collector. Each fraction was measured for ^{125}I radioactivity by 1275 MINIGAMMA gamma counter and for protein amounts by BCA assay.

5.3 CHARACTERISATION OF DRVs ENTRAPPING MANNOSYLATED HBsAg

The mannosylated HBsAg was entrapped into liposomes composed of PC or DSPC with equimolar Chol and DSPC/DOPE (2:1 molar ratio) by dehydration-rehydration method to yield DRVs. These DRVs were characterized with entrapment efficiency of mannosylated HBsAg, vesicle size distribution and morphology.

5.3.1 Entrapment efficiency of mannosylated HBsAg into DRVs

The entrapment of mannosylated HBsAg into DRVs was carried out by using different lipid compositions and preparation procedures. Moreover, DRVs encapsulating mannosylated HBsAg were prepared in the presence of sucrose to allow the reduction of their vesicle size upon rehydration. The entrapment efficiency of mannosylated HBsAg into DRVs prepared in the presence of sucrose was also characterized.

5.3.1.1 Effect of preparation procedures

The entrapment of mannosylated HBsAg into DRVs was attempted by controlling each step in the following preparation procedures. DRVs prepared in the absence of sonication exhibited a different entrapment efficiency of antigen into DRVs. Indeed, the entrapment efficiency of mannosylated HBsAg into PC/Chol DRVs decreased from 41% to 36%, but that in DSPC/Chol DRVs increased from 33% to 38% (Figure 5.5). However, the additional sonication step in the DRV preparation method had no effect on the entrapment efficiency of mannosylated HBsAg into the DSPC/DOPE DRVs. Furthermore, the sonication procedure led to preparations exhibiting the same contribution to the entrapment efficiency of HBsAg and

mannosylated HBsAg (Figure 3.3 and 5.5). Generally, the effect of sonication on DRV preparation for antigen entrapment into DRVs depended on their lipid compositions.

The DRVs prepared by rehydration at different temperature did not show different entrapment efficiency values of mannosylated HBsAg expect in the case when the liposomes were made of DSPC/DOPE. Rehydration of DSPC/DOPE freeze-dried cake at 60°C increased the entrapment efficiency of mannosylated HBsAg from 35% to 42% by comparison to those obtained after rehydration at 25°C. The argument of the mannosylated HBsAg entrapment into DRVs at 60°C might be due to the properties of DSPC/DOPE lipid bilayers (Webb *et al.*,1993). The entrapment efficiency of HBsAg into DSPC/Chol DRVs increased with the resealing at the temperature above the phase transition temperature. However, the entrapment efficiency of mannosylated HBsAg in DSPC/DOPE vesicles could not be improved in this way. These results suggested that the temperature control during the preparation of DRVs entrapping mannosylated HBsAg was important for the DSPC/DOPE DRVs, but not for PC/Chol or DSPC/Chol DRVs.

The mannosylated HBsAg was added during the rehydration step to form DRVs. In this approach, the entrapment efficiency of mannosylated HBsAg into liposomes was reduced for PC/Chol and DSPC/DOPE DRVs (Figure 5.5(c)).Results indicated that the temperature of reconstitution and sonication had different effect on the entrapment efficiency of mannosylated HBsAg in DRVs composed of various compositions. Moreover, the addition of mannosylated HBsAg in precursor SUVs could enhance its entrapment efficiency for PC/Chol or DSPC/DOPE DRVs.

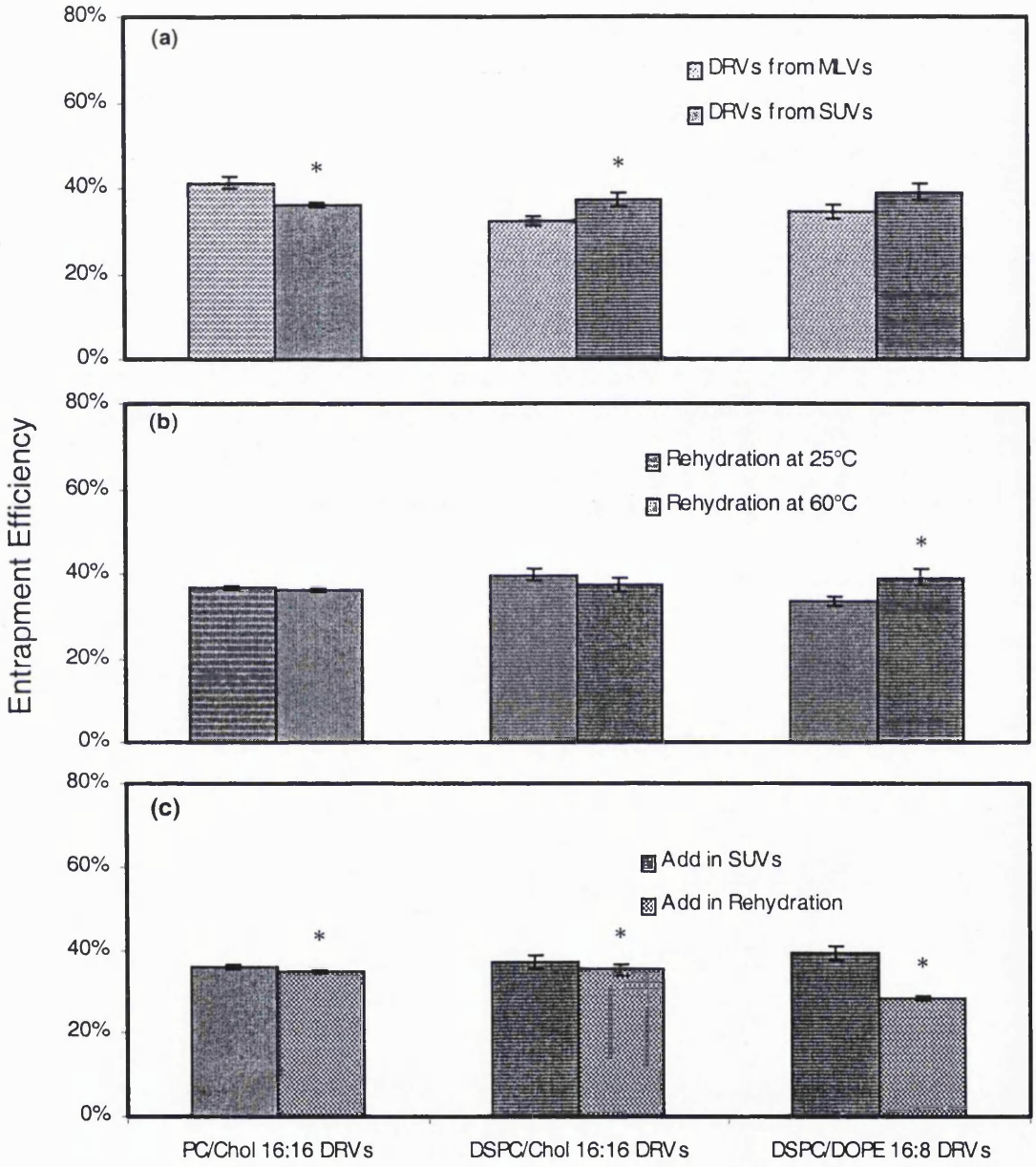

Figure 5.5: Comparison of the entrapment efficiency of mannosylated HBsAg into DRVs with the various preparation procedures.10 μg of mannosylated HBsAg was entrapped into DRVs with different compositions, and the preparation procedures were controlled by (a) sonication, (b) rehydration temperature, and (c)addition procedure of antigens. The entrapment efficiency of mannosylated HBsAg was evaluated by the method described in Figure 3.3. Bars are mean ± SD of three experiments. *Statistical differences are determined by Mann-Whitney U test, $U \leq 0$ (critical value) was considered significant.

5.3.1.2 Effect of HBsAg mannosylation

The property of HBsAg was modified by mannosylation (Figure 5.1). Mannosylated HBsAg may therefore exhibit different entrapment values by comparison to native HBsAg incorporation into DRVs. In order to understand the effect of HBsAg mannosylation on the protein entrapment into DRVs, amounts of HBsAg and mannosylated HBsAg encapsulated into liposomes were compared.(Figure 5.6).

The loading amount of mannosylated HBsAg into DRVs was less than that of native HBsAg when the same amount of antigens were used for entrapment (Figure 5.6). When the amount of proteins applied increased up to 40 μg, the amount of HBsAg entrapped into DRVs was up to around 15 μg, but the amount of mannosylated HBsAg entrapped into liposomes was 7 μg. This might result from the interaction between protein and lipid in liposomes. The entrapment of HBsAg into liposomes depended on the incorporation of proteins into lipid bilayers. However, the mannosylated HBsAg prefers to exist in the aqueous phase and inter-bilayer spaces in DRVs. Consequently, the amount of mannosylated HBsAg entrapped into DRVs was less than that of HBsAg entrapped into DRVs. In addition, the loading amount of mannosylated HBsAg in PC/Chol DRVs was less than that in DSPC/Chol or DSPC/DOPE DRVs. This showed that the liposomes composed of saturated lipids (DSPC) may have the advantages to entrap this antigen. Generally, the loading amount of mannosylated HBsAg in DRVs is less than that of HBsAg. The reduction of the amount of the entrapped antigens changed the lipid to protein ratio in the liposomal formulations, possibly modifying the liposomal adjuvanticity.

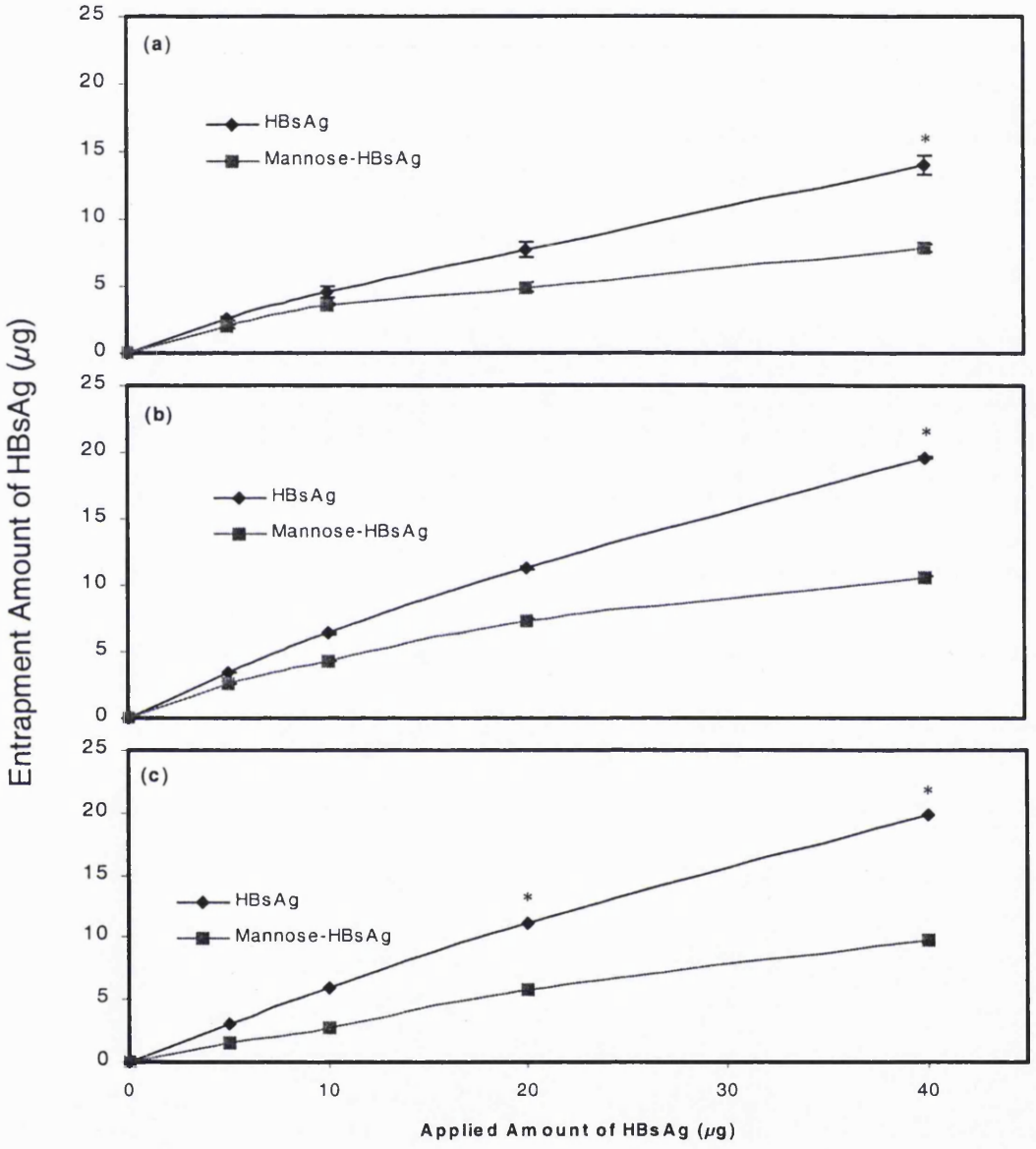

Figure 5.6: Comparison of HBsAg and mannosylated HBsAg entrapment in DRVs. Various amount (5-40μg) of HBsAg or mannosylated HBsAg were entrapped into DRVs composed of PC/Chol (16:16μmoles) (a), DSPC/Chol (16:16μmoles) (b) and DSPC/DOPE (16:8) (c). DRVs were prepared by dehydration-rehydration method rehydrated at 60°C. The entrapment yield of mannosylated HBsAg was evaluated by the radioactivity of ^{125}I-labelled proteins in a 1275 MINIGAMMA gamma counter. Data shown are mean ± SD of three experiments.* Significantly different from DRVs entrapping mannosylated HBsAg: U≤0 (critical value) by the Mann-Whitney U test.

5.3.1.3 Effect of the presence of sucrose during DRV preparation

In Chapter 4, HBsAg-containing DRVs prepared in the presence of sucrose were of smaller vesicle size. These formulations induced higher antibody titres in immunized mice. The same entrapment procedure was used in the preparation of DRVs entrapping mannosylated HBsAg.

The efficiency of entrapment of mannosylated HBsAg into DRVs was affected by the lipid compositions and the amount of sucrose used. With PC/Chol DRVs, entrapment of mannosylated HBsAg was reduced from 25% to 5% when adding 0.05 to 0.5 mMole of sucrose to the precursor SUVs (Figure 5.7(a)). On the other hand, the efficiency of entrapment of mannosylated HBsAg in DSPC/Chol or DSPC/DOPE DRVs was reduced only slightly with increasing amounts of sucrose (Figure 5.7(b), (c)). As anticipated (Zadi and Gregoriadis, 2000), increasing amounts of sucrose also led to reduced vesicle sizes (see 5.3.2). Previous studies (Adrian and Huang, 1979) had shown that the apparent decrease in entrapping efficiency of the proteins in small liposomes can be accounted for the combination of the bound water layer at the internal surface of vesicle and the steric hindrances when protein is captured during vesicle formation.

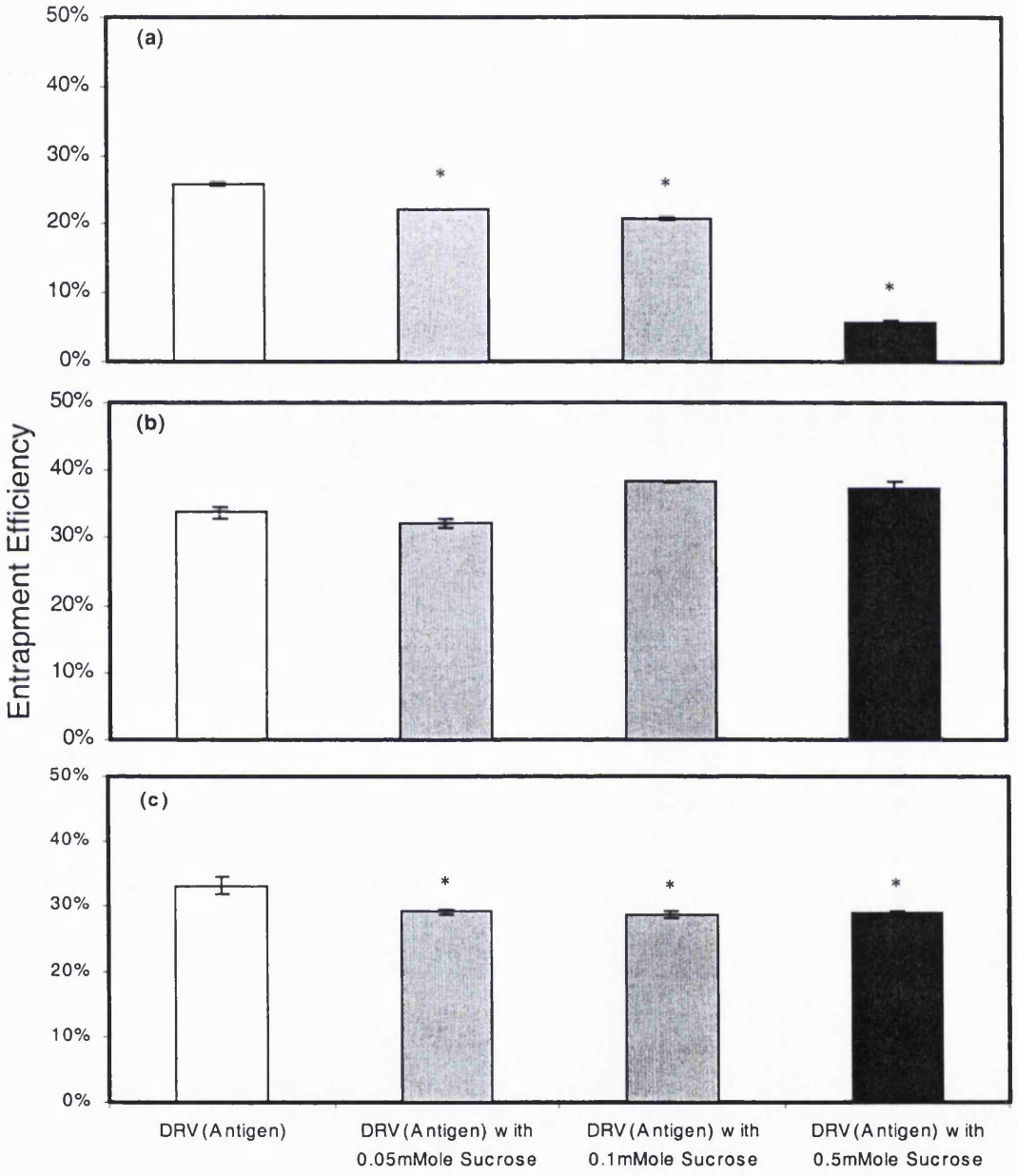

Figure 5.7: Entrapment efficiency of mannosylated HBsAg into DRVs prepared in the presence of sucrose. 10μg of HBsAg mannosylated HBsAg was entrapped into DRV composed of PC/Chol(16:16 μmoles) (a) and DSPC/Chol(16:16 μmoles) (b) and DSPC/DOPE (16:8 μmoles) (c) DRVs were prepared in the presence of various amounts of sucrose. The entrapment efficiencies of proteins were evaluated by the radioactivity of ^{125}I-labelled mannosylated HBsAg Each bar represents mean ± SD of three experiments. *Statistical differences with DRVs prepared in the absence of sucrose were determined by Mann-Whitney U test, U≤0 (critical value) was considered significant.

5.3.2 Vesicle size distribution of DRVs entrapping mannosylated HBsAg

5.3.2.1 Effect of preparation procedures

The characteristics of DRVs entrapping mannosylated HBsAg were affected by the preparation procedures. The vesicle size and zeta potential of DRVs entrapping mannosylated HBsAg prepared by using the various procedures were carried out (Table 5.1). The results showed that the preparation procedures and lipid compositions could alter the properties of these DRVs.

Table 5.1: Characteristics of DRVs entrapping mannosylated HBsAg prepared by different procedures.

Liposomal Compositions	Preparation Procedure	Volume Mean Diameter (μm)	Zeta Potential (mV)
PC/Chol (16:16 μmoles) DRVs entrapped 5μg of mannosylated HBsAg	from MLVs	11.92±1.98 (10.35±1.49)	-33.5±0.4
	rehydration at 25°C	7.81±0.81	-38.1±0.1
	rehydration at 60°C	8.76±0.82	-38.4±0.2
	Add protein during rehydration	10.09±0.91	-40.1±0.2
DSPC/Chol (16:16 μmoles) DRVs entrapped 5μg of mannosylated HBsAg	from MLVs	11.20±1.13 (11.27±1.14)	-22.6±0.4
	rehydration at 25°C	7.83±0.82	-30.5±1.1
	rehydration at 60°C	9.92±0.93	-25.1±0.9
	Add protein during rehydration	7.69±1.82	-29.6±0.7
DSPC/DOPE (16:8 μmoles) DRVs entrapped 5μg of mannosylated HBsAg	from MLVs	14.39±1.26 (29.05±1.46)	-16.8±1.0
	rehydration at 25°C	29.24±4.46*	-7.2±0.4
	rehydration at 60°C	11.38±2.07	-10.1±0.3
	Add protein during rehydration	8.27±1.37	-5.7±2.4

Data are shown with mean ± SD of three experiments. Number in parentheses denote the volume mean size of MLVs. *Significantly different from DRVs prepared in the rehydration at 60°C: $U \leq 0$ (critical value) in Mann-Whitney U test.

The vesicle size of DRVs prepared by different methods depended on the lipid composition, especially for the DSPC/DOPE formulation. Regarding the volume mean sizes of PC and DSPC/Chol DRVs, the results did not show significant differences except in the case of the DRVs prepared in the absence of sonication. However, the volume mean diameter of DSPC/DOPE DRVs increased from 11.38 to 29.24μm when the rehydration step was carried out at 25°C. Studies of the phase diagram of the DOPE/water system have suggested that hydration of DOPE is significantly less than other phospholipid species (such as PC) which exhibit a more efficient hydration when in equilibrium with water(Webb *et al.*, 1995). The DOPE/water system is in the H_{II} (inverted hexagonal) phase above 25°C, but this system will be a mixture of the L_{α} (lamellar liquid crystalline phase) and the H_{II} phase between 0 and 25°C (Seddon *et al.*, 1984). This could make the dispersion of the freeze-dried DSPC/DOPE mixtures in water more difficult and lead to DRV aggregation/fusion toward a larger volume mean diameter (Table 5.1).

The zeta potentials of DRVs entrapping mannosylated HBsAg prepared from the procedure in the absence of sonication were less negative for cholesterol-containing DRVs, but they were more negative for DOPE-containing DRVs. Zeta potentials of DRVs entrapping mannosylated HBsAg prepared in the absence of prior sonication varied from -38 to -33 mV for PC/Chol DRVs and from -30 to -22mV for DSPC/Chol DRVs. However, they shifted from -7 to -17 mV for DSPC/DOPE DRVs (Table 5.1). These alternations may be due to the rearrangement of lipids during the preparation of DRVs (Labhasetwar *et al.*, 1994). Results showed that the preparation procedures of DRVs could decide of their electrostatic properties.

5.3.2.2 Effect of the presence of sucrose in DRVs

The vesicle sizes of DRVs entrapping mannosylated HBsAg prepared in the presence of sucrose were reduced when increasing amount of sucrose was added (Table 5.2). The size reduction of these DRVs prepared in the presence of sucrose depended on lipid compositions and the amount of sucrose added. The vesicle size of DSPC/DOPE DRVs entrapping mannosylated HBsAg prepared in the presence of 0.5 mMole of sucrose was decreased down to 0.67 μm. This size was larger than the cholesterol-containing DRVs prepared in the presence of the same amount of sucrose. These results showed that the effect of vesicle size reduction by using sucrose was more important for cholesterol-containing DRVs at high molar ratio of sucrose to lipid.

The zeta potential of PC/Chol DRVs entrapping mannosylated HBsAg were changed with increasing amount of sucrose used. However, the zeta potentials of DSPC-containing DRVs prepared in the presence of sucrose was not significantly affected. Then, results indicated that lipid compositions may decide the zeta potentials of DRVs prepared in the presence of sucrose.

Table 5.2: Characteristics of DRVs entrapping mannosylated HBsAg prepared in the presence of various amount of sucrose (Data shown are mean ± SD of three experiments).

Liposomal Formulation	Amount of sucrose in preparation	Volume Mean and Z-Average Diameter (μm)	Zeta Potential (mV)
PC/Chol DRVs (16:16 μmoles) entrapping 5μg of mannosylated HBsAg	-	8.76±0.82	-38.5±0.4
	0.05 mMole	5.48±0.40	-32.4±1.0
	0.1 mMole	2.92±0.34*	-20.2±1.7*
	0.5 mMole	0.102±0.001*	-3.9±0.5*

Liposomal Formulation	Amount of sucrose in preparation	Volume Mean and Z-Average Diameter (μm)	Zeta Potential (mV)
DSPC/Chol DRVs (16:16 μmoles) entrapping 5 μg of mannosylated HBsAg	-	9.92±0.93	-25.2±0.4
	0.05 mMole	5.05±0.30*	-24.3±0.8
	0.1 mMole	4.93±0.27*	-21.6±0.1
	0.5 mMole	0.163±0.001*	-20.8±0.6
DSPC/DOPE DRVs (16:8 μmoles) entrapping 5 μg of mannosylated HBsAg	-	11.38±2.07	-10.1±0.3
	0.05 mMole	4.10±0.71*	-10.3±1.2
	0.1 mMole	3.44±0.38*	-11.1±0.7
	0.5 mMole	0.67±0.03*	-14.2±0.7

*Significantly different from DRVs prepared in the absence of sucrose: $U \leq 0$ (critical value) in Mann-Whitney U test.

5.3.3 Morphology of DRVs entrapping mannosylated HBsAg

Photographs of DRVs entrapping mannosylated HBsAg examined under TEM showed that DRVs prepared in the presence of sucrose had smaller size than control groups. The DSPC/DOPE DRVs were close to the polyhedral structure(Figure 5.8 E, F), which had been found to be exhibited by niosomes, non-ionic surfactant containing vesicles (Uchegbu *et al.*, 1997). HBsAg-containing DRVs (Figure 3.4, 4.8) exhibited different morphology by entrapping mannosylated HBsAg for liposomes composed of same compositions. This alteration may result from the modification of the interaction between the modified protein and lipid molecules. Photographs of liposomes examined under transmission electron microscopy confirmed that the size of vesicles prepared in the presence of sucrose had been reduced effectively.

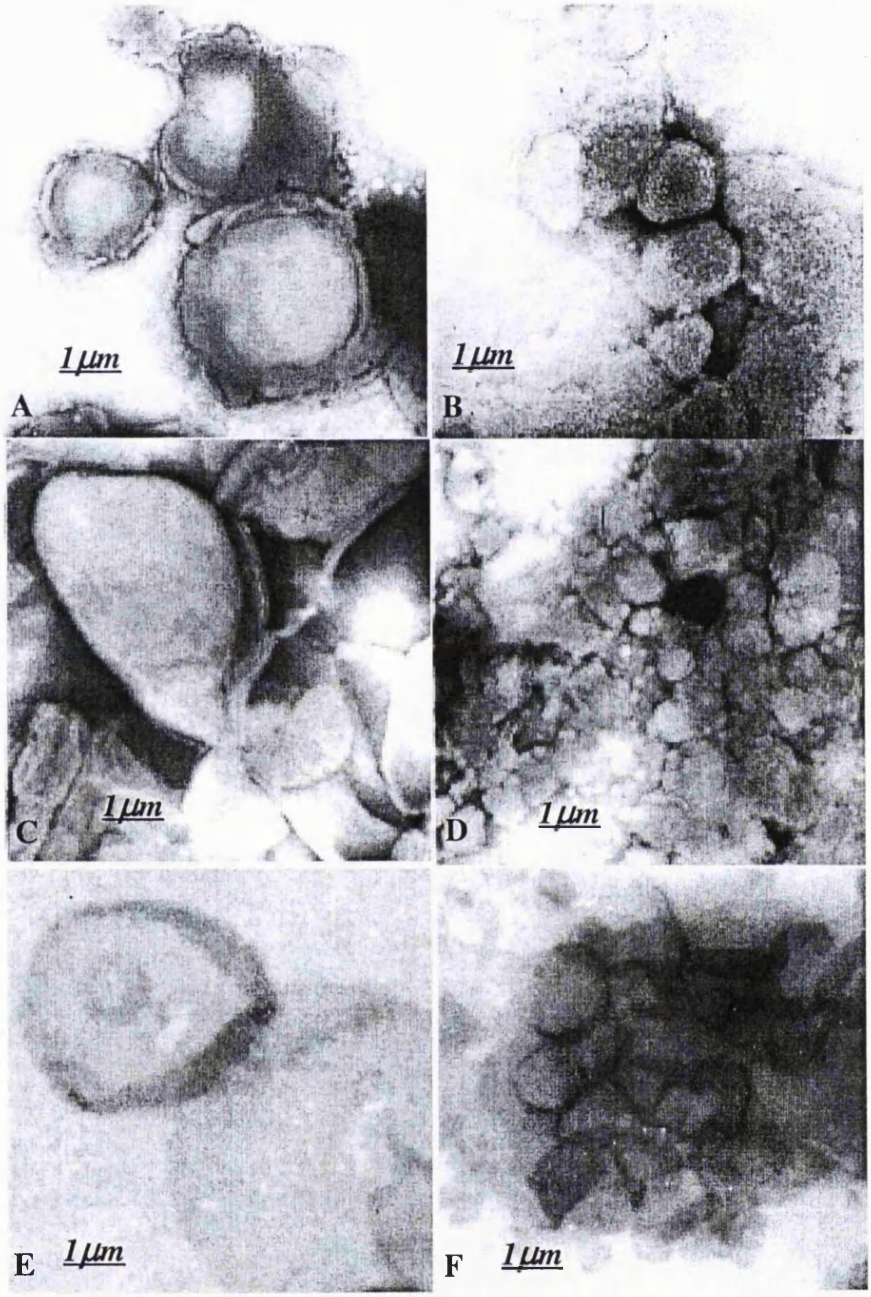

Figure 5.8: Photographs of DRVs entrapping mannosylated HBsAg under transmission electron microscopy. A. PC/Chol (16:16μmoles)(5μg of mannosylated HBsAg) DRVs, B. PC/Chol (16:16μmoles)(5μg of mannosylated HBsAg)DRVs with 0.5mMole of sucrose, C. DSPC/Chol (16:16μmoles)(5μg of mannosylated HBsAg) DRVs, D. DSPC/Chol (16:16μmoles)(5μg of mannosylated HBsAg) DRVs with 0.5mMole of sucrose, E. DSPC/DOPE (16:8μmoles)(5μg of mannosylated HBsAg) DRVs, F. DSPC/DOPE (16:8μmoles)(5μg of mannosylated HBsAg) DRVs with 0.5mMole of sucrose. DRVs were examined in a Philips 201 transmission electron microscope, and the photos were taken at 100kV.

5.3.4 Stability of DRVs entrapping mannosylated HBsAg

The stability of DRVs entrapping mannosylated HBsAg was evaluated by measuring the retention of proteins by DRVs as well as their vesicle sizes and the surface zeta potentials for a time period of eight weeks. In addition, the release amounts of mannosylated HBsAg from these DRVs in PBS or muscular extract were measured for 24 hours.

5.3.4.1 Retention of mannosylated HBsAg in DRVs on storage at 4°C

The antigen retention by DRVs, their corresponding vesicle sizes and zeta potentials were monitored during eight weeks' time period (Table 5.3). The data in these experiments were triplicated for each sample at the same condition in different batches prepared.

Table 5.3: Stability of DRVs entrapping mannosylated HBsAg on storage at 4°C.

Formulation	Storage Time	Retention (%)	Volume Mean or Z-Average Diameter (μm)	Zeta potential (mV)
PC/Chol (16:16 μmoles) (5 μg of mannosylated HBsAg) DRVs	1 week	66.77±1.13	8.13±1.22	-39.4±0.5
	2 weeks	57.56±1.01	9.60±1.19	-36.4±1.1
	4 weeks	36.23±0.51	9.74±1.17	-35.1±1.5
	6 weeks	26.59±0.32	9.94±1.02	-30.1±0.2
	8 weeks	16.04±0.77	10.20±2.48	-30.1±0.3
PC/Chol (16:16 μmoles) (5μg of mannosylated HBsAg)DRVs in the presence of 0.5 mMole of sucrose	1 week	80.61±0.51	0.106±0.002	-6.4±1.0
	2 weeks	61.09±1.34	0.159±0.006	-24.6±2.7
	4 weeks	34.37±1.80	0.193±0.010	-30.9±1.1
	6 weeks	21.69±0.50	0.220±0.004	-36.0±0.2
	8 weeks	13.12±0.60	0.233±0.035	-40.7±1.7

Formulation	Storage Time	Retention (%)	Volume Mean or Z-Average Diameter (μm)	Zeta potential (mV)
DSPC/Chol (16:16 μmoles) (5 μg of mannosylated HBsAg) DRVs	1 week	89.34±1.25	9.80±2.06	-25.4±0.5
	2 weeks	83.86±1.36	10.15±2.21	-30.4±1.1
	4 weeks	74.74±1.55	10.83±1.49	-31.1±1.5
	6 weeks	64.91±1.55	11.45±1.44	-31.5±0.2
	8 weeks	55.33±1.78	11.56±2.01	-35.5±0.8
DSPC/Chol(16:16 μmoles) (5μg of mannosylated HBsAg)DRVs in the presence of 0.5 mMole of sucrose	1 week	95.69±1.05	0.128±0.008	-19.9±0.2
	2 weeks	85.09±0.59	0.129±0.011	-21.7±1.7
	4 weeks	52.84±0.29	0.137±0.013	-30.1±2.1
	6 weeks	35.79±0.31	0.139±0.012	-33.0±0.7
	8 weeks	23.13±1.17	0.148±0.010	-34.3±0.3
DSPC/DOPE (16:8 μmoles) (5 μg of mannosylated HBsAg) DRVs	1 week	94.16±0.47	11.32±2.27	-10.6±0.5
	2 weeks	89.00±0.32	11.98±1.47	-13.7±0.9
	4 weeks	82.37±0.48	12.12±1.88	-19.6±0.6
	6 weeks	75.79±0.97	12.52±1.47	-20.6±0.3
	8 weeks	66.82±1.63	12.92±1.71	-21.0±0.3
DSPC/DOPE(16: 8 μmoles) (5μg of mannosylated HBsAg)DRVs prepared in the presence of 0.5 mMole of sucrose	1 week	93.54±1.48	0.672±0.021	-14.6±0.5
	2 weeks	85.22±1.43	0.862±0.082	-15.9±0.6
	4 weeks	69.69±1.03	11.19±1.73	-17.4±1.7
	6 weeks	57.77±0.66	15.98±1.98	-18.7±1.1
	8 weeks	42.98±0.29	24.96±1.83	-21.2±0.3

(Data shown are mean ±SD of three experiments)

The retention of the mannosylated HBsAg in DRVs was monitored on storage at 4°C through a period of two months. The data indicated that mannosylated HBsAg leaked easily from PC/Chol DRVs and small vesicles prepared in the presence of sucrose than from other liposomes.

Only 20% of entrapped mannosylated HBsAg was retained in DRVs after storage for eight weeks. Compared to the stability results obtained form HBsAg-containing DRVs (Table 3.2), the retention of HBsAg is higher than mannosylated HBsAg in DRVs during the storage period. This instability suggested that these formulations should be kept as a freeze-dried powder of precursor SUVs and rehydrated just before use.

The characteristics of DRVs entrapping mannosylated HBsAg on storage did not change dramatically except for the DSPC/DOPE DRVs prepared in the presence of sucrose. The size of these vesicles increased from 0.7 to 25 μm and creaming phenomena happened in the dispersion. The liposomal size increased with membrane fusion may be induced by the leakage of co-entrapped sucrose. In addition, protein leakage out from DRVs did not affect the vesicle sizes. This indicated that the vesicle structure was maintained in these formulations but could not maintain the protein inside the vesicles. These results suggested that these formulations could be freshly prepared and the freeze-dried cake may be more suitable for storage than the rehydrated colloidal dispersion.

5.3.4.2 Release profiles of mannosylated HBsAg from DRVs at 37°C

The release profiles of mannosylated HBsAg from these formulations had been evaluated at 37°C. The data showed that mannosylated HBsAg is released more easily from the DRVs prepared in the presence of sucrose than from those DRVs prepared in the absence of sucrose.

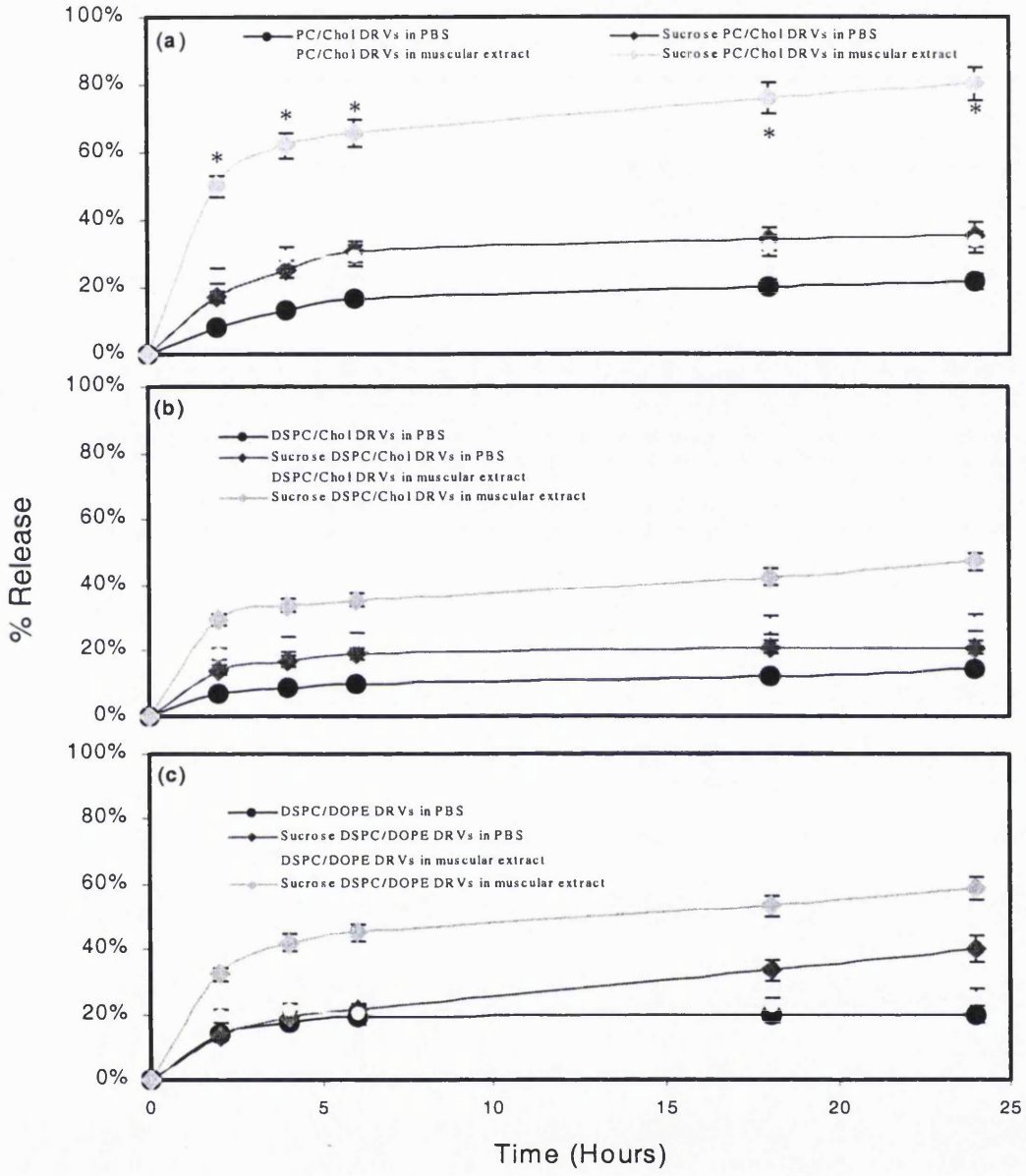

Figure 5.9: Release profiles of mannosylated HBsAg from DRVs prepared in the presence or absence of sucrose *in vitro*. (a) PC/Chol (16:16 μmoles) DRVs containing 5 μg of mannosylated HBsAg, (b) DSPC/Chol (16:16 μmoles) DRVs containing 5 μg of mannosylated HBsAg, and (c) DSPC/DOPE (16:8 μmoles) DRVs containing 5 μg of mannosylated HBsAg prepared in the presence or absence of 0.5 mMole of sucrose were resuspended in PBS or 10% muscular extract in PBS at 37°C. The factions were taken in different time points and centrifuged by ultra-centrifuge to evaluate the release ratio in the supernatant by measuring ^{125}I radioactivity. Data shown are mean ± SD of three experiments. *Significant different from the release from the DRVs prepared in the absence of sucrose: $U \leq 0$ (critical value) in Mann-Whitney U test.

The release profiles of mannosylated HBsAg from DRVs prepared in the absence of sucrose did not show significant difference for DRVs composed of different lipid compositions (Figure 5.9). However, the release kinetics of mannosylated HBsAg from the DRVs prepared in presence of sucrose showed a difference for PC/Chol DRVs. This may be due to the modification of lipid bilayers in PC/Chol DRVs prepared in the presence of sucrose. The release of mannosylated HBsAg from the DRVs prepared in the presence of sucrose was more than that observed from the DRVs prepared in the absence of sucrose.

5.4 IMMUNE RESPONSES OF DRVs ENTRAPPING MANNOSYLATED HBsAg

The mannosylated HBsAg changed the antigen form from whole entity to monomer (Figure 5.1 and 5.3). In immunisation, HBsAg had been shown to induce immune responses without using adjuvants. Previous results had confirmed that liposomes can present HBsAg B-epitope peptide with the T-epitope peptide co-entrapment (Gregoriadis *et al.*, 1993). Furthermore, other studies showed that the entrapment of water-soluble antigens into liposomes for the induction of CTL responses (Huang *et al.*, 1992). However, the mannosylated HBsAg contains the B-epitope, T-epitope and CTL epitope and may induce the antibody responses or cellular immunity. In this study, mannosylated HBsAg was entrapped into DRVs composed of various compositions. These formulations were injected intramuscularly into the mice to induce the antibody responses. The effect of liposomes as vaccine adjuvants for mannosylated HBsAg was preformed. In addition, the adjuvanticity of DRVs prepared in the presence of sucrose for mannosylated HBsAg was carried out as well.

5.4.1 Effect of HBsAg mannosylation

The total IgG responses in Balb/c mice induced by mannosylated HBsAg were weaker than those elicited by HBsAg. The mannosylation of HBsAg decreased the mean of log values of serum IgG titres from 2.7 to 2.2 in the primary responses and reduced those values of IgG levels from 3.7 to 2.4 (log_{10}) in the secondary responses (Figure 5.10). For mannosylated HBsAg, the secondary IgG responses were almost identical to the primary responses. This indicated that mannosylated HBsAg might not trigger the immunological memory in the immunisation.

These liposomal formulations inhibited the primary responses induced by mannosylated HBsAg in Balb/c mice. However, the mice immunised with DSPC/DOPE DRVs entrapping mannosylated HBsAg enhanced the secondary antibody responses. The adjuvanticity of these DRVs for mannosylated HBsAg was different from that for HBsAg. The antibody titres induced by these vesicles were much lower than those elicited by HBsAg-containing DRVs. DSPC/DOPE DRVs could increase the antibody titres elicited by mannosylated HBsAg from 2.4 to 3.2 (log_{10}) in the secondary responses. However, they could not increase the mean of log values of IgG titres induced by HBsAg in the secondary responses. The fusogenic property of DOPE in liposomes may have improved the delivery of this antigen into APCs. Consequently, the possibility of this antigen processed by the cells would be increased. However, the antibody responses induced by DRVs entrapping mannosylated HBsAg did not achieve the levels as those obtained from mice immunised with native HBsAg. Generally, fusogenic liposomes might work as the carriers to deliver this antigen into APCs for antibody induction. In summary, only DSPC/DOPE DRVs could improve the antibody responses for mannosylated HBsAg.

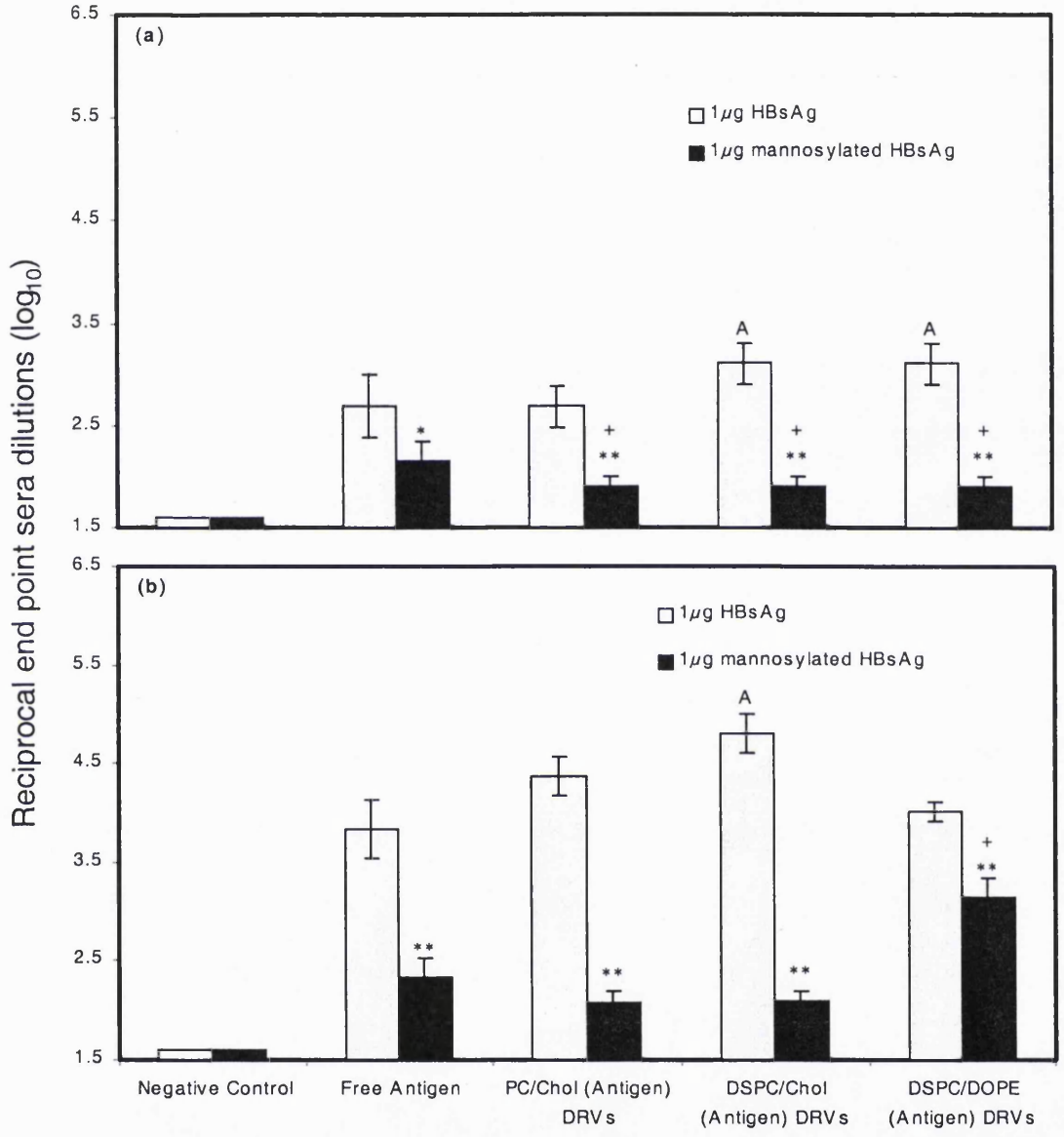

Figure 5.10: Comparison of total IgG responses to the liposomal formulations of HBsAg and mannosylated HBsAg in Balb/c mice. The mice were immunised intramuscularly with free HBsAg or mannosylated HBsAg (1μg) and their liposome-entrapped formulations. The sera taken at 28 days after first IM injection were the primary responses (a). Those bled at 10 days after another boosting on 28 days were the secondary responses (b). The IgG titres were measured as the previous chapter. Each bar represents mean ± SD (n=5). Significantly different from the mice immunised with HBsAg and its formulations: *$p<0.05$; **$p<0.005$. ^{A}Statistical different from the mice treated with HBsAg, $p<0.05$ was considered significant. +$p<0.05$ compared to the mice injected with mannosylated HBsAg (Student's unpaired *t*-test).

The subclass IgG responses induced by mannosylated HBsAg and its liposomal formulations in Balb/c mice were much weaker than those induced by free HBsAg and its DRV form (Figure 5.11 and 5.12). In the primary responses, the mannosylated HBsAg and its liposomal formulations almost had no antibody responses (Figure 5.11). However, these formulations still did not induce high titres in the secondary responses except for DSPC/DOPE DRVs entrapping mannosylated HBsAg (Figure 5.12).

The subclass IgG responses induced by mannosylated HBsAg and its liposomal formulations in Balb/c mice depended on the lipid compositions for the primary or secondary responses. Generally, the mice immunized with mannosylated HBsAg and its liposomal formulations induced low antibody titres. The liposomal formulations didn't enhance the antibody titres at all in the primary responses. Furthermore, most of the secondary antibody titres induced by the mannosylated HBsAg and its liposomal formulations were lower than those elicited by HBsAg-containing DRVs. In the primary IgG_1 and IgG_{2b} responses, mannosylated HBsAg and its liposomal formulations could not induce any titre in the immunized mice. However, the mice vaccinated with these formulations induced a low titre achieving the same level as that elicited by HBsAg-containing DRVs in the primary IgG_{2a} responses. Moreover, the secondary subclass antibody responses in the mice treated with mannosylated HBsAg showed that DSPC/DOPE DRVs increased the IgG_1 responses from 1.9 to 2.9 on the mean of $\log_{10}$ values in immunised mice. However, none of these formulations to induced stronger IgG_{2a} and IgG_{2b} responses for mannosylated HBsAg in immunized mice. These results are different from those obtained from immunisation with HBsAg-containing DRVs which could induce higher IgG_{2b}.

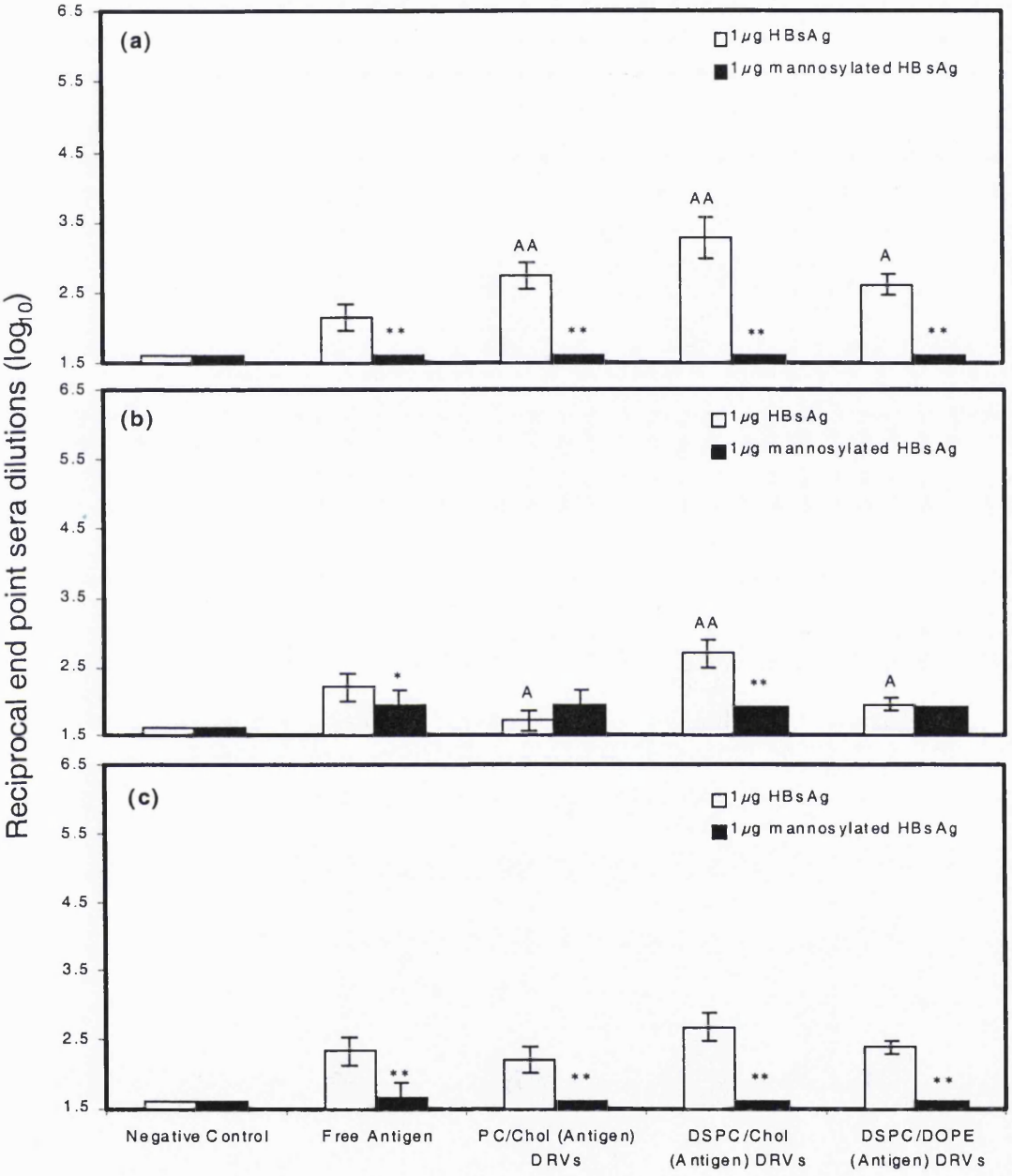

Figure 5.11: Comparison of the effect of liposomal formulations on the primary subclass IgG responses to HBsAg and mannosylated HBsAg in Balb/c mice. The sera taken at 28 days after first immunisation by IM injection were the primary responses. The sera were tested by ELISA for (a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b} responses against HBsAg. The values are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach reading of about 0.2 (n=5). *p<0.05, **p<0.005 compared to the groups immunised with HBsAg and its liposomal formulations. Significantly different from the mice treated with free HBsAg: $^{A}p<0.05$; $^{AA}p<0.005$(Student's unpaired *t*-test).

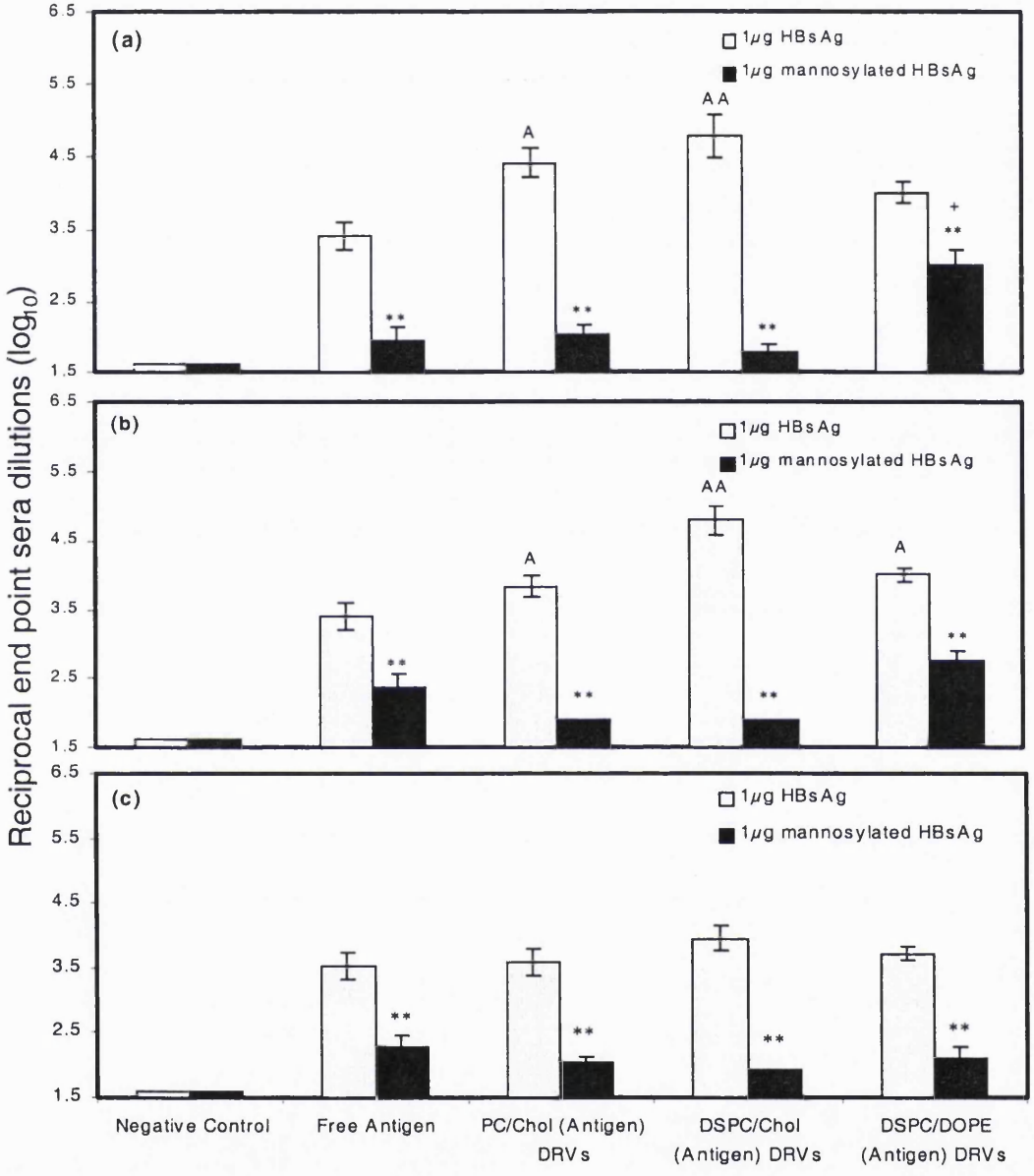

Figure 5.12: Comparison of the effect of liposomal formulations on secondary subclass IgG responses to HBsAg and mannosylated HBsAg in Balb/c mice. The sera taken at 10 days after the second boosting by IM injection were the secondary responses. These sera were tested by ELISA for (a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b} responses against HBsAg. The values are mean ± SD of $\log_{10}$ of reciprocal end point serum dilutions required for OD_{492} to reach reading of about 0.2 (n=5). *p<0.05, **p<0.005 compared to the groups immunised with HBsAg and its liposomal formulations. Significantly different from the groups treated with HBsAg: $^{A}p<0.05$; $^{AA}p<0.005$(Student's unpaired *t*-test).

In summary, the mannosylation of HBsAg reduced the ability of this antigen to induce the antibody responses. Liposomes exhibited different adjuvanticity for mannosylated HBsAg by comparison to that for HBsAg. DSPC/DOPE DRVs can enhance the secondary IgG_1 response for mannosylated HBsAg in the immunized mice. However, the PC/Chol or DSPC/Chol DRVs might not increase any subclass IgG titres for these immunised animals. Moreover, IgG_1 dominance in HBsAg-containing DRVs was observed in the mice immunized with DSPC/DOPE DRVs entrapping mannosylated HBsAg. However, this was not observed in those vaccinated with the DSPC/Chol DRVs entrapping mannosylated HBsAg. Generally, HBsAg mannosylation did not enhance the antibody responses in the immunisation study. Some liposomal formulations lost the ability to increase the antibody titres induced by mannosylated HBsAg in immunisation.

5.4.2 Effect of the presence of sucrose in DRVs

When the mannosylated HBsAg was entrapped into the DRVs, the conjugated mannose may be masked by the lipid bilayers and thus losing the ability of being recognised by macrophages, which contains mannose receptors on its surface (Mullin *et al.*, 1994). In Chapter 4, the DRVs prepared in the presence of sucrose had their vesicle size reduced successfully. The mannosylated HBsAg was entrapped into the DRVs prepared in the presence or absence of sucrose. These formulations were studied in the immunisation of Balb/c mice. Firstly, the total IgG responses were examined for the mice immunised with the formulations prepared in the presence or absence of sucrose. Then, the subclass IgG responses induced by these DRVs in vaccinated animals were tested to investigate the effect of sucrose on the dominant of IgG subclasses.

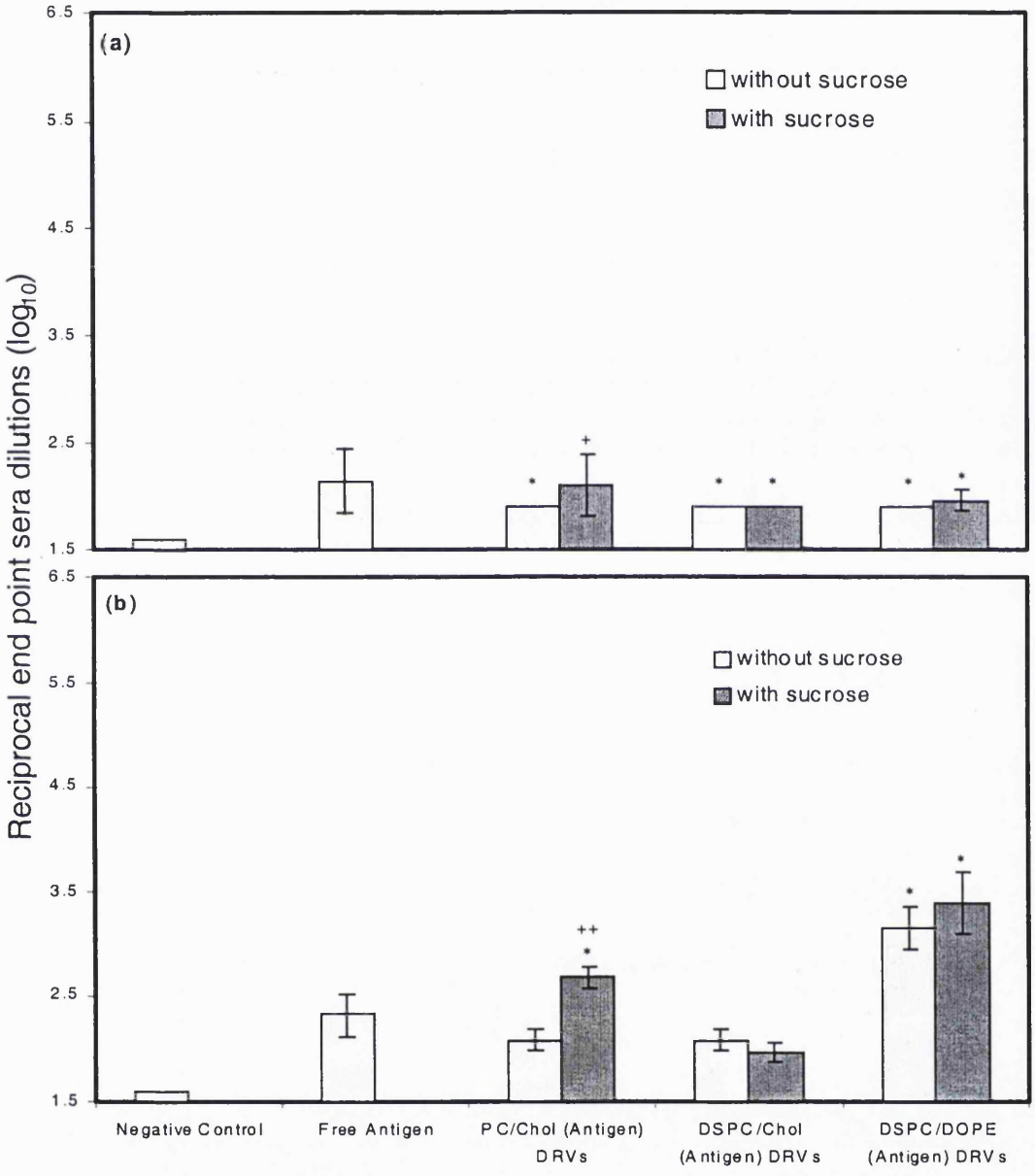

Figure 5.13: Comparison of total IgG responses induced by DRVs entrapping mannosylated HBsAg prepared in the presence or absence of sucrose in Balb/c mice. The mice were immunized with 1 μg of mannosylated HBsAg and its liposomal formulations prepared in the presence or absence of sucrose (0.021mMole/16μmole phospholipids). The sera taken at 28 days after first IM injection were the primary responses (a) and those bled at 10 days after second boosting were the secondary responses (b). The IgG titres were measured as described previously. Each bar represents mean ± SD (n=5). *p<0.05, **p<0.005 compared to the mice immunised with mannosylated HBsAg. Significantly different from the groups treated with DRVs entrapping mannosylated HBsAg: $^{+}p<0.05$; $^{++}p<0.005$(Student's unpaired *t*-test).

PC/Chol DRVs prepared in the presence of sucrose did not show adjuvanticity for entrapped mannosylated HBsAg in the primary responses. However, the secondary IgG titres elicited by entrapped mannosylated HBsAg were increased in the mice treated with sucrose presented PC/Chol or DSPC/DOPE DRVs (Figure 5.13).

The mice immunised with the DSPC/DOPE DRVs prepared in the presence of sucrose exhibited an enhanced primary and secondary IgG_1 response to mannosylated HBsAg(Figure 5.14 and 5.15). These subclass IgG responses induced by DRVs entrapping mannosylated HBsAg were totally different from those elicited by the HBsAg-containing DRVs in Balb/c mice. Only in mice treated with DSPC/DOPE DRVs entrapping mannosylated HBsAg, the mean of $\log_{10}$ values of serum IgG_1 titres induced by this antigen increased from 1.8 to 3.2. These results showed that vesicle size is not an important factor to control the liposomal adjuvanticity for mannosylated HBsAg. Moreover, DSPC/DOPE DRVs could increase the IgG_1 titre but not IgG_{2a} or IgG_{2b} in the immunisation with mannosylated HBsAg. This indicated that these DRVs exhibited Th2 dominance in antibody induction. However, they could not improve Th2 and Th1 pathways simultaneously as HBsAg-containing DRVs prepared in the presence of sucrose. In summary, DSPC/DOPE DRVs could change the subclass IgG responses from Th1 dominance (higher IgG_2 response) to Th2 dominance (higher IgG_1 response) for mannosylated HBsAg.

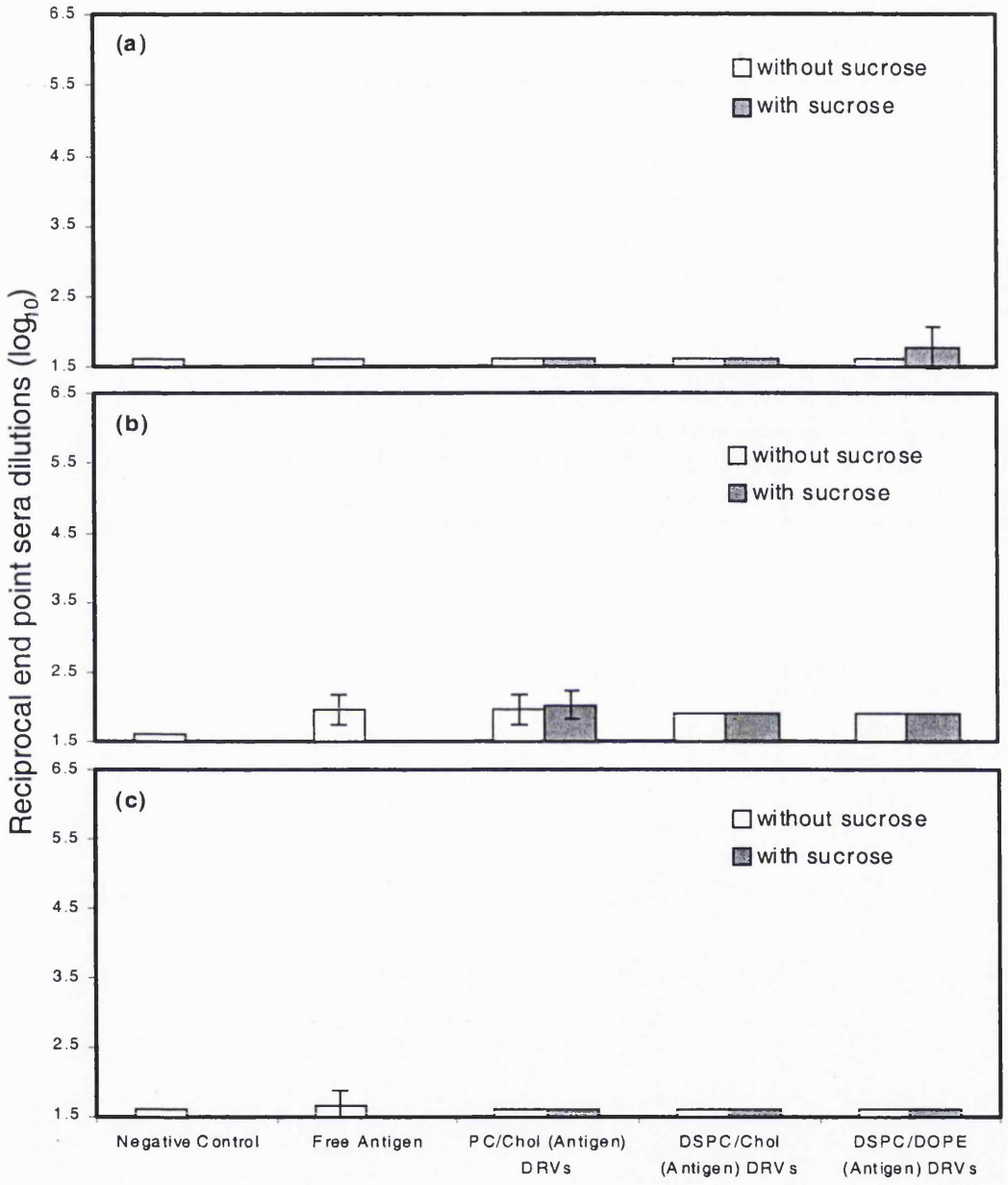

Figure 5.14: Comparison of the primary subclass IgG responses induced by DRVs entrapping mannosylated HBsAg prepared in the presence or absence of sucrose in Balb/c mice. The mice were immunized with 1 μg of mannosylated HBsAg and its liposomal formulations prepared in the presence or absence of sucrose (0.5mMole/ 16μMole phospholipid). The sera taken at 28 days after first IM injection were the primary responses. they were tested by ELISA for (a) IgG_1, (b) IgG_{2a} and (c) IgG_{2b} responses to HBsAg. The values demonstrated are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach reading of about 0.2 (n=5).

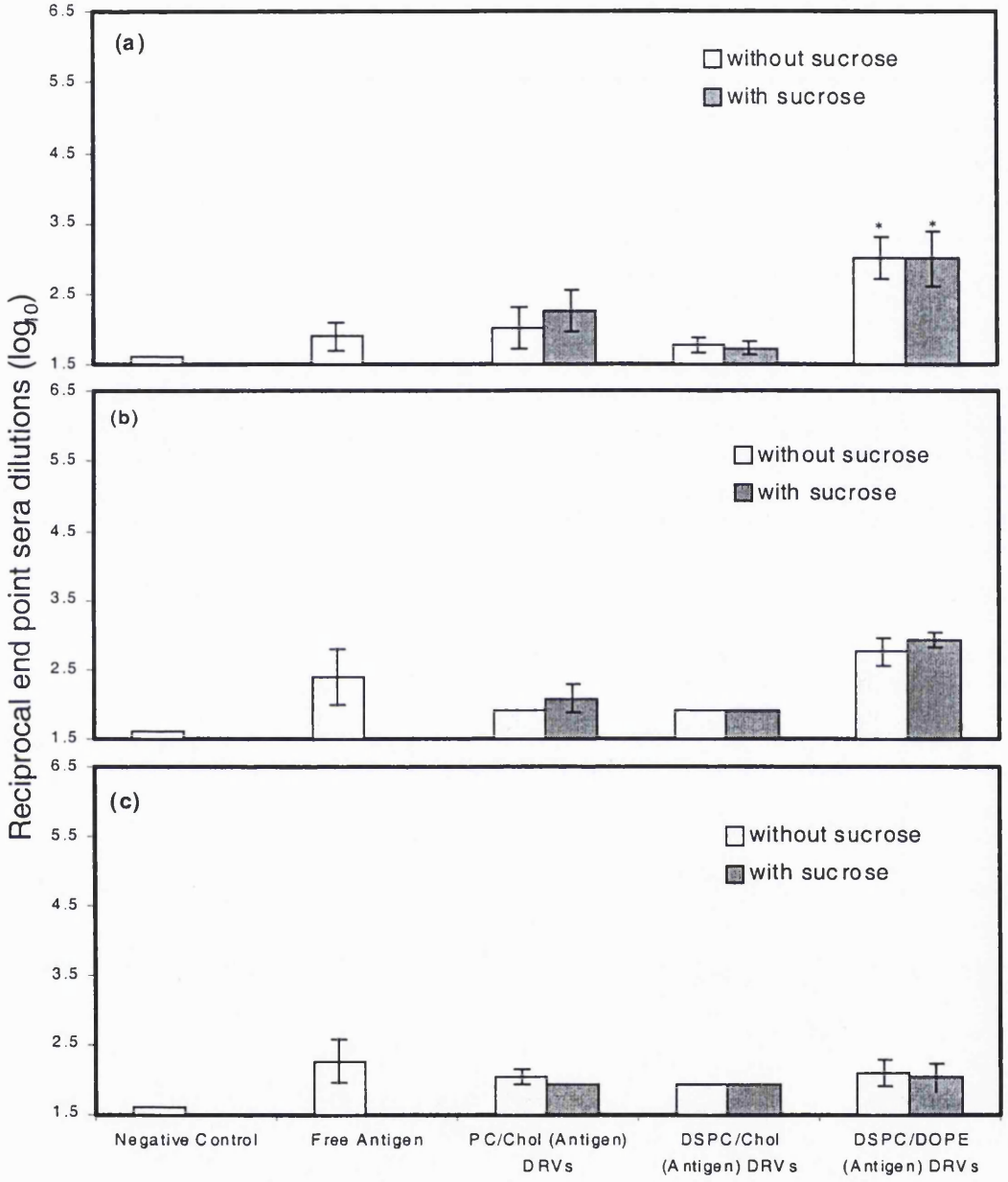

Figure 5.15: Comparison of secondary subclass IgG responses induced by DRVs entrapping mannosylated HBsAg in the presence or absence of sucrose in Balb/c mice. The mice were immunized with 1 μg of mannosylated HBsAg and its liposomal formulations prepared in the presence or absence of sucrose (0.5 mMole/16μMole phospholipid). The sera taken at 10 days after second IM injection were the secondary responses. They were tested by ELISA for (a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b} responses against HBsAg. The values demonstrated are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach reading of about 0.2 (n=5). *denoted values for the groups treated with DRV(mannosylated HBsAg) were significant higher ($p<0.05$) than those immunised with mannosylated HBsAg (Student's unpaired *t*-test).

5.5 DISCUSSION AND CONCLUSIONS

In this study, mannosylated HBsAg had been entrapped into the DRVs exhibiting different compositions and sizes and injected intramuscularly to the Balb/c mice to induce the antibody responses. The mannosylation of HBsAg reduced the antibody titres induced by this antigen in immunised Balb/c mice. Liposomes, such as DSPC/DOPE DRVs, could act as vaccine adjuvants for mannosylated HBsAg as judged by the antibody responses. This indicated that fusogenic liposomes may play a crucial role in inducing the IgG responses for mannosylated HBsAg. The characteristics of liposomes entrapping mannosylated HBsAg and their adjuvanticity for this antigen are discussed as follows.

HBsAg is a complex macromolecular aggregate composed of a major protein and lipids. The recombinant product from yeast is a kind of the natural vesicle (Diminsky *et al.*, 1996). These antigen particles can induce high antibody titres by administration in the absence of any adjuvant. However, HBsAg was reconstructed into the liposomes to enhance the antibody response (Chapter 3 and 4). In immunology, the antigens are processed by antigen-presentation cells and the B-epitope peptides are presented on the MHC II molecules to activate the B-cell for antibody production. Previous studies stated that liposomes can entrap both B-epitope and T-epitope peptides to induce antibody response by T-B cooperation (Gregoriadis *et al.*, 1993). This denoted that liposomes may act as immunological adjuvants for HBsAg peptide chain and help the modified HBsAg to induce antibody responses. Furthermore, HBsAg was conjugated to mannose to produce mannosylated antigen allowing the delivery of the antigen molecules to the macrophages, which contains a lot of mannose receptors on their surface (Mullin *et al.*, 1994).

Macrophages can present the processed foreign antigens to T-lymphocytes allowing the development of a specific immune response (Sato and Beutler, 1993). The characteristics of mannosylated HBsAg showed that HBsAg change from a whole entity to monomers after mannosylation (Figure 5.3). Consequently, mannosylated HBsAg was selected to study liposomal adjuvanticity in immunised mice. The results indicated that adjuvant effects of liposomes for HBsAg and mannosylated HBsAg are different.

The entrapment of mannosylated HBsAg into DRVs showed that the preparation procedure is important for optimum entrapment efficiency (Figure 5.5). In addition, the entrapped amount of mannosylated HBsAg was less than that of HBsAg into DRVs (Figure 5.6). Mannosylated HBsAg was more hydrophilic than the native one. HBsAg, a membrane protein, can incorporate into the lipid bilayers, but the mannosylated protein will be entrapped into the aqueous spaces of DRVs. In addition, the vesicle size of DRVs entrapping mannosylated HBsAg prepared in the presence of sucrose was smaller than those of the DRVs prepared in the absence of sucrose. However, the entrapment efficiency of mannosylated HBsAg in these liposomes only decreased in PC/Chol DRVs prepared in the presence of sucrose. The mechanism of protein entrapment into DRVs is still not clear. Results suggested that the lipid composition of liposomes affected the entrapment efficiency of mannosylated HBsAg in the DRVs prepared in the presence of sucrose. In summary, the entrapment of mannosylated HBsAg was controlled by the lipid composition, the preparation procedures, the loading amount of antigens and the presence or absence of sucrose during DRV preparation.

The vesicle size of DRVs entrapping mannosylated HBsAg is dependent on the preparation procedures (Table 5.1) and the amount of sucrose used (Table 5.2). The preparation procedures affected the lipid arrangement and bilayer structure in DRVs (Winterhalter and Lasic, 1993). For the DRVs entrapping mannosylated HBsAg, the DRV prepared involving the sonication step exhibited smaller size. In addition, the rehydration temperature was another significant factor affecting the size of DSPC/DOPE DRVs entrapping mannosylated HBsAg. The large size of DSPC/DOPE DRVs obtained from the rehydration at room temperature exhibited clearly the temperature effect. The hydration potential of DOPE is significantly lower than other phospholipid species such as PC, which shows higher hydration potential when in equilibrium with water (Webb *et al.*, 1995). The phase behaviour of DOPE in water may be related to the existence of polyhedral structure exhibited by the vesicles observed under transmission electron microscopy (Figure5.8). Furthermore, the DRVs prepared in the presence of sucrose have reduced vesicle sizes when increasing amount of sucrose is added. The results were similar to those of HBsAg-containing DRVs prepared in the presence of sucrose. Generally, the vesicle size distribution of DRVs entrapping mannosylated HBsAg was controlled by the preparation procedures. The extent of size reduction of these vesicles prepared in the presence of sucrose depended on the lipid compositions and the amount of sucrose added.

The stability studies of these vesicles upon storage at 4°C showed that the lipid compositions as well as vesicle sizes are important factors to control the retention of mannosylated HBsAg in DRVs. The vesicle size of these formulations did not increase upon storage expect DSPC/DOPE DRVs prepared in the presence of sucrose. The retention of

mannosylated HBsAg in DRVs was possibly decided by the integrity of lipid bilayers. However, membrane properties of liposomes prepared in the presence of sucrose were possibly altered. These data demonstrated that the formulations entrapping mannosylated HBsAg were unstable upon storage at 4°C. It suggested that these formulations should be stored in freeze-dried cake form of precursor SUVs.

The release profiles of mannosylated HBsAg from DRVs depended on the lipid compositions as well as the vesicle sizes of these formulations (Figure 5.9). The amount of mannosylated HBsAg released from PC/Chol DRVs was less than that of HBsAg released from a similiar formulations (Figure 3.7 and 5.9). The location of antigens in DRVs may be the major reason leading to this observed difference. Furthermore, the release profiles could not show that the protection effect of liposomes in the delivery pathways. However, the behaviour of liposomes *in vivo* after intramuscular injection is still unclear. Hence, the protection effect of liposomes during the antigen delivery is not the only factor to control its adjuvanticity. Consequently, these results estimated the antigen characteristics in delivery process by intramuscular injection. However, the relationship between release data and the immune responses induced by these formulations required more experiments to characterise.

The antibody responses induced by mannosylated HBsAg in Balb/c mice were less than those elicited by the native HBsAg. In the mice immunised with these formulations, only the mice immunised with DSPC/DOPE DRVs entrapping mannosylated HBsAg could enhance the antibody response induced by this antigen. These results showed that mannosylation made

HBsAg lose its ability to induce antibody responses. However, the antigen processing and presentation suggested that the amino acid sequence of epitope is a key point for induction of immune responses (Blum *et al.*, 1997). Possibly, the antigen passed through the processing to present on MHC I molecules for CTL induction, amino acid sequence of antigen may be more important. However, the antibody responses were induced by the antigen binding on the surface of APCs, and triggered the release of antibodies from B-cells. Hence, the modified HBsAg may be unable to bind on the APCs and lose its ability to induce antibody responses. In addition, another possibility is that the modified HBsAg, containing B, T and CTL epitope, can induce the CTL responses. The activated CTL killed the APCs presenting the HBsAg epitope. Consequently, the antibody responses elicited by mannosylated HBsAg may be deceased by this mechanism.

In conclusion, HBsAg mannosylation generated a new antigen, which exhibited different properties when encapsulated into DRVs as well as immunisation by using these DRVs. As judged by antibody responses, there is an indication that native HBsAg particles are more suitable than mannosylated HBsAg for liposomal formulations in the development of a vaccine against hepatitis B. However, the mechanism of antibody induction in the mice immunised with mannosylated HBsAg is still unclear. In order to understand all immune responses elicited by these formulations, the cellular immunity induced by these formulation is required to be investigated in further research.

Chapter 6

Cholera Toxin B Subunit Conjugated with Liposomes as Oral Vaccine Adjuvants: Oral Immunisation for HBsAg

6.1 INTRODUCTION

Most of the vaccines are available in an injectable dosage form. However, they are almost ineffective at inducing mucosal responses (Kaul and Ogra, 1998). The target organ of hepatitis B viruses is the liver, so the induction of immune responses in the gastrointestinal tract may pass through enterohepatic circulation to protect the liver from viral infection. However, oral immunisation with purified antigens often leads to weak immune responses probably due to their digestion and limited adsorption of antigens by the Peyer's patch, a major inductive site of mucosal responses in the gut environment. Therefore, various antigen delivery systems and mucosal adjuvants are under investigation for developing effective mucosal vaccines (Eyles *et al.*, 2000). Liposomes have also been studied for their effectiveness as oral antigen delivery systems (Childers *et al.*, 1991; Michalek *et al.*, 1989). They may protect antigens from digestion in the gut and contribute to their immunogenicity by presenting the antigens in a particulate form. This can enhance antigen uptake by the M cells of Peyer's patches in the gastrointestinal tract (Michalek *et al.*, 1994) and may also act as an antigen depot (Gregoriadis, 1990) containing a sufficient amount of antigens to induce specific secretory immune responses via the common mucosal immune system. Targeting of liposomes to specific tissue sites using cell surface ligands has also been proposed as useful means to enhance the efficiency of liposomes as a vaccine delivery or immunological adjuvant system (Leserman *et al.*, 1980).

A novel approach to enhance mucosal immune responses for orally administered antigens by co-administration of cholera toxin (CT) or its B subunit (CTB). CT, the primary enterotoxin produced by *Vibrio cholerae*, consists of two components (Gill, 1976): the A subunit (CTA)

which is toxic and the non-toxic pentamer CTB which mediates the binding of CT to the surface of cells through interaction with G_{M1} ganglioside (Cuatrecasas, 1973). Although the function of CTB as a mucosal adjuvant is questionable (Elson and Ealding, 1984b), it may act via its targeting to cell surface G_{M1} ganglioside (Holmgren *et al.*, 1993).

In this study, the CTB was coupled covalently to the HBsAg-containing DRVs via a thioether bond and these formulations were applied by oral administration. Firstly, the conjugation of CTB was checked in each step of chemical reactions. Then, these DRVs conjugated CTB were characterized by the amount of HBsAg in these vesicles and their vesicle size. Moreover, the stability of these vesicles was monitored upon storage and the release profiles of HBsAg from DRVs incubated with PBS or gastrointestinal extract fluid were tested. Finally, these vesicles were applied into the mice with oral administration to check the humoral (IgG titres) and mucosal immunity (sIgA titres).

6.2 CONJUGATION OF CTB SUBUNIT TO HBsAg-CONTAINING DRVs

6.2.1 Preparation of DSPE-MCC conjugates

DSPE-MCC was prepared by mixing 50 mg of DSPE with 35.8 mg of SMCC in 3.6 ml of chloroform/methanol (9:1) as described previously (2.2.8.1). The product was concentrated *in vacuo*, resuspended in 1 ml of chloroform and passed through a silica column (12×0.8 cm). The purified product was analysed by thin layer chromatography and identified with iodine. This product was also identified by using UV spectroscopy.

6.2.1.1 Purification of DSPE-MCC conjugates

The preparation was dried under a nitrogen stream to avoid oxidation of products and lyophilized under high vacuum for one hour. Then, the dried film was resuspended in 1 ml of chloroform and eluted with chloroform through a silica column. Finally, the purified reaction product was analysed by thin layer chromatography on silica plates by using chloroform/ methanol/ water (65:25:4) as solvent. The fractions were examined by UV spectroscopy and the absorbances at 205 nm and 300nm were measured to estimate the amount for the DSPE and conjugated MCC (Figure 6.1). DSPE was eluted out in the first peak and DSPE-MCC was in the following one.

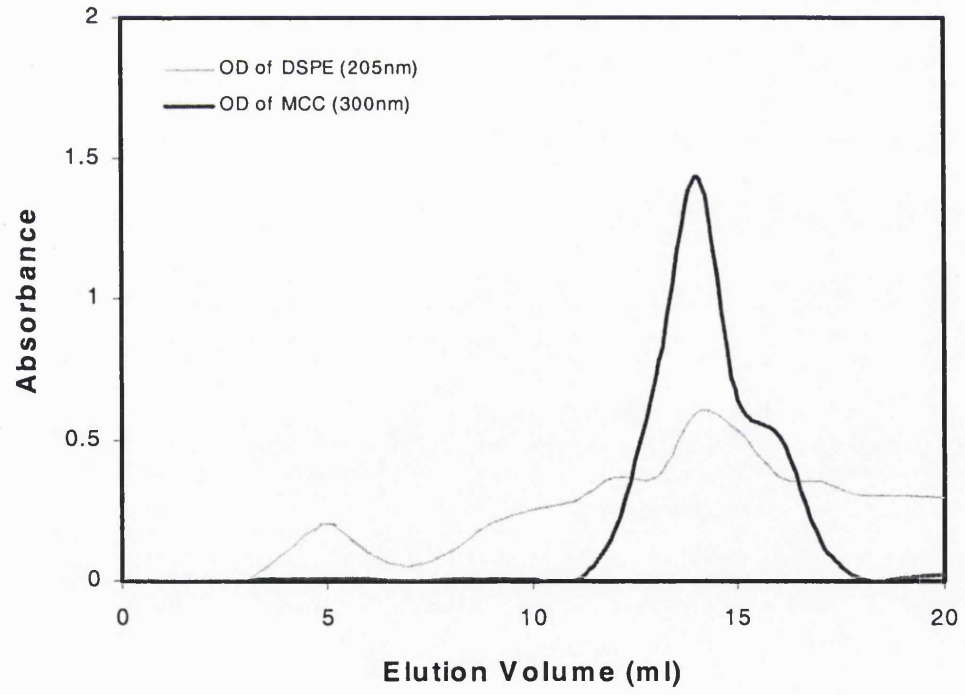

Figure 6.1: Chromatograms of the purification of DSPE-MCC conjugates. Fractions (one ml each) were collected and measured for DSPE and MCC at 205nm or 300nm respectively.

6.2.1.2 Characterisation of DSPE-MCC conjugates

The purified product of DSPE-MCC was scanned by UV spectroscopy from 200 to 400 nm, and the spectrum was compared with that of DSPE (Figure 6.2). The SMCC conjugation on DSPE led to a broad peak around the wavelength at 300 nm. This is the absorption band of the N-maleimidomethyl group. This group contains a double bond conjugated with a carbonyl group which gave the broad adsorption band. The spectra confirmed that the MCC group was conjugated with DSPE.

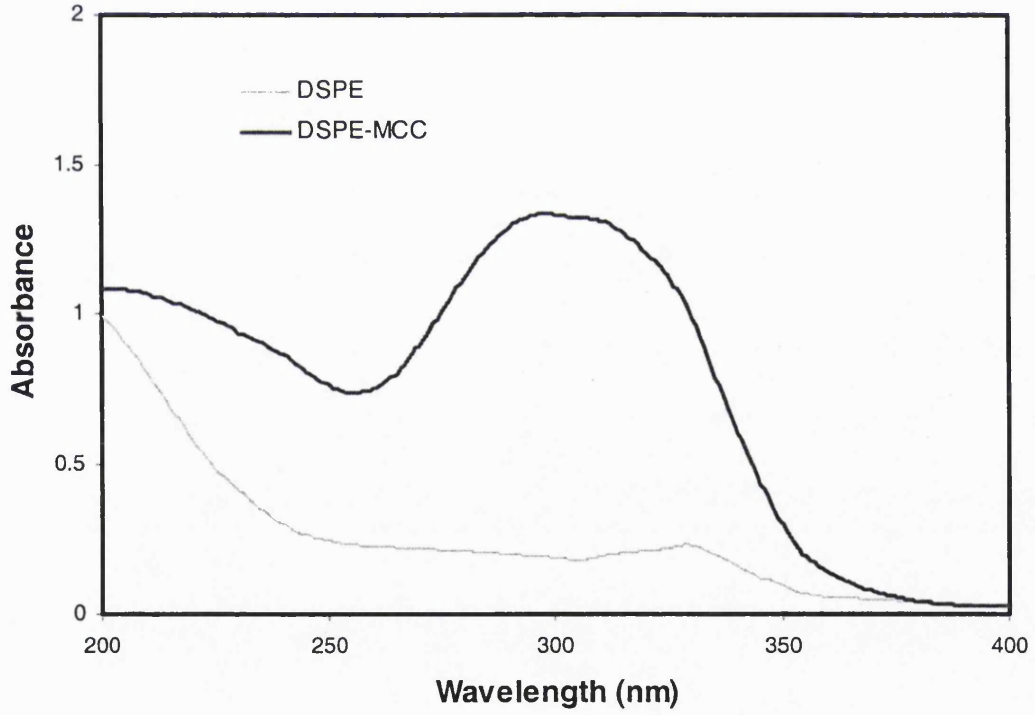

Figure 6.2: UV absorption spectra of DSPE and DSPE-MCC. 1 mg of DSPE and DSPE-MCC were dissolved in 2 ml of chloroform, and scanned by UV spectroscopy at a wavelength range of 200nm to 400nm. The adsorption band around 300 nm from purified DSPE-MCC indicates the presence of conjugated SMCC.

6.2.2 Incorporation of DSPE-MCC conjugates into HBsAg-containing DRVs

The purified DSPE-MCC was incorporated into the HBsAg-containing DRVs for further conjugation with CTB. The changes in the entrapment of HBsAg into the DRVs, their vesicle size and zeta potentials were observed (Table 6.1). When the DSPE was incorporated into liposomal formulations, the entrapment efficiency of HBsAg into DRVs increased from 45.5% to 60.8% and their zeta potentials were changed from -25mV to -41mV. By comparison with DSPE-bearing DRVs, the entrapment of HBsAg and the zeta potential did not change significantly when DSPE-MCC conjugates were incorporated into DRVs entrapping HBsAg. The results exhibited that the lipid compositions changed the entrapment of HBsAg in DRVs and the surface charge of these vesicles. These entrapment data were used to decide the dose for oral immunisation.

Table 6.1: Effect of DSPE-MCC incorporation in HBsAg-containing DRVs.

Liposomal composition	Entrapment amount (μg/10μg)	Volume Mean Diameter (μm)	Zeta potential (mV)
DSPC/Chol (16:16 μmole)	4.55 ± 0.11	5.06 ± 0.47	-24.5 ± 0.8
DSPE/DSPC/Chol (4:12:16 μmole)	6.08 ± 0.18*	5.67 ± 0.67	-41.4 ± 0.3*
DSPE-MCC/DSPC/Chol (4:12:16 μmole)	5.62 ± 0.24*	5.22 ± 0.64	-37.8 ± 0.6*

Data shown are mean ± SD of three experiments. *U≤0(critical value) compared to the conjugation on DSPC/Chol DRVs (Mann-Whitney U test).

6.2.3 Preparation of CTB-SPDP conjugates

The amine-reactive reagent SPDP was used to add thiol groups to the lysine residues of CTB. The thiol groups were necessary for reaction with the maleimide group of DSPE-MCC on liposomes. Briefly, CTB was dissolved in and dialyzed against Hepes buffer and subsequently incubated with SPDP at molar ratios of 1:50 (CTB:SPDP) in the dark at room temperature for 24 hours. The reaction product was then monitored by SDS-PAGE (15% Gel). Figure 6.3 shows that the molecular weight of CTB-SPDP conjugates ranged from about 30 to 70kD suggesting that the CTB (monomer 11.6 kD) was cross-linked to trimers and other higher molecular weight oligomers in the presence of SPDP. However, the CTB molecules kept on the top of separation gel of SDS-PAGE. Consequently, SPDP conjugation led to dissociation of the assembly of CTB pentamers.

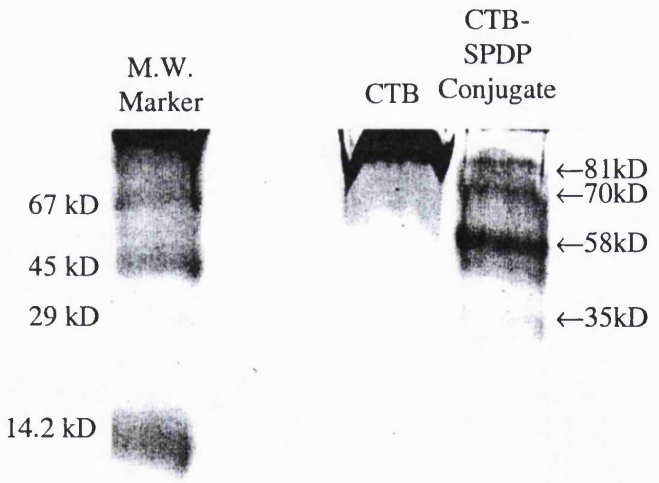

Figure 6.3: SDS-PAGE of CTB-SPDP conjugates. CTB and its SPDP conjugates were checked with SDS-PAGE (15% gel). The molecular weight markers included bovine serum albumin (67 kD), chicken egg albumin (45 kD), carbonic anhydrase (29 kD) and α-lactalbumin (14.2kD). The band of CTB suggests that its molecular weight is more than 67kD, but the CTB-SPDP conjugates are around 30-80kD.

6.2.4 Conjugation of CTB to the surface of HBsAg-containing DRVs

Conjugation of ^{125}I-labelled CTB to liposomes was carried out by incubating the reduced protein with a colloidal dispersion of DSPE-MCC bearing liposomes at 4°C for overnight. The coupling reaction was stopped by adding 10 μl of L-cysteine buffer to the reaction solution. The liposomes were separated from the unconjugated proteins and washed twice with Hepes buffer by centrifugation at 27,300g for 30 minutes. The amount of CTB in the washed liposome pellet was determined by the radioactivity. The amount of CTB conjugation depended on the applied amount of CTB-SPDP conjugates. Moreover, the DSPE-MCC bearing DRVs had more conjugation amounts than the control groups (Figure 6.4). The results showed that the spacer, MCC, improved the CTB conjugation on the liposomal surface.

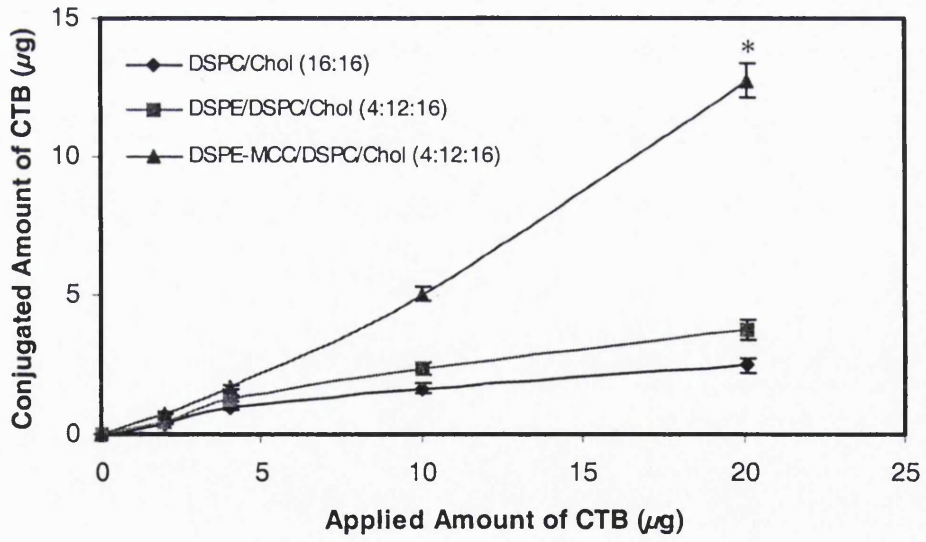

Figure 6.4: Conjugation of CTB to the HBsAg-containing DRVs with different lipid compositions. Various amounts (2, 4, 10 and 20 μg) of CTB were applied to conjugate with the HBsAg-containing DRVs composed of DSPC/Chol (16:16 μmoles); DSPE/DSPC/Chol (4:12:16 μmoles); DSPE-MCC/DSPC/Chol (4:12:16 μmoles)). The conjugated amount was evaluated by radioactivity. Data shown are mean ± SD of three experiments. *U≤0(critical value) compared to the conjugation on DSPC/Chol DRVs (Mann-Whitney U test).

6.3 CHARACTERISATION OF CTB CONJUGATED DRVs ENTRAPPING HBsAg

The CTB conjugated HBsAg-containing DRVs were prepared by the methods mentioned above. CTB conjugation changed the properties of HBsAg-containing DRVs, such as entrapment efficiency of HBsAg, vesicle size and surface property. The characteristics of DSPE-MCC bearing HBsAg-containing DRVs were determined after CTB conjugation. Firstly, the vesicle properties were carried out to show the effect of CTB conjugation on the HBsAg entrapment and the vesicle size distribution of these vesicles. Then, photos of HBsAg-containing DRVs conjugated with FITC-labelled CTB-SPDP were taken under the fluorescence microscopy to clarify the CTB on the surface of the CTB-conjugated DRVs. Moreover, the photographs of the DRVs and CTB-conjugated DRVs under cryo-scanning electron microscopy were examined to confirm the modified surface. Finally, the stability of CTB-conjugated DRVs entrapping HBsAg were monitored in the retention of conjugated CTB and entrapped HBsAg upon storage at 4°C. Furthermore, the release profiles of HBsAg from these DRVs were checked in the phosphate buffered saline, gastric and intestinal extracts to evaluate the possibility of delivering the HBsAg through GI tract for oral immunisation.

6.3.1 Vesicle properties of CTB-conjugated DRVs entrapping HBsAg

In order to deliver the HBsAg-containing DRVs to the mucosal tissue by targeting in the gastrointestinal tract, the CTB was conjugated on the surface of HBsAg-containing DRVs. The HBsAg amounts were monitored before and after the conjugation with CTB to confirm the effect of this modification on the entrapment of HBsAg into DRVs. Moreover, the vesicle properties were examined to investigate the effect of CTB conjugation on liposomal characteristics.

Table 6.2: Effect of CTB conjugation on HBsAg-containing DRVs.

Liposomal compositions	Conjugation amount of CTB (μg)	Entrapment amount of HBsAg (μg/10μg)	VolumeMean Diameter (μm)	Span	Zeta potential (mV)
DSPE-MCC/DSPC/Chol (4:12:16 μmoles)	0	5.62±0.24	5.22±0.64	1.37 ± 0.04	-37.8±0.6
DSPE-MCC/DSPC/Chol (4:12:16 μmoles) + 2 μg of CTB	0.766±0.078	4.97±0.23	4.13±0.60	1.48 ± 0.06	-39.7±0.6
DSPE-MCC/DSPC/Chol (4:12:16 μmoles) + 4 μg of CTB	1.669±0.120	4.89±0.21	3.61±0.56	1.60 ± 0.05	-39.2±0.2
DSPE-MCC/DSPC/Chol (4:12:16 μmoles) + 10 μg of CTB	5.017±0.388	4.84±0.22	3.56±0.6	1.86 ± 0.08*	-39.2±0.1
DSPE-MCC/DSPC/Chol (4:12:16 μmoles) + 20 μg of CTB	12.739±1.138	4.81±0.25	3.26±0.44 *	2.03 ± 0.07*	-39.3±0.2

Values shown are mean ± SD of three experiments. *Significantly different from HBsAg-containing DRVs: U≤0 (critical value) in Mann-Whitney U test.

6.3.1.1 Entrapment efficiency of HBsAg in CTB-conjugated DRVs entrapping HBsAg

The amount of HBsAg entrapped in DRVs did not decrease significantly when DRVs were conjugated to CTB (Table 6.2). This indicated that CTB conjugation did not destabilize the structure of HBsAg-containing DRVs. In addition, the slight reduction of HBsAg entrapment only occurred with CTB conjugation (Table 6.2) suggesting that CTB displaced some of the HBsAg on the outer layers of DRVs. DRVs containing the same amount of HBsAg and CTB were selected for the further studies in morphology, stability and immunization.

6.3.1.2 Vesicle size distribution of CTB-conjugated DRVs entrapping HBsAg

When HBsAg-containing DRVs conjugated with CTB, the volume mean of vesicle size decreased but the span value of vesicle size distribution increased (Table 6.2). However, their zeta potentials did not show any significant change. The surface modification may supply the

steric hindrance to inhibit the liposomal aggregation and keep the vesicle size be smaller. However, the conjugation of CTB did not change the zeta potential significantly. These results indicated that CTB conjugation made the vesicle size be smaller by the steric hindrance but not by electrostatic repulsion among the vesicles.

6.3.2 Morphology of CTB conjugated DRVs entrapping HBsAg

The FITC-labelled CTB was incubated with the HBsAg-containing DRVs for overnight and washed twice by phosphate-buffered saline to remove free FITC-labelled CTB. The samples were examined under fluorescence microscopy and the photographs showed the adsorption of protein on the surface of CTB-conjugated DRVs HBsAg entrapping HBsAg (Figure 6.5). The photographs confirmed that the CTB conjugation on the surface of HBsAg-containing DRVs. The specific identification of CTB on the liposomal surface needs the ganglioside G_{M1} labelled with fluorescence marker in further studies.

After examining these vesicles using cryo-SEM, the polydispersity of CTB-conjugated DRVs encapsulating HBsAg was found to be larger than that of HBsAg-containing DRVs (Figure 6.6). This result is in agreement with the data obtained from the measurement by the Mastersizer (Table 6.2). Some of the CTB-conjugated DRVs entrapping HBsAg were larger than the unmodified DRVs. However, the volume mean diameter of CTB-conjugated DRVs measured by the Mastersizer was smaller than that of unmodified DRVs (Table 6.2). The results indicated that CTB-conjugation to HBsAg-containing DRVs shifted the vesicle population towards a smaller size and reduced the volume mean diameter.

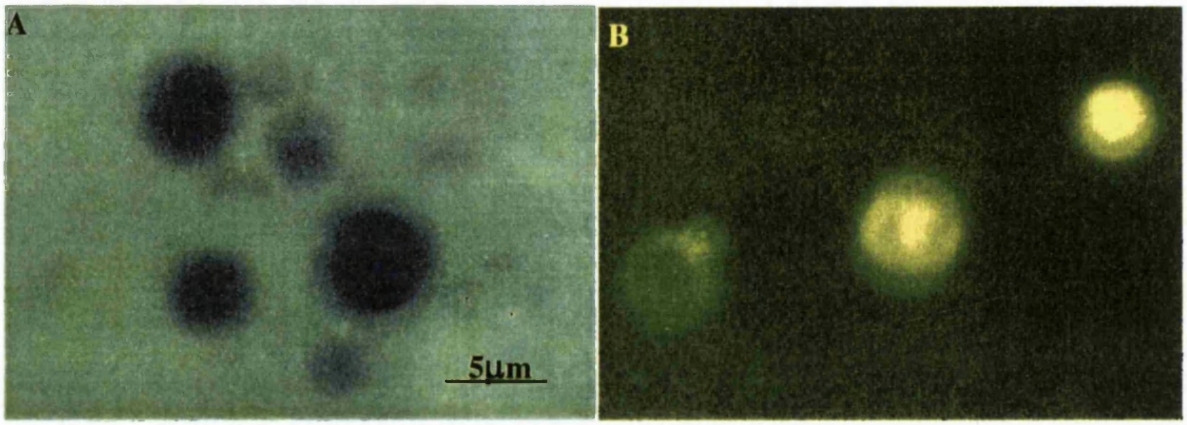

Figure 6.5: Photographs of CTB-conjugated DRVs entrapping HBsAg under fluorescence microscopy. (A) HBsAg-containing DRVs and (B) FITC-labelled CTB conjugated to HBsAg-containing DRVs.

The conjugation of CTB onto the surface of HBsAg-containing DRVs may change the surface of these vesicles. However, the examination of these vesicles under cryo-scanning electron microscopy did not show significant differences between conjugated and unconjugated DRVs (Figure 6.6).

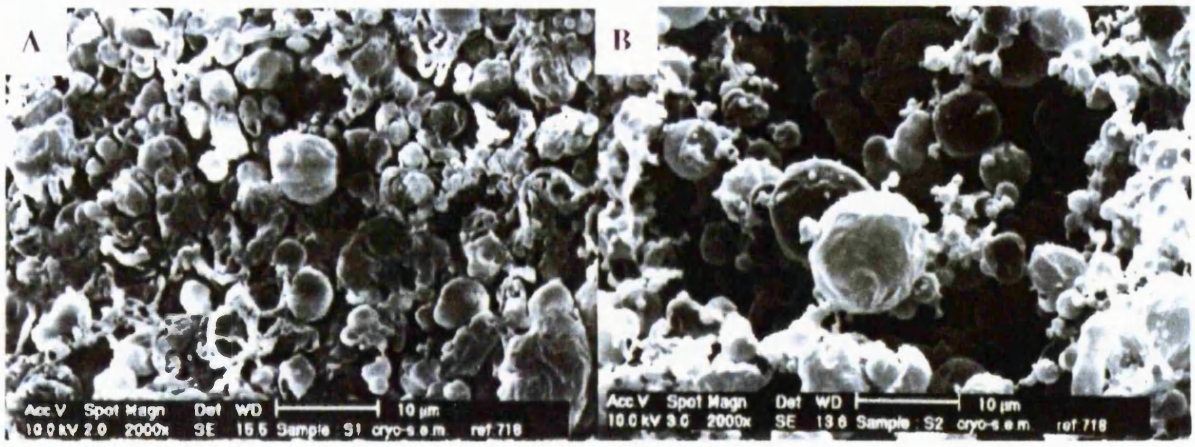

Figure 6.6: Photographs of CTB-conjugated DRVs entrapping HBsAg examined under cryo-scanning electron microscopy. (A) HBsAg-containing DRVs and (B) CTB-conjugated DRVs entrapping HBsAg. DRVs were frozen in liquid nitrogen slush and examined in a Philips XL20 scanning electron microscope.

6.3.3 Stability of CTB-conjugated DRVs entrapping HBsAg

In order to evaluate the stability of these vesicles on storage, the characteristics of CTB-conjugated DRVs entrapping HBsAg was monitored at 4°C for several weeks. The retention of entrapped HBsAg and conjugated CTB in DRVs, their vesicle size and zeta potential were measured at different time points to examine the stability of these vesicles on storage. In addition, the release profiles of HBsAg from DRVs were carried out in the buffer, the gastric and intestinal extracts to study the protection effect of these vesicles for oral delivery. This data evaluated the stability of these vesicles on storage and the possibility for oral administration.

6.3.3.1 Retention of CTB on CTB-conjugated DRVs entrapping HBsAg on storage at 4°C

The ability of these liposomes targeting to musosal surfaces depended upon the characteristics of conjugated CTB on their surface. Thus, the retention of conjugated CTB on the HBsAg-containing DRVs was monitored for several weeks at 4°C. The results showed that the conjugated CTB left from the HBsAg-containing DRVs with one week's half-life on storage at 4°C. The dissociation of conjugated CTB from the surface of DRVs changed the value of their zeta potential from -39 to -42 mV(Table 6.3). However, their vesicle sizes did not show significant alteration. After storage at 4°C for eight weeks, the properties of these CTB-conjugated vesicles were similiar to the DSPE/DSPC/Chol DRVs in the absence of CTB conjugation. These results indicated that the conjugated CTB was not stable on the surface of HBsAg-containing DRVs and suggested that CTB should be conjugated on these vesicles before immunisation.

Table 6.3: Stability of CTB-conjugated DRVs entrapping HBsAg on storage at 4°C.

Storage time	Retention of conjugated CTB (%)	Retention of HBsAg (%)	Volume Mean Diameter (μm)	Zeta potential (mV)
1 week	53.59 ± 0.88	97.59 ± 1.01	3.56 ± 0.62	-39.2 ± 1.4
2 weeks	25.16 ± 1.66	95.76 ± 0.90	3.84 ± 0.78	-39.7 ± 1.7
4 weeks	9.78 ± 0.68	93.15 ± 1.20	4.15 ± 0.83	–40.0 ± 1.9
6 weeks	6.18 ± 1.24	90.70 ± 1.10	4.41 ± 0.79	-40.9 ± 2.3
8 weeks	3.45 ± 2.05	87.77 ± 1.10	4.60 ± 0.81	-41.9 ± 2.8

Retention of conjugated CTB on these vesicles was carried out by monitoring ^{125}I-labelled CTB conjugated DRVs entrapping HBsAg. Retention of HBsAg on these vesicles was carried by measuring ^{125}I-labelled HBsAg in CTB-conjugated DRVs. Values shown are mean ± SD of three experiments.

6.3.3.2 Retention of HBsAg on CTB-conjugated DRVs on storage at 4°C

When these formulations were stored at 4°C, the retention of HBsAg in CTB-conjugated DRVs was quite stable and the shelf-life of the retention of entrapped HBsAg in DRVs was around 6 weeks. After 8 weeks at 4°C, there is still 88% of entrapped HBsAg remained in CTB conjugated DRVs (Table 6.3). High retention of entrapped HBsAg in CTB-conjugated DRVs can be explained by the interaction between HBsAg and DSPE-MCC. The interaction between HBsAg and DSPE-MCC also increased the entrapment efficiency of HBsAg in DSPE-MCC bearing DRVs (Table 6.1). Consequently, a stable liposomal product for HBsAg could be developed. However, a detailed mechanism of HBsAg entrapment and retention in DRVs required more experiments to carry out. This result provided a possible method to improve the retention of entrapped HBsAg in DRVs and might be suitable for developing as a new formulation for this vaccine.

6.3.3.3 Release profiles of HBsAg from CTB-conjugated DRVs at 37°C

The release of ^{125}I-labelled HBsAg from these vesicles in PBS and gastrointestinal extract depended on the lipid composition of liposomes and the media that DRVs were incubated in (Figure 6.7). In order to evaluate the stability of liposomes in the gastrointestinal tract, the HBsAg-containing liposomes were resuspended in the gastrointestinal extract for various time intervals (2, 4, 6, 18 and 24 hours) and centrifuged at 35000g for 30 minutes to monitor the release profile of HBsAg. The gastrointestinal extracts were obtained from fresh stomach or intestinal tissues, homogenized in 0.9% sodium chloride solution (1 to 10 dilution) and centrifuged to remove cell debris. The supernatants were then adjusted to pH 2.0 for stomach extract with hydrochloric acid and with sodium bicarbonate to pH 7.4 for the intestinal extract. The release ratio of HBsAg over 24 hours of incubation in intestinal extract was 20% for CTB conjugated DSPE-MCC/DSPC/Chol DRVs, 38% for DSPE/DSPC/Chol DRVs and 52% for DSPC/Chol DRVs. The release ratios of HBsAg in gastric extract were less than 15% in each case and similar to those observed in PBS (Figure 6.7). The components in the intestinal extract, such as bile salts, known to destroy the lipid bilayers, are likely to account for the reduced stability of DRV. Therefore, HBsAg released from DRVs given orally could be digested in the intestine before they can be taken up by the Peyer's patches or M cells in the intestinal mucosa for antigen processing. The results (Figure 6.7) suggested that CTB-conjugated DRVs can protect HBsAg in the intestine thus increasing the chances of absorption of HBsAg administrated orally and antibody responses induced by HBsAg.

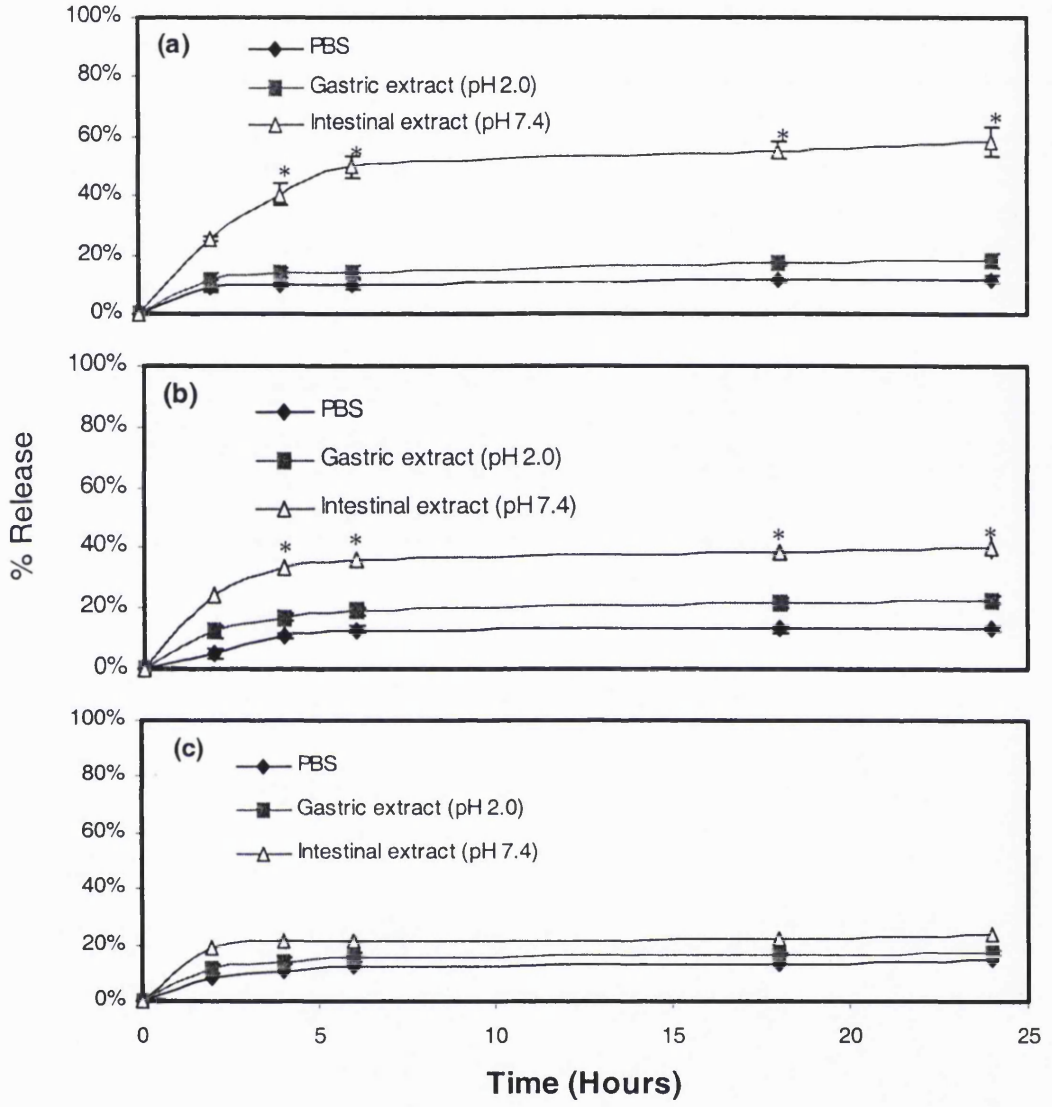

Figure 6.7: Release profiles of ^{125}I-labelled HBsAg from DRVs in PBS and gastrointestinal extract *in vitro*. (a) DSPC/Chol (16:16 μmoles) DRVs entrapping 4.5 μg of HBsAg, (b) DSPE/DSPC/Chol (4:12:16 μmoles) DRVs entrapping 6.1 μg of HBsAg and (c) 5.0 μg of CTB conjugated DSPE-MCC/DSPC/Chol (4:12:16 μmoles) DRVs entrapping 4.8 μg of HBsAg were incubated in PBS and 10% gastric or intestinal extracts in PBS at 37°C. Aliquots were taken at different time intervals (2, 4, 6, 18 and 24 hours) and the release ratio of HBsAg into the supernatant was monitored by measuring the radioactivity. Data shown are mean ± SD of three experiments. *Significantly different from the released amount of HBsAg from the DRVs incubated in PBS: $U \leq 0$ (critical value) in Mann-Whitney test.

6.4 IMMUNE RESPONSES OF CTB-CONJUGATED DRVs ENTRAPPING HBsAg

Previous studies showed that the CTB can bind with the GM_1 ganglioside (Cuatrecasas, 1973) in the intestinal muscosa, so the CTB conjugated DRVs might deliver the antigen to the intestine by targeting. The mice immunised with these formulations by oral administration were shown and compared with those treated by the formulations without CTB conjugation. DSPC/Chol liposomes had been applied in oral immunisation by using ovalbumin as model protein and induced the antibody responses (Aramaki *et al.*, 1993). In this study, CTB had been conjugated on the liposomal surface and these liposomal formulations for HBsAg had been applied to immunize the Balb/c mice by oral administration. The antibody responses induced by these formulations had been measured to evaluate the effect of liposomes and CTB conjugation on oral immunisation.

The male Balb/c mice were immunised with free HBsAg, HBsAg-containing DRVs by oral administration at days 0 and 28. The sera were taken at days 28 and 38 as the primary and secondary antibody responses. Although the mucosal immunity is not important for preventing the infection of Hepatitis B, the feces were collected at days 28 and 38 to measure the secretory-IgA titres. The antibody responses of the mice immunised with these liposomal formulations orally were measured and the properties of liposomes as oral vaccine adjuvants were characterized.

6.4.1 Total IgG responses by oral immunisation

In the primary responses of total IgG induced by oral immunisation, the mice immunised with liposomal HBsAg did not show stronger antibody responses than those treated with free antigens. However, the mice vaccinated with liposomal HBsAg enhanced the mean of $\log_{10}$ values of serum IgG from 2.5 to 4.0 in the secondary responses (Figure 6.8). The mice immunised with CTB-conjugated DRVs elicited higher titres than those treated with DRVs entrapping HBsAg and CTB. This indicated that the CTB conjugated DRVs had more potential to induce the antibody responses by oral administration. However, the DRVs entrapping HBsAg and CTB could not induce higher titres than the HBsAg-containing DRVs by oral vaccination. Thus, the enhancement of antibody response might not result from the existence of CTB in the liposomal formulations, but from the targeting effect of CTB-conjugated DRVs in GI tract.

By comparison with the immune responses in the mice with IM immunisation, the secondary antibody responses induced by oral vaccination were weaker than those in IM immunisation (Figure 3.9). The bioavailability of antigens by oral administration is one of the problems for oral vaccines. Low absorption efficiency of antigens from the oral routes may result from the protein digestion and poor absorption by GALT. The CTB-conjugated DRVs could increase the mean of $\log_{10}$ values of the IgG titres to 4.0 in the immunised mice. This anti-HBsAg titres were similar to the titres induced by free HBsAg in IM immunisation. These results suggested that CTB conjugated DRVs might increase the bioavailability of HBsAg by oral administration. Consequently, these vesicles can improve the antibody responses by oral vaccination.

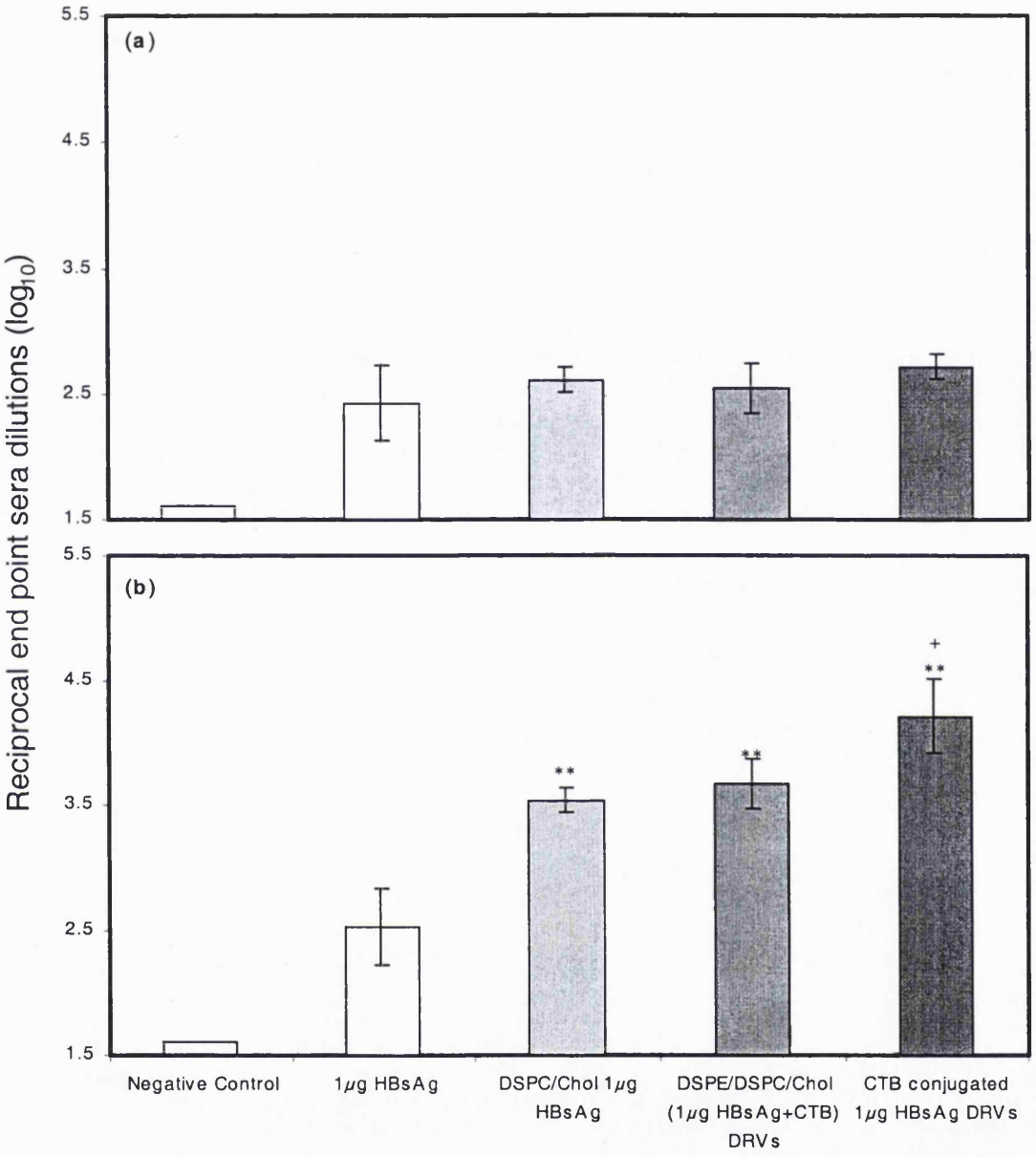

Figure 6.8: Total IgG responses to liposomal formulations of HBsAg by oral immunisation in Balb/c mice. The sera taken at 28 days after first oral administration were the primary responses (a) and those bled at 10 days after fed on 28days were the secondary responses (b). The antibody titres were measured by ELISA described previously. Each bar represents mean ± SD (n=5). **p<0.005 compared to the mice immunised with free HBsAg. Significantly different from the groups treated with the DSPC/Chol HBsAg DRVs: +p<0.05 (Student's unpaired *t*-test).

6.4.2 Subclass IgG responses by oral immunisation

The subclass IgG responses in Balb/c mice induced by these formulations by oral immunisation showed that the mice immunised with liposomal HBsAg produced higher titres than those treated with free antigens in IgG_1, IgG_{2a} and IgG_{2b} subclasses. The mice vaccinated with liposomal HBsAg orally increased the mean of $\log_{10}$ values of IgG_1 and IgG_{2b} titres from 1.6 to 2.5 in the primary responses (Figure 6.9). In addition, the mean of $\log_{10}$ values of IgG_1, IgG_{2a} and IgG_{2b} titres increased from 2.0 to 3.5 in the secondary responses of the immunised mice (Figure 6.10). For the primary responses of oral vaccination, HBsAg only induced the IgG_{2a} response. In the secondary responses of oral immunisation, HBsAg did not engender higher titres than the primary responses except the IgG_{2b} subclass. However, liposomal formulations enhanced the responses in each IgG subclass in the secondary responses of oral vaccination. Moreover, the mice immunised with CTB-conjugated DRVs by oral administration induced higher IgG_1 responses in secondary responses than other groups. In conclusion, liposomes improved antigens to generate the immunological memory and conjugated CTB modified the regulation in IgG subclass responses in oral immunisation.

By comparison with the results obtained from IM immunisation, the liposomal formulations increased the IgG_{2a} and IgG_{2b} titres as IgG_1 responses at the oral vaccination in Balb/c mice. Liposomal HBsAg only increased the mean of $\log_{10}$ values of IgG_1 or IgG_{2b} titres from 3.5 to 4.5 in the secondary responses (Figure 3.10). However, they increased the mean of $\log_{10}$ values of IgG_1, IgG_{2a} and IgG_{2b} titres from 2.3 to 3.4 in the secondary responses of oral immunisation. These enhancements might result from the protection effect of the DRVs on

HBsAg but not from the regulation of T-helper cell activation.

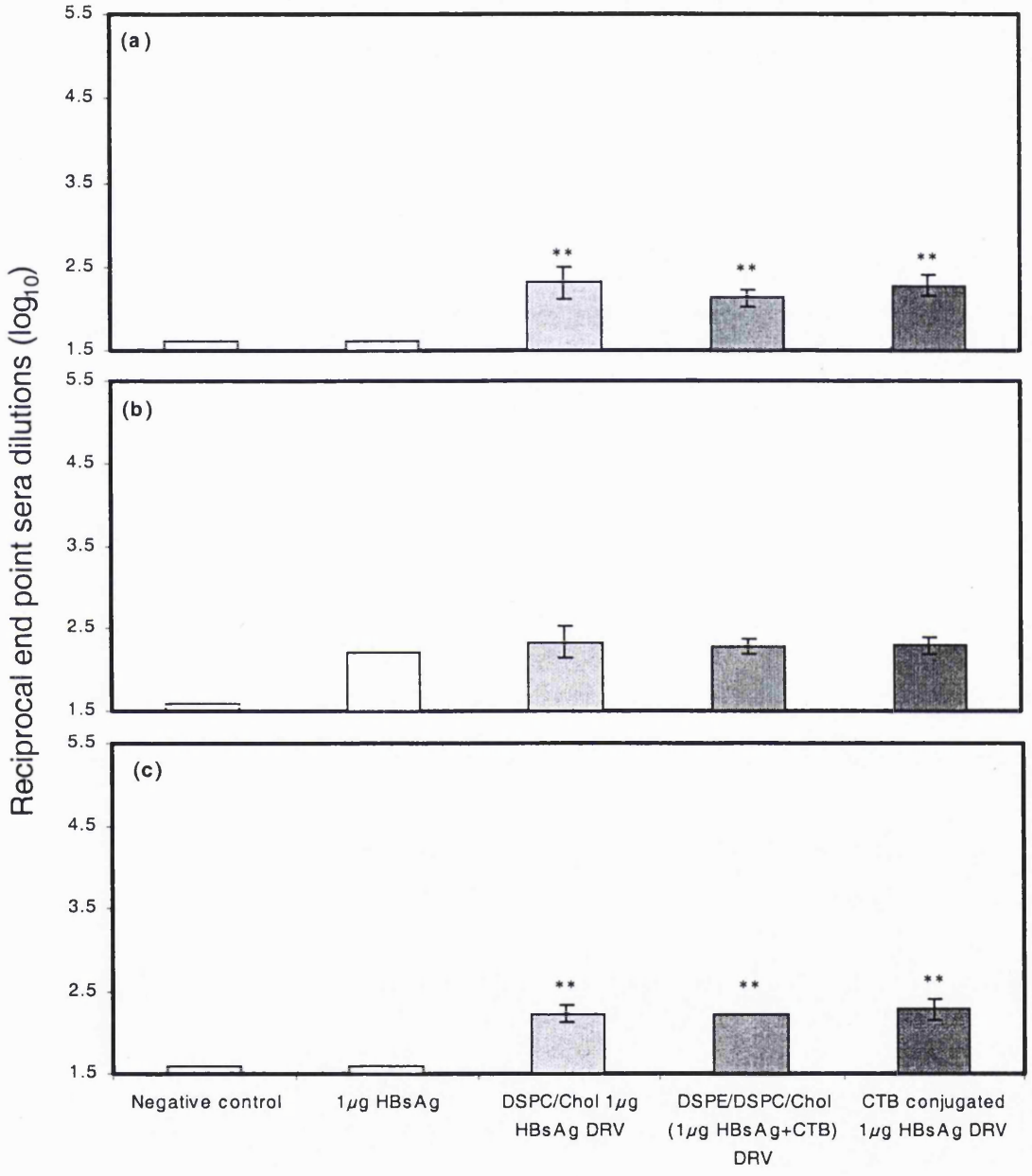

Figure 6.9: Comparison of the primary subclass IgG responses induced by oral vaccination with HBsAg and its liposomal formulations. The sera taken at 28 days after first immunisation by oral administration were the primary responses. The sera were tested with ELISA for (a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b} responses against HBsAg. The values shown are mean ± SD of log_{10} in reciprocal end point sera dilutions required for OD_{492} less than 0.2(n=5). Significantly different from the groups immunised with free HBsAg: **p<0.005 (Student's unpaired *t*-test).

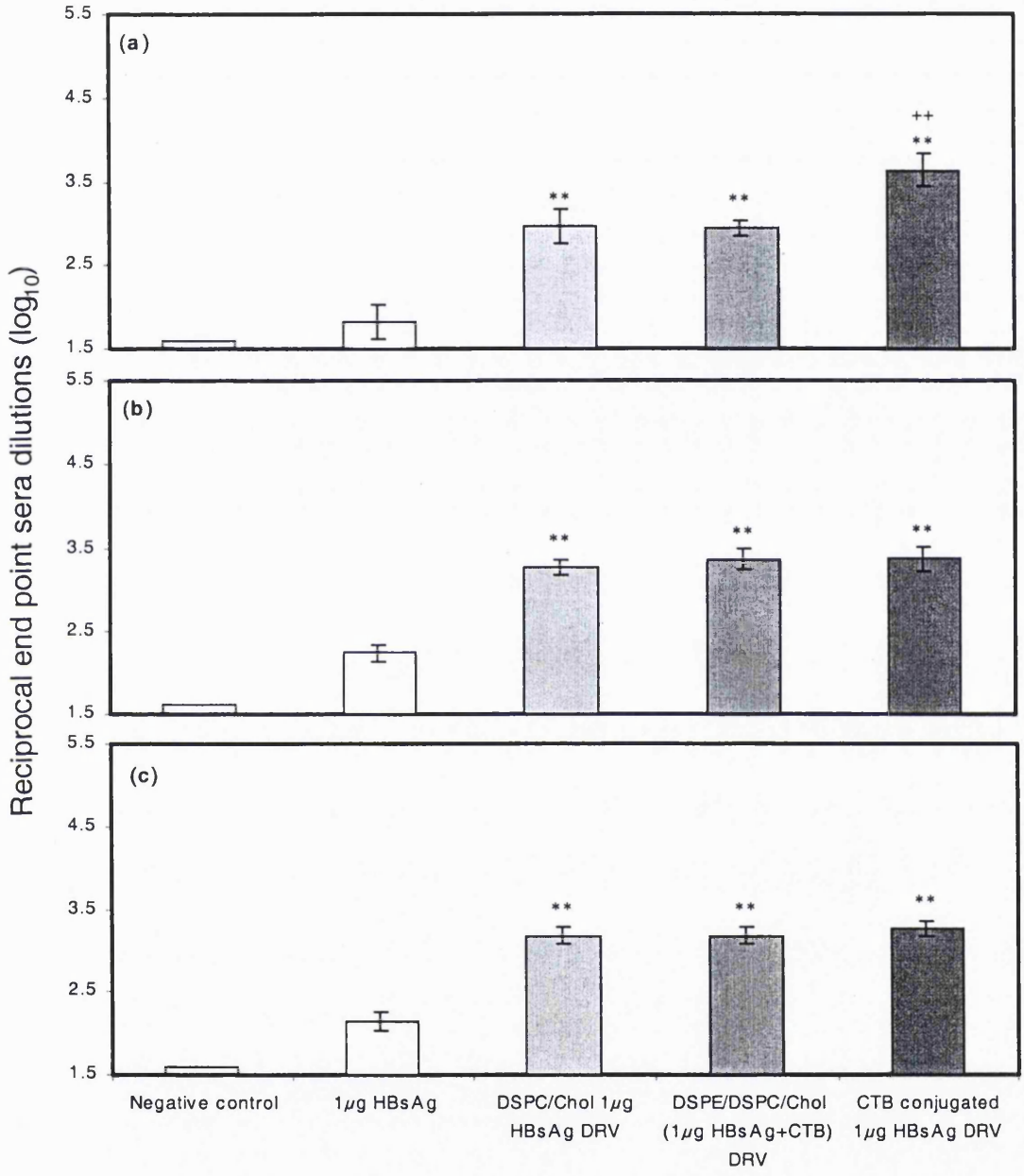

Figure 6.10: Comparison of the secondary subclass IgG responses induced by oral vaccination with HBsAg and its liposomal formulations. The sera taken at 10 days after second immunisation by oral administration were the secondary responses. The sera were tested with ELISA for (a) IgG_1, (b) IgG_{2a}, (c) IgG_{2b} responses against HBsAg. Data shown are mean ± SD of log_{10} in reciprocal end point sera dilutions required for OD_{492} less than 0.2 (n=5). **$p<0.005$ compared to the mice treated with free HBsAg. Significantly different from the groups immunised with DSPC/Chol (HBsAg) DRV: $^{++}p<0.005$ (Student's unpaired *t*-test).

6.4.3 sIgA responses by oral immunisation

The mice fed with liposomal formulations gave higher sIgA titres in the secondary response and the group treated with CTB-conjugated DRVs entrapping HBsAg induced higher sIgA titre than other groups (Figure 6.11). This indicated that the conjugated CTB on liposomal surface can induce stronger sIgA responses than that entrapped inside the liposomes. However, hepatitis B is transmitted by direct inoculation but probably almost as frequently by nonparenteral routes and is usually endemic (Deinhardt, 1976). Thus, mucosal immunity is not important to prevent the infection of hepatitis B viruses. The sIgA responses induced in these formulations exhibited the possibility of CTB-conjugated DRVs entrapping HBsAg for mucosal immunity, but might not have the advantage to prevent this disease.

Generally, the mice treated with CTB-conjugated DRVs entrapping HBsAg induced higher antibody titres in mice by oral administration than other formulations. The effect of CTB on the mucosal immunity show the CTB-conjugated DRVs may be a promising adjuvant for oral vaccine. The application of these vesicles in hepatitis B vaccines for oral vaccination may not be suitable, but these DRVs provide a possibility to develop the liposomes as oral vaccine adjuvants. Previous study had tried to use liposomes as oral vaccine carriers (Childers *et al.*, 1991), but these results demonstrated the systemic antibody responses induced by oral administration with CTB-conjugated liposomes. The results exhibited the possibility of applying the surface modified liposomes as antigen carriers by oral delivery.

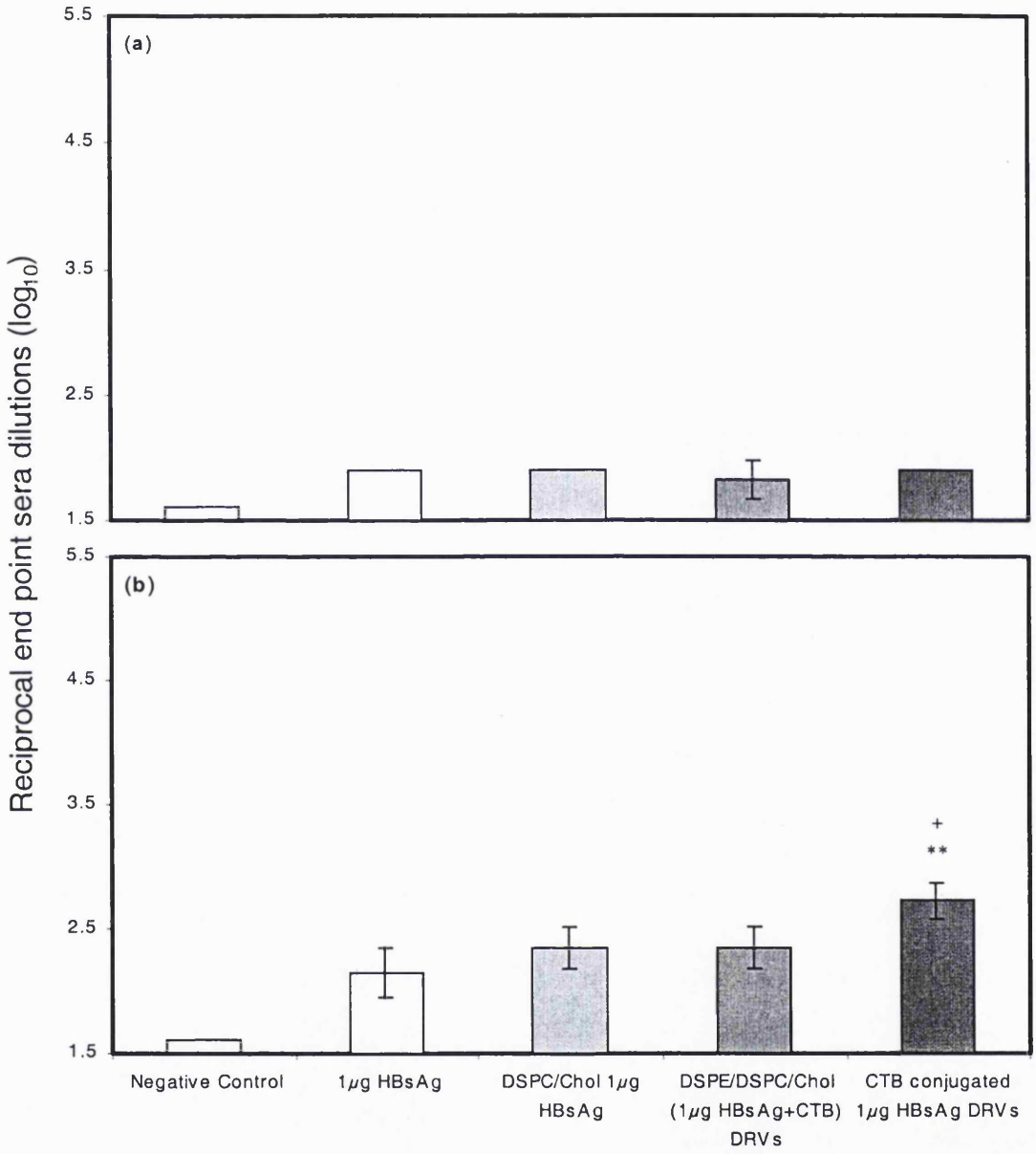

Figure 6.11: Secretory IgA responses to liposomal formulations of HBsAg by oral immunisation in Balb/c mice. The sera taken at 28 days after first oral administration were the primary responses(a) and those bled at 10 days after fed on 28days were the secondary responses (b). The antibody titres were measured by ELISA described previously. Each bar represents mean ±SD (n=5). **$p<0.005$ compared to the mice treated with free HBsAg. Significantly different from the groups treated with the DSPC/Chol (HBsAg) DRVs: +$p<0.05$ (Student's unpaired *t*-test).

6.5 DISCUSSION AND CONCLUSIONS

In this study, CTB was conjugated to the surface of HBsAg-containing DRVs with a thioether bond by the previous method (Harokopakis *et al.*, 1995) and applied to immunize the male Balb/c mice by oral administration. Liposomes had been applied as vaccine adjuvant for more than thirty years, but their application in oral vaccination just started from several years ago (Michalek *et al.*, 1989). The observation that CT could act as an adjuvant for antibody responses following intravenous delivery was first reported in 1972 (Northrup and Fauci, 1972). However, it was the subsequent finding that such adjuvant effects were evident following oral administration which established the importance of CT as an immunological tool (Elson and Ealding, 1984a). Subsequent works demonstrated that the adjuvant activity of low doses of CT could be markedly enhanced by addition of purified CTB (Wilson *et al.*, 1990). Consequently, CTB was chosen to conjugate onto HBsAg-containing DRVs. The preparation, characterization and oral immune responses of CTB-conjugated DRVs were accomplished and the details are discussed below.

In the conjugation of CTB to the surface of HBsAg-containing DRVs, the spacer SMCC and SPDP were used to link CTB with the DRVs. To link the CTB covalently with the HBsAg-containing DRVs, the DSPE conjugated with SMCC to form the DSPE-MCC conjugates firstly. Then, these conjugates were incorporated into HBsAg-containing DRVs by adding them into SUV precursors. Some of the conjugates buried inside of the DRVs and cannot conjugate with the SPDP-CTB. However, the conjugation amount of CTB by this method is still more than the DSPE-bearing DRVs without spacer SMCC (Figure 6.4). It indicated that the reaction on the

thioether bond formation is more active than that on peptide bond construction. This is the reason why this method was used to conjugate CTB on the surface of HBsAg-containing DRVs.

The results from SDS-PAGE showed that the SPDP conjugation made CTB dissociate from its pentamer entity. SPDP-CTB became cross-linked trimers and higher oligomers of CTB. This change may influence the interaction between conjugated CTB and the GM1 on the intestinal mucosa. Thus, the conjugated CTB may lose its targeting ability to GI tract. However, the immune responses induced by CTB-conjugated DRVs entrapping HBsAg still gave higher antibody titres than those elicited by other formulations. These results indicated that the targeting capacity of CTB may not be lost after it is conjugated to the surface of HBsAg-containing DRVs.

In order to confirm the CTB conjugation to the HBsAg-containing DRVs, the FITC-labelled CTB was used to conjugate with HBsAg-containing DRVs and the conjugated amount of CTB was evaluated by the ^{125}I-labelled CTB (Table 6.2). Photographs under fluorescence microscopy showed that the protein coated around the CTB-conjugated DRVs entrapping HBsAg (Figure 6.5). The cryo-SEM photographs of these vesicles exhibiting the vesicle structure of CTB-conjugated DRVs did not change after conjugation with CTB (Figure 6.6). These results illustrated that CTB conjugation to the HBsAg-containing DRVs could be done and that the conjugation with CTB might change the surface property of the vesicles but not their structure.

The stability of CTB-conjugated DRVs was demonstrated by their stability to retain more entrapped HBsAg than DRVs in the absence of CTB conjugation did (Table 6.3). However, the conjugation of CTB to the surface of HBsAg-containing DRVs was very unstable. The half-life of conjugated CTB lost from the surface of HBsAg-containing DRVs is about one week (Table 6.3). The reason of this instability of conjugated-CTB is unclear. The long spacer between CTB and liposomal surfaces might make the linkage more vulnerable hydrolysis under storage. These results showed that CTB conjugation with the surface of HBsAg-containing DRVs is unstable and suggest that the conjugation must be carried out preferably just before application.

In the release kinetics of HBsAg from HBsAg-containing DRVs, most of entrapped HBsAg released from the DRVs in the intestinal extracts except the CTB-conjugated DRVs (Figure 6.7). The bile salts in the intestine are the major component that may destroy the liposomal integrity by oral administration. The conjugated CTB may be able to protect the liposomes from the attack of bile salts or enzymes and keep the entrapped HBsAg molecules. This is the advantage of CTB conjugation for the HBsAg delivery to the M cells on the intestinal muscosa. Hence, CTB-conjugated DRVs increased the antigen amount to be processed and presented. Furthermore, the absorption of these vesicles *in vivo* showed that the CTB-conjugated DRVs can be absorbed more than other formulations. Consequently, CTB-conjugated DRVs provided the protection and targeting function to deliver HBsAg by oral administration.

In the immunisation studies, these formulations were applied to immunize the male Balb/c mice by oral administration. The antibody responses induced by these liposomal

formulations were similar to the results obtained from the previous studies (Harokopakies *et al.*,1998). The mice treated with CTB- conjugated DRVs induced higher titres than other groups. This might be contributed by the protection and targeting effect of these vesicles in oral administration. The mice treated with CTB- conjugated DRVs induced higher antibody responses than those immunised with the CTB co-entrapped ones. This implied that the importance of the CTB exposure outside the DRVs for targeting delivery. The antibody responses engendered by these formulations entrapping HBsAg in mice can match with the release profiles and retention of antigens in the gastrointestinal tract. This data suggested that the protection and targeting to mucosa were the optimisation strategy to develop the carrier systems for oral vaccines.

The subclass IgG responses in this study showed that the enhancement in IgG_1, IgG_{2a} and IgG_{2b} in the mice fed with these liposomal formulations. These results were different from the intramuscular immunisation. Regarding the vaccination by IM injection, the mice treated with liposomal HBsAg elicited higher IgG_1 and IgG_{2b} titres, but not IgG_{2a} response. However, compared with the results in IM vaccination, the antibody titres induced by these formulations orally were still low. Thus, the enhancement of the titres in oral immunisation may result from the low titres induced by free HBsAg. This antigen may be digested through the delivery in gastrointestinal tracts. Furthermore, the mice immunised with CTB-conjugated HBsAg-containing DRVs induced higher S-IgA titre than other groups. It showed its mucosal immunity as the report used CTB as oral adjuvant previously. Results indicated that conjugated CTB still kept the activity to induce the mucosal immune responses.

In conclusion, the CTB-conjugated DRVs showed the possibility to be the HBsAg adjuvants by oral administration. Their adjuvant effect may result from the antigen protection and targeting effect on the oral delivery. However, the CTB conjugation to the surface of HBsAg-containing DRVs is unstable upon storage. This suggested that the CTB conjugation should be done before the immunisation, or another method could be used to stabilize the CTB conjugation on the liposomal surfaces. Results indicated that the CTB-conjugated DRV is a promising candidate for oral vaccine carrier for HBsAg. This study exhibited the possibility of using liposome to promote antibody responses for HBsAg by oral administration.

Chapter 7

Liposomes Mediated DNA Immunisation :Effect of Liposome-entrapped Calcium Phosphate-DNA Complexes

7.1 INTRODUCTION

Advances in recombinant DNA, monoclonal antibody technology, the understanding of the immunological structure of proteins and factors regulating immune responses witnessed in the last two decades. These techniques have led to a new generation of recombinant subunit and synthetic peptide vaccines (Newman and Powel, 1995) that mimic small regions of microbial proteins. A novel and exciting concept now developed, namely de novo production of the required vaccine antigen by the host's cells *in vivo*, promises to revolutionize vaccination. The concept entails the direct injection of antigen-encoding plasmid DNA which, following its uptake by cells, finds its way to the nucleus where it transfects the cells episomally. Produced antigen is subjected to pathway similar to those undergone by the antigens of internalized viruses (but without pathogenic effects) leading to protective humoral and cell-mediated immunity (Davis *et al.*, 1993a). Thus, a succession of publications from 1992 onwards established first the ability of plasmid DNA to induce an antibody response to the encoded foreign protein. Then, that immunity was both humoral and cell-mediated and also protective in mice challenged with the viruses (Ulmer *et al.*,1993; Fynan *et al.*,1993). However, vaccination with naked DNA by the intramuscular route relies on the ability of myocytes to engulf the plasmid. It follows that approaches to protect DNA from the extracellular biological milieu, introduce it into cells more efficiently or target it to specific cells (e.g., antigen presentation cells) expressed the plasmid should contribute to optimal DNA vaccine design.

Viral DNA can be delivered into host cells by virus particle for infection and the enveloped viruses have better infection efficiency than non-enveloped viruses (Patterson and

Oxford, 1986). From the structure of enveloped viruses, lipid bilayers in the virus envelope may play a special role to deliver viral nucleic acid into the target cells. Based on these viral properties, using liposomes to entrap DNA-complex for genetic vaccination may be a promising approach for DNA vaccination. However, DNA, a kind of polyelectrolyte with negative charge, can interact with the cationic substances to form DNA complex and be applied in the cell culture for gene transfection. The step that DNA interacts with counter-ionic substances calls the DNA condensation, which is a complicated phenomenon for polyelectrolyte solution (Fixman, 1979 and Fenley *et al.*, 1990). Furthermore, calcium ions, cationic lipids, and polymers with positive charge can condense the DNA to form the DNA-complexes. Many substances had been tried *in vitro* or *vivo* as non-viral vectors for gene therapy. Of these substances, calcium phosphate (CaPi) has been applied for more than 25 years (Graham *et al.*,1973) to precipitate DNA in cell culture and also tried in clinical trials (Anderson, 1992). Although the clinical trials failed, calcium phosphate is still used as a vaccine adjuvant similarly to aluminum hydroxide (Aggerbeck, *et al.*, 1996). Thus, its biocompatibility is better than other substances used and may be potentially used as an adjuvant for DNA vaccination.

The CaPi-mediated DNA transfection *in vitro* had been studied for a couple of years, and widely applied in the studies of molecular biology (Chen and Okayama, 1987). Previous work (Yang and Yang, 1997) suggested that CaPi can deliver DNA by releasing it under low pH conditions. However, the precipitate of CaPi and DNA will aggregated into large particles (up to 10μm) and sedimented down and crystallized in a medium containing serum. These phenomena may have caused in the failure of the clinical trials mentioned above and ways should

be found to formate it appropriately. Hence, the CaPi-DNA complexes were entrapped into liposomes by dehydration-rehydration methods. These formulations were then studied in terms of structural characteristics and also in immunisation studies. The immunization results obtained from DNA vaccination were compared with the data from protein immunisation to study the antibody responses induced by direct (protein vaccination) or indirect (protein expression from DNA) approach.

7.2 CHARACTERISATION OF CaPi-DNA COMPLEXES

The properties of the CaPi-DNA complexes have been characterized several years ago. Here, this method had been applied to condense the plasmid pRc/CMV-HBS DNA and the characteristics, such as DNA loading, morphology and DNA release from the complexes, were carried out.

7.2.1 DNA loading into CaPi-DNA complexes

In order to load the DNA into calcium phosphate complexes, a various amount of DNA was mixed with the 2.5 μmole of calcium phosphate and the loading yield was evaluated by the radioactivity of ^{35}S-labelled DNA. The data showed that the 2.5 μmoles of CaPi can precipitate up to 50 μg of DNA, with excess of DNA existing in the solution in the free form. Furthermore, this complex can be scaled up with the same ratio of CaPi to DNA, but the toxicity from the overdose of calcium *in vivo* should be considered. This procedure was applied to condense plasmid pRc/CMV-HBS DNA for further studies.

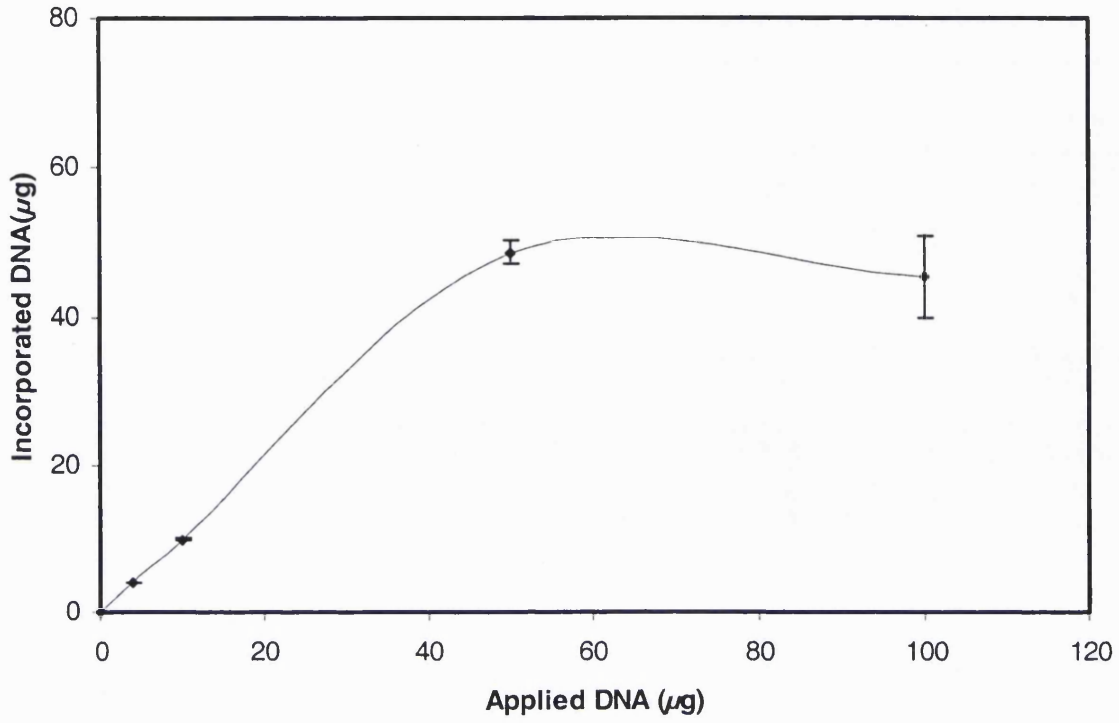

Figure 7.1: Loading of plasmid pRc/CMV-HBS DNA in the CaPi-DNA complexes. A various amount of plasmid pRc/CMV-HBS DNA were complexed with 2.5 μmole of calcium phosphate and the incorporated DNA were evaluated by ^{35}S-labelled DNA radioactivity (Data shown are mean ± SD, n=5).

7.2.2 Morphology of CaPi-DNA complexes

CaPi-DNA complexes precipitated in the medium. They showed the spherical shape under the transmission electron microscopy but aggregation in dispersion(Figure 7.2). The particles exhibited similar morphology as previously (Yang and Yang,1997). The property of these complexes depended on the CaPi but not on the size of DNA molecules. The size of each particle is around 200nm, but the particle size can reach up to 10 μm in the medium due to aggregation. The zeta potential of the complexes was found to be around -2 mV. This neutral surface charge induced aggregation of these particles. However, this aggregation may decrease

the cellular uptake. Thus, the size control in the complex is an important criterion to improve the efficiency of DNA delivery into the cells (Jordan *et al.*, 1996).

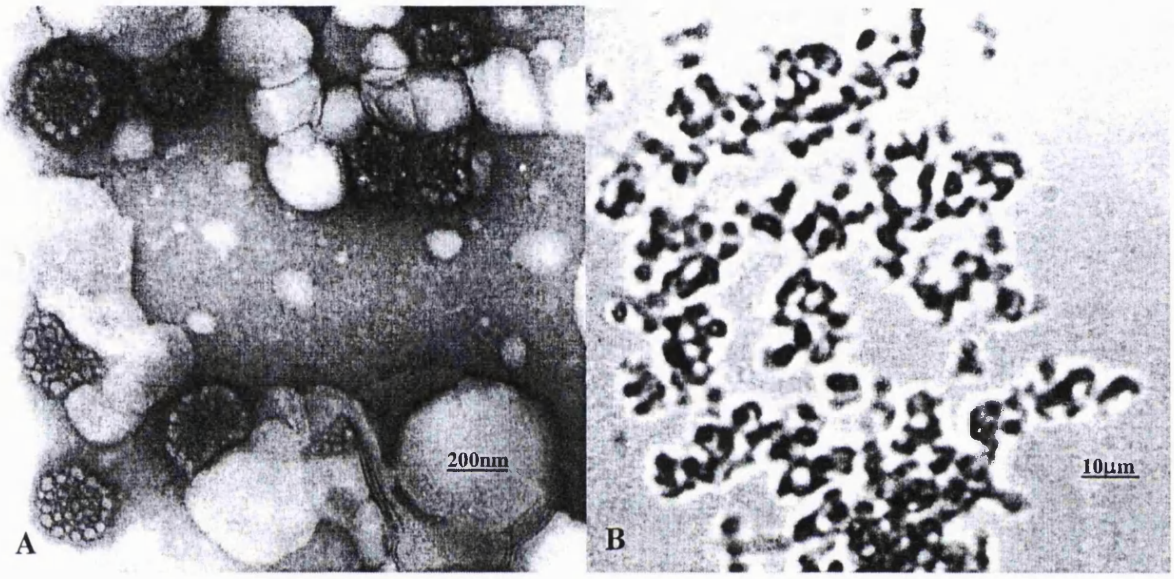

Figure 7.2: Photographs of CaPi-DNA complexes under microscopy. 50 μg of plasmid pRc/CMV-HBS DNA was precipitated with 2.5 μmole of calcium phosphate and the complexes were examined in a Philips 201 transmission electron microscopy (A) or light microscopy (B).

7.2.3 Release of DNA from CaPi-DNA complexes

CaPi can be dissolved in a medium below pH 5.0 (Owens *et al.*,1986) and DNA release from complexes was studied (Loyter *et al.*,1982). The CaPi-pRc/CMV-HBS DNA complexes were resuspended in PBS with varying pH. The particle size distribution and released ratio of DNA was characterized (Figure 7.3). When the pH value of the medium was below 5.5, DNA release from these complexes were up to 84%. In addition, the particle size distributions showed the disappearance of smaller particles (Figure 7.3(a)). This may result from the phosphate ionization in different pH conditions. These phosphoric ions can form complexes with calcium

ions (Chughtai, *et al.*,1968). For the intracellular delivery of DNA, the viruses can deliver DNA through attachment, internalization, uncoating and DNA release in the nucleus (Marsh and Helenius, 1990). CaPi-DNA complexes could dissociate in acidic media (pH 5.0) and DNA would release from these complexes (Figure 7.3(b)). Thus, CaPi may has the potential to be developed as gene carrier by protecting DNA in physiological condition and releasing in the late endosomes (pH 4.5) when it passed through the cellular uptake (Orrantia and Chang, 1990). Furthermore, the pH-dependent characteristics of these complexes allowed the removal of unentrapped complexes in liposomal preparation procedures. Moreover, CaPi had been applied to prepare adsorbed vaccines in immunization (Relyveld, 1986).Therefore, the CaPi was selected to condense DNA for the formulation of DNA vaccination.

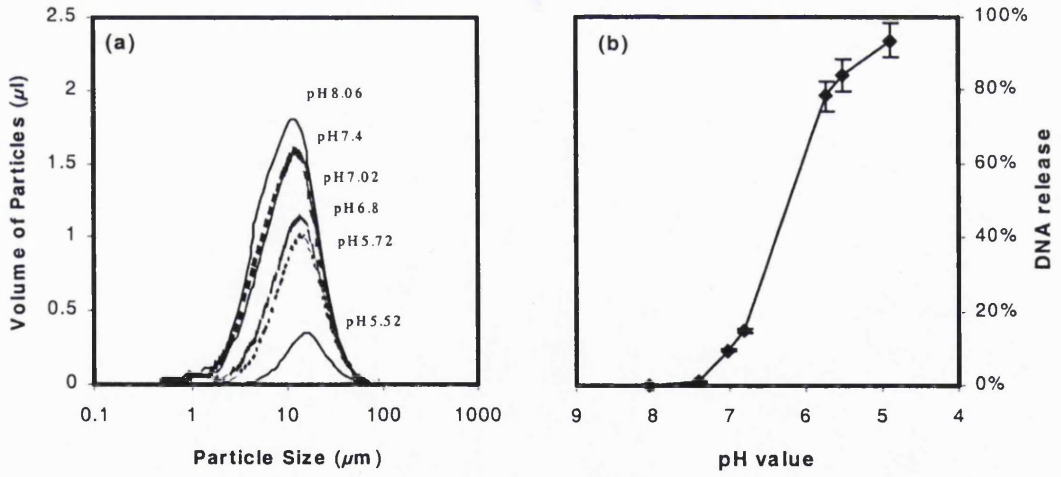

Figure 7.3: Release of pRc/CMV-HBS DNA from CaPi-DNA by acidification. 50 μg of plasmid pRc/CMV-HBS DNA were precipitated with 2.5 μmole of calcium phosphate and the complexes were resuspended in the 0.015M PBS solutions of varying pH. The particle size distribution (panel (a)) was measured by a Mastersizer and the release of DNA (panel (b)) was evaluated by the ^{35}S radioactivity in the supernatant (Data shown are mean ± SD of three experiments).

7.3 CHARACTERISATION OF DRVs ENTRAPPING CaPi-DNA COMPLEXES

The CaPi can interact with phospholipid *in vivo* (Schewe *et al.*, 1992), so the CaPi-DNA complexes were able to be entrapped into the liposomes by the dehydration-rehydration method. Liposomes may prevent the aggregation of these complexes by coating them with lipid bilayers. This methodology is mediated by CaPi to entrap DNA into liposomes, so these liposomes may be termed as "capisomes". Capisomes are different from the traditional cationic liposomes and the calcium-containing liposomes, which entrap the DNA into the liposomes without condensed precursor of DNA. There are two advantages in preparing capisomes for DNA delivery. Firstly, it will combine the high efficiency form liposome and high colony stability from CaPi-mediated gene transfection. Secondly, it will reduce the aggregation of CaPi-DNA complexes to increase the possibility of this system to be applied *in vivo*. The characteristics of capisomes were studied in terms of their ability to entrap DNA, vesicle size distribution and the stability of DNA entrapment. The results are illustrated in the following section.

7.3.1 Entrapment of CaPi-DNA complexes into DRVs

In order to confirm that the DNA complexes are entrapped into the DRVs, ethidium bromide labelled pRc/CMV-HBS DNA and 6-carboxyfluorescein were co-entrapped into the liposomes. Fluorescence microscopy photographs were taken before and after the pellet was washed with the 0.1N of hydrochloric acid solution. These pictures showed that the adsorbed CaPi-DNA complexes were removed after treatment by 0.1N of hydrochloric acid, and the entrapped complexes remained within the DRVs (Figure 7.4).

By comparison with DNA entrapment into cationic liposomes, entrapment of CaPi-DNA complexes into DRVs provided a more accurate approach to entrap DNA molecules into DRVs and could control the surface charges of capisomes by using appropriate lipid compositions. However, the entrapment efficiency of DNA into capisomes was lower than in cationic liposomes. The cationic lipids interacted with DNA molecules to form the cationic lipid-DNA complexes with spiral structure in liposomes (Thierry, et al., 1997). However, it is difficult to remove adsorbed DNA from the surface of cationic liposomes. Consequently, capisomes showed the advantage to entrap DNA into liposomes without cationic lipids.

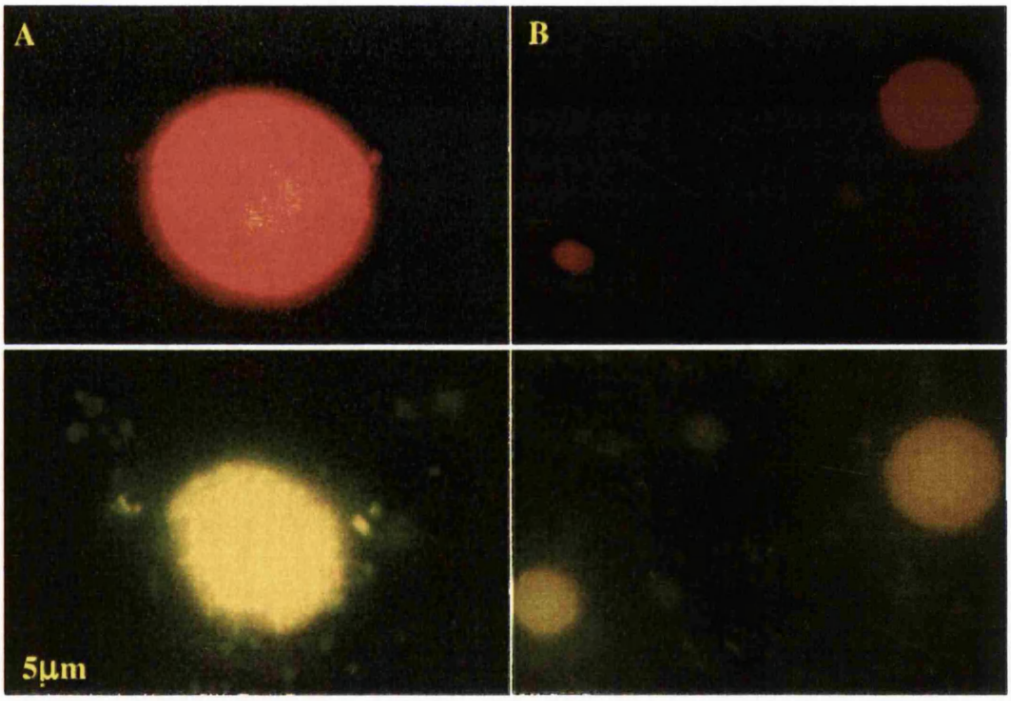

Figure 7.4: Photographs of DNA entrapment into the capisomes under fluorescence microscopy. DNA was labelled with ethidium bromide. CaPi-DNA complexes and 6-carboxyfluorscein were co-entrapped into PC/Chol DRVs. The samples were examined under the fluorescence microscopy with two different filters to show the ethidium bromide labelled DNA (red, up) and the carboxyfluorscein (yellow, bottom). The sample (A) is the capisome before treated with 0.1 N of hydrochloric acid, but (B) is the sample after washed with 0.1 N of hydrochloric acid.

The photographs of capisomes under the fluorescence microscopy showed that DNA can be entrapped into the DRVs by this method. The treatment with 0.1N of hydrochloric acid dissolved the CaPi-DNA complexes adsorbed on the surface of the liposomes. The photographs were taken at the same condition with different fluorescence microscopical filters. The photos showed that liposomal region (yellow color) was smaller than area of CaPi-DNA complexes (red color) before treated with acid. However, it was larger than area of DNA complexes after the capisomes washed by acid solution. This indicated that the adsorbed CaPi-DNA complexes on the liposomal surface were removed by the acid treatment. This result had confirmed with the data of particle size distribution. Therefore, calcium phosphate can mediate DNA entrapment into the DRVs and the non-entrapped complexes can be removed by the treatment with acid.

7.3.2 Entrapment efficiency of DNA into DRVs

Entrapment efficiency of DNA into the capisomes depended on the calcium phosphate-DNA complexes, lipid compositions, vesicle sizes and co-entrapped substances. Firstly, the entrapment efficiency of DNA into capisomes composed of various lipid compositions was carried out. Then, capisomes were prepared in the presence of sucrose to reduce the vesicle size. Furthermore, HBsAg was co-entrapped into DRVs to produce the combined vaccine against Hepatitis B. The entrapment efficiency of DNA into DRVs prepared by these methods was measured and compared with each other.

7.3.2.1 Effect of lipid composition

The complexes were entrapped into the liposomes composed of various lipid

compositions to prepare capisomes with negative, neutral or positive charges. In addition, the liposomes composed of the same compositions were applied to entrap the pRc/CMV-HBS DNA and the entrapment efficiency of DNA into liposomes were compared to those in capisomes. In PC/Chol DRVs, only 38% of naked DNA was entrapped, but CaPi-DNA complexes increased the entrapment efficiency of DNA into these liposomes up to 82% (Figure 7.5). Furthermore, entrapment of DNA through CaPi-DNA complexes increased the entrapment efficiency of DNA into PC/DOPE DRVs from 51% to 89%. However, the method by CaPi-DNA to entrap DNA into liposomes could not increase the entrapment efficiency of DNA into cationic PC/DOPE/DC-Chol DRVs. The data suggested that electrostatic interactions between DNA and cationic substances (calcium and DC-Chol) were a crucial factor for DNA entrapment into liposomes.

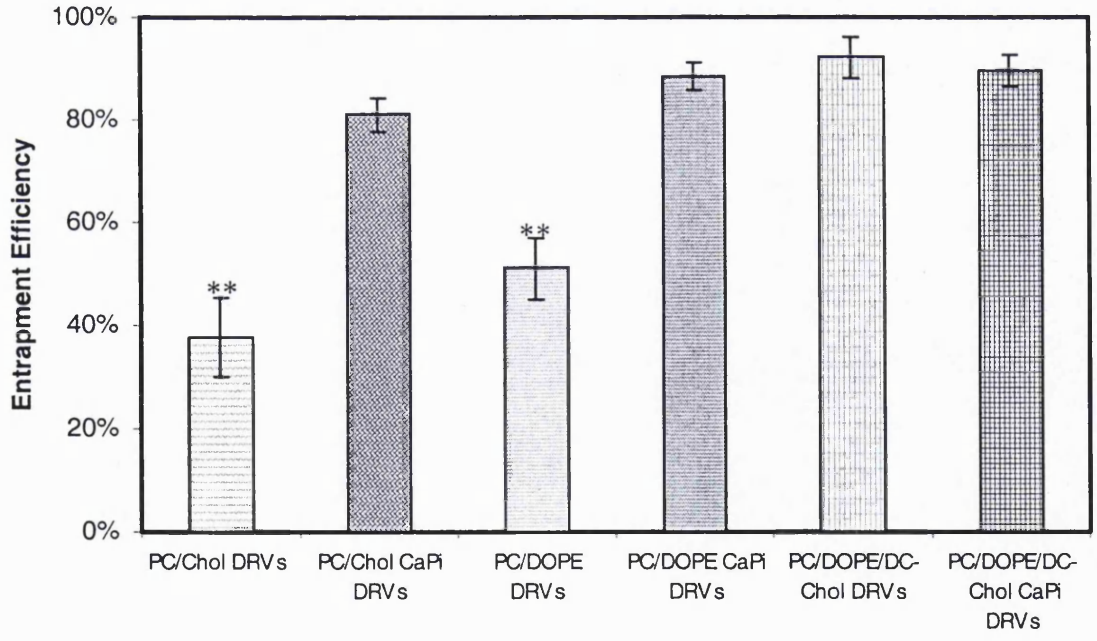

Figure 7.5: Entrapment efficiency of pRc/CMV-HBS DNA in DRVs composed of various compositions. 100 μg of pRc/CMV-HBS DNA or its CaPi complexes were entrapped into DRVs and the entrapment efficiencies were evaluated by radioactivity of ^{35}S-labelled DNA tracers. Data shown are mean ± SD (n=5). **p<0.005 compared to capisomes (Student's unpaired *t*-test).

7.3.2.2 Effect of the presence of sucrose during DRV preparation

The DRVs prepared in the presence of sucrose had been used to reduce the vesicle size in the previous chapters and induced stronger antibody responses in some cases. Hence, capisomes were prepared in the presence of sucrose. The entrapment efficiency of pRc/CMV-HBS DNA into capisomes was evaluated with ^{35}S-labelled DNA tracers. There is no significant alteration in DNA entrapment into capisomes prepared in the presence of sucrose (Figure 7.6).

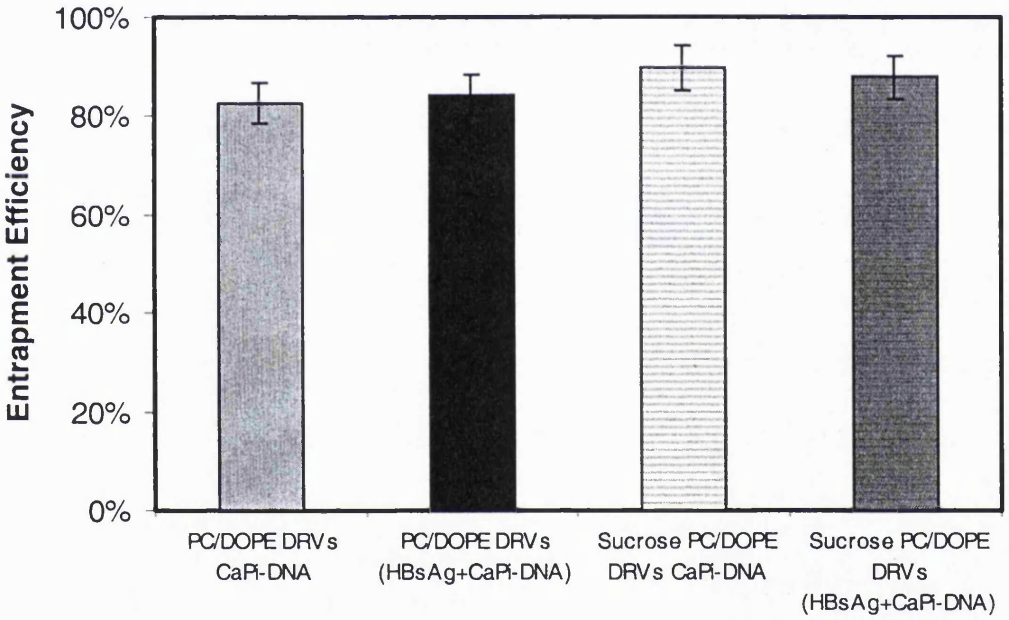

Figure 7.6: Entrapment efficiency of pRc/CMV-HBS DNA into DRVs prepared in the presence of sucrose or HBsAg. 100 μg of pRc/CMV-HBS DNA was precipitated with calcium phosphate and co-entrapped with 0.5 mMole of sucrose or 2 μg of HBsAg into the DRVs, and the entrapment efficiencies were evaluated by radioactivity of ^{35}S-labelled DNA tracers (Data shown are mean ± SD, n=5).

7.3.2.3 Effect of the presence of HBsAg during DRV preparation

In order to combine the protein and DNA for single-dose immunisation, HBsAg was co-entrapped with the calcium phosphate-pRc/CMV-HBS DNA complexes into the DRVs. In

addition, these formulations were prepared in the presence or absence of sucrose for controlling the final size. The capisomes entrapping HBsAg did not change the entrapment efficiency of DNA into these formulations significantly. The entrapment efficiency of DNA into capisomes was as same as those prepared in the presence of sucrose (Figure 7.6). These results suggested that the entrapped HBsAg in capisomes did not alter DNA entrapment into these vesicles. The entrapment of pRc/CMV-HBS DNA into capisomes prepared in the presence of HBsAg made the protein and DNA combined vaccine be possible.

7.3.3 Vesicle size distribution of DRVs entrapping CaPi-DNA complexes

The vesicle characteristics of the DNA-containing DRVs were carried out with the vesicle size distribution and the zeta potential as previously. Firstly, the liposomes composed of various lipid compositions had different surface charge and vesicle size. Then, capisomes prepared in the presence of sucrose had smaller size. Their characteristics were checked over in terms of vesicle size and zeta potential. Finally, the properties of the capisomes co-entrapping HBsAg were characterized to investigate the effect of the presence of HBsAg on the size of capisomes.

7.3.3.1 Effect of lipid composition

The lipid composition of vesicles affected the size distribution of DNA-containing DRVs. The vesicle size of DNA-containing DRVs was reduced by entrapping CaPi-DNA complexes into DRVs (Table 7.1). The zeta potentials of PC/Chol DRVs entrapping DNA were -27mV. However, the zeta potentials of PC/DOPE DRVs entrapping DNA were -4 mV. Entrapping DNA into liposomes with CaPi-DNA complexes, the zeta potential of these particles changed from -27

to -38mV for PC/Chol DRVs, from -4 to -8mV for PC/DOPE DRVs and from 36 to 33 mV for PC/DOPE/DC-Chol DRVs (Table 7.1). Generally, the neutral surface charge made the vesicle aggregate more easily. The volume mean size of the PC/DOPE DRVs entrapping DNA can increase up to 25 μm. The reduction in the electrostatic repulsion among these vesicles made these vesicles gather in a mass. The vesicle sizes of DNA-containing DRVs prepared from DNA condensation precursors were smaller than those DRVs entrapping DNA directly. Furthermore, the surface charge of DNA-containing vesicles depended on the lipid compositions of DRVs and related to the aggregation of these vesicles. Results indicated that the existence of cationic substances (DC-Chol, Calcium) in DNA-containing DRVs can make their size be smaller. The zeta potential of PC/Chol DRVs entrapping CaPi-DNA complexes was more negative than that of PC/Chol DRVs entrapping DNA.

Table 7.1: Characteristics of DNA-containing DRVs.

Liposomal Compositions	DNA Form	Volume Mean Diameter (μm)	Zeta Potential (mV)
PC/Chol (16:16 μmole)	100 μg DNA	5.57 ± 2.31	-26.8 ±1.6
PC/Chol (16:16 μmole)	CaPi-100 μg DNA	3.50 ± 1.35	-37.9 ±0.2*
PC/DOPE (16:8 μmole)	100 μg DNA	24.91 ± 3.84	-4.3 ±0.5
PC/DOPE (16:8 μmole)	CaPi-100 μg DNA	8.15 ± 2.78*	-7.7 ±0.3
PC/DOPE/DC-Chol (16:8:4 μmole)	100 μg DNA	2.51 ± 1.24	35.6 ±0.7
PC/DOPE/DC-Chol (16:8:4 μmole)	CaPi-100 μg DNA	2.79 ± 1.31	33.4 ±1.2

Values shown are mean ± SD of three experiments. *Significantly different from the control groups: $U \leq 0$ (critical value) in Mann-Whitney U test.

7.3.3.2 Effect of the presence of sucrose in DRVs

Calcium phosphate-DNA complexes were entrapped into DRVs prepared in the presence of sucrose. The average vesicle size of these capisomes reduced down to 90 nm (Table 7.2). Compared to the mannosylated HBsAg entrapped in these DRVs (Table 5.2), the size of DRVs entrapping CaPi-DNA complexes prepared in the presence of sucrose is similar to those of DRVs encapsulating mannosylated HBsAg and sucrose. Consequently, CaPi did not affect the lipid bilayer aggregation and/or fusion in the formation of DRVs prepared in the presence of sucrose. These data suggested the vesicle sizes were reduced in capisomes prepared in the presence of sucrose. It indicated that the mechanism of size reduction in DRVs prepared in the presence of sucrose would not be affected by co-entrapping with CaPi-DNA complexes.

7.3.3.3 Effect of the presence of HBsAg in DRVs

Liposome technology can be applied to co-entrap various antigens to produce the combined vaccines (Khemka *et al.*, 1998). Combining protein and DNA vaccines may have the advantages to develop a single-dose vaccine. The HBsAg was co-entrapped with the CaPi-DNA complexes into the liposomes by the dehydration-rehydration method. When the CaPi-DNA complexes was entrapped into PC/DOPE DRVs prepared in the presence of HBsAg, the vesicle size of capisomes was enlarged. In addition, the surface charge of these vesicles became more negative (Table 7.2). These results showed that the HBsAg changes the characteristics of lipid bilayer in liposomes. This alternation made the size of DRVs be larger even the DRVs prepared in the presence of sucrose.

Table 7.2: Characteristics of DNA-containing capisomes prepared in the presence of sucrose or HBsAg.

Liposomal Compositions	Entrapment of Sucrose	Entrapment of HBsAg	Volume Mean or Z-Average Diameter (μm)	Zeta Potential (mV)
PC/DOPE (18:9 μmole) with CaPi-100 μg DNA	0	0	8.15 ± 2.78	-7.7 ± 0.3
PC/DOPE (18:9 μmole) with CaPi-100 μg DNA	0	1μg /1.3μg	9.90 ± 3.05	-15.4 ± 0.4
PC/DOPE (18:9 μmole) with CaPi-100 μg DNA	0.02125/ 0.5 mMole	0	0.09 ± 0.01	-2.1 ± 0.5
PC/DOPE (18:9 μmole) with CaPi-100 μg DNA	0.02125/ 0.5 mMole	1μg /1.4μg	0.18 ± 0.04	-3.6 ± 0.4

Data shown are mean ± SD (n=3).

7.3.4 Morphology of DNA-containing DRVs

The DNA complexes containing DRVs were examined under transmission electron microscopy (Figure 7.7). Photos of capisomes exhibited different morphology from that of cationic liposomes. Cationic liposomes entrapped DNA showed the lamellar structure in these vesicles (Figure 7.7 D). The capisomes did not have the lamellarity in their particles. Previous study examined calcium phosphate precipitation in aqueous suspensions of multilamellar liposomes as a possible *in vitro* model for matrix vesicle mineralization (Eanes *et al.*, 1984). In the absence of entrapped phosphate ions, calcium ions taken up by the liposomes were largely bound to inner membrane surfaces. With the presence of phosphate ions, calcium ion uptake increased as much as sixfold with maximum accumulations well above values sufficient for solid formation. Precipitated solids appeared to be located predominantly in the aqueous intermembranous spaces of the liposomes. This precipitation made capisomes lose the lammellarity as shown in photos(Figure 7.7 A, B and C).

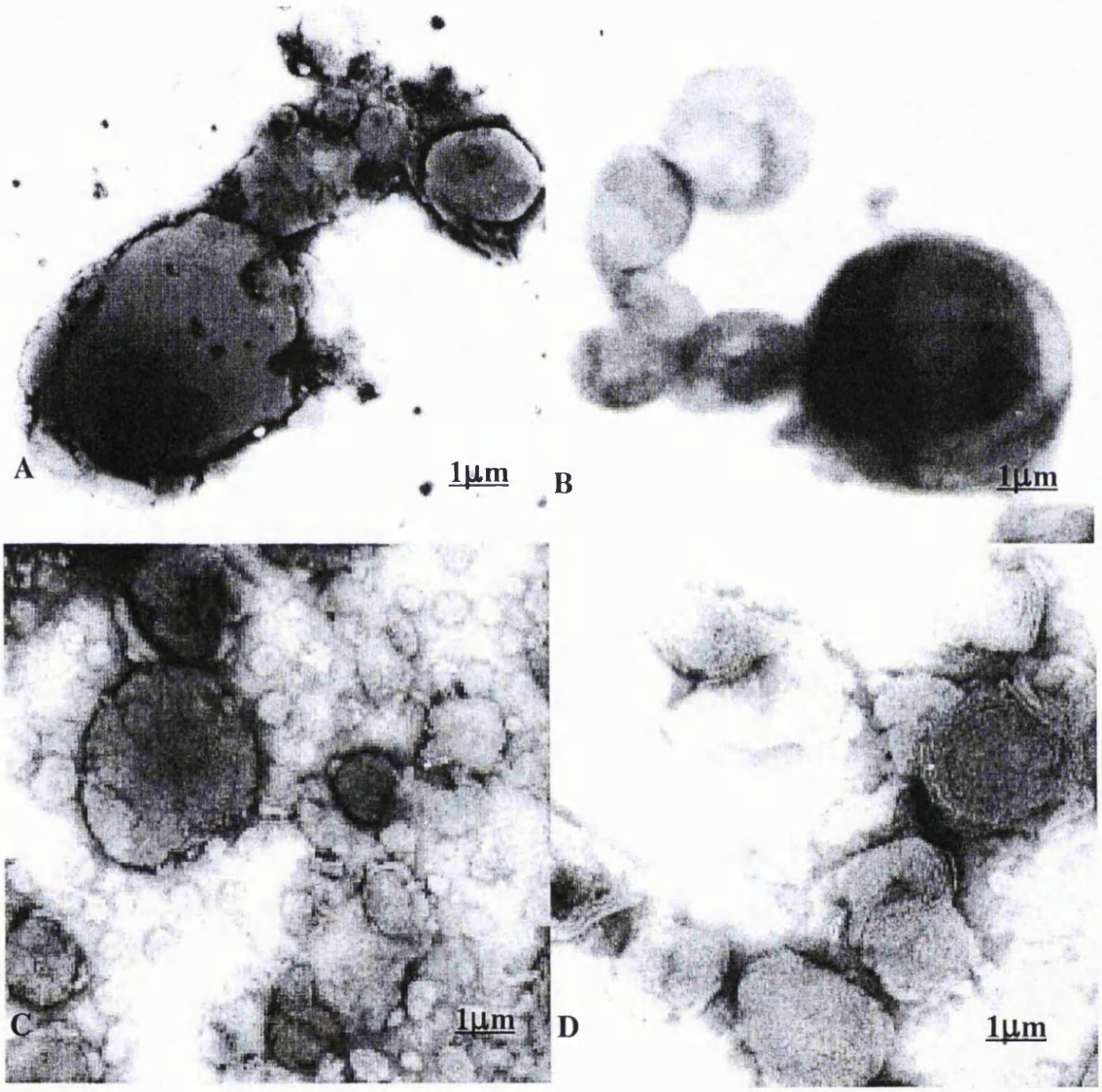

Figure 7.7: Photographs of DNA-containing DRVs under transmission electron microscopy. (A) calcium phosphate(CaPi)-DNA complexes in PC/Chol DRVs, (B) CaPi-DNA complexes in PC/DOPE DRVs, (C) CaPi-DNA complexes in PC/DOPE/DC-Chol DRVs, and (D) DNA in PC/DOPE/DC-Chol DRVs. DRVs were examined in a Philips 201 transmission electron microscope, and the photos were taken at 100kV.

7.3.5 Stability of DNA-containing DRVs

7.3.5.1 Retention of DNA in DRVs on storage at 4°C

To evaluate the stability of DNA in the DRVs, the retention of DNA in cationic liposomes and capisomes was monitored at different time courses. Firstly, the retention of DNA in the DRVs composed of various compositions was quantified and the vesicle size, zeta potential of these DRVs measured at the same time. Then, the DNA-containing PC/DOPE capisomes prepared in the presence of sucrose or HBsAg were also monitored to study the effect of sucrose and HBsAg on the stability of these capisomes.

The DNA retention in the calcium phosphate-DNA complexes and DRVs entrapping DNA in PBS showed the liposomal formulations retained more DNA than the complexes and that the vesicles did not aggregate to larger size (Table 7.3). Monitoring at different time courses through 8 weeks showed that liposomal formulations retained more DNA in the formulations by comparing with the CaPi-DNA complexes. The CaPi-DNA complexes retained 44% DNA in the particles, whereas capisomes retained 47% to 59% DNA in the formulations and PC/DOPE/DC-Chol DRVs kept 73% DNA after storage at 4°C for 8 weeks. Furthermore, DNA formulations with liposomal entrapment made the formulation be stable on storage and prevented the particle size growth by aggregation. The particle size of CaPi-DNA complexes reached up to 60μm after the storage under 4°C for 8 weeks. However, this aggregration did not occur in the liposome-entrapped DNA complexes. The surface charge of CaPi-DNA complexes is near neutral. Little electrical repulsion between particle and particle made them be easy to aggregate.

Table 7.3: Stability of DNA complexes and their DRVs on storage at 4°C.

Formulation	Storage Time	DNA Retention (%)	Volume Mean Diameter (μm)	Zeta potential (mV)
CaPi-DNA complexes	1 week	76.13 ± 2.59	7.32 ± 0.34	-2.4 ± 0.7
	2 weeks	55.48 ± 9.06	10.07 ± 1.96	-2.2 ± 0.8
	4 weeks	49.31 ± 8.52	15.32 ± 1.52	-2.5 ± 0.6
	6 weeks	45.43 ± 3.07	18.68 ± 0.79	-2.9 ± 0.7
	8 weeks	43.79 ± 6.11	60.42 ± 1.34	-14.1 ± 1.6
PC/Chol DRVs entrapped CaPi-DNA complexes	1 week	86.35 ± 4.60	5.71 ± 0.91	-37.9 ± 0.2
	2 weeks	75.23 ± 3.13	6.28 ± 1.10	-41.0 ± 0.8
	4 weeks	68.90 ± 1.13	6.68 ± 0.95	-44.0 ± 1.7
	6 weeks	60.77 ± 6.38	6.67 ± 1.44	-42.6 ± 2.4
	8 weeks	58.93 ± 3.75	6.52 ± 1.61	-41.3 ± 1.4
PC/DOPE DRVs entrapped CaPi-DNA complexes	1 week	84.62 ± 3.01	5.23 ± 0.66	-7.7 ± 0.3
	2 weeks	77.11 ± 2.12	5.67 ± 1.56	-7.2 ± 0.4
	4 weeks	69.34 ± 3.29	5.54 ± 1.66	-6.3 ± 0.5
	6 weeks	50.77± 8.38	5.67 ± 1.44	-6.2 ± 1.4
	8 weeks	47.45 ± 8.70	5.23 ± 1.34	-6.6 ± 0.5
PC/DOPE/DC-Chol DRVs entrapped CaPi-DNA Complexes	1 week	87.40 ± 2.52	2.78 ± 1.31	33.4 ± 1.2
	2 weeks	64.92 ± 4.01	2.76 ± 1.29	30.8 ± 1.1
	4 weeks	52.20 ± 7.06	2.74± 1.63	26.9 ± 0.2
	6 weeks	50.14 ± 6.32	2.42 ± 0.86	25.4 ± 0.8
	8 weeks	48.03 ± 5.77	2.18 ± 0.51	24.3 ± 1.6
PC/DOPE/DC-Chol DRVs entrapped DNA	1 week	91.53 ± 0.28	2.51 ± 1.42	34.9 ± 0.7
	2 weeks	88.76 ± 1.28	2.59 ± 0.75	24.7 ± 0.8
	4 weeks	84.75 ± 0.90	2.62 ± 0.84	12.7± 0.6
	6 weeks	80.86 ± 1.64	2.64 ± 0.98	10.2 ± 0.7
	8 weeks	72.64 ± 3.58	2.73 ± 1.34	8.1 ± 1.6

Data shown are mean ± SD of three experiments.

When capisomes entrapping DNA prepared in the presence of sucrose or HBsAg, the retention of DNA molecules in capisomes on storage was modified (Table 7.4). The capisomes prepared in the presence of HBsAg did not change the DNA retention in these formulations on storage. However, capisomes prepared in the presence of sucrose retained less DNA after storing at 4 °C for one week. The leakage of entrapped substances from DRVs prepared in the presence of sucrose had happened on HBsAg-containing DRVs. The substances leaking from DRVs prepared in the presence of sucrose could be explained by the modification of lipid bilayers in DRVs entrapping sucrose. Despite the presence of the sucrose, repacking of the bilayer components takes place both during and after rehydration (Zhang *et al.*, 1997). Thus, DRVs prepared in the presence of sucrose were unstable to retain the entrapped substances in the dispersion. As previous study in DRVs entrapping sucrose, the freeze-dried powder of precursor SUVs and sucrose was recommended to apply in the practical application. Furthermore, the release of DNA from these formulations led the surface charge of these vesicles prepared in the presence of sucrose to be more negative in DRVs. These phenomena could be explained by the exposure of DNA molecules on the surface of the vesicles. However, the capisomes prepared in the absence of sucrose did not change their zeta potentials when DNA leaked out from them. Therefore, DNA leaked out from capisomes prepared in the presence of sucrose may interact with these capisomes and adsorb on their surface to increase the negative charge. Results indicate that capisomes prepared in the presence of HBsAg did not alter the DNA retention in these formulations. However, the retention of entrapped DNA in capisomes prepared in the presence of sucrose were as same as HBsAg in sucrose co-entrapped HBsAg-containing DRVs. They did not retain the entrapped substances, such as protein and DNA, in the formulations upon storage.

Table 7.4: Stability of DNA-containing capisomes prepared in the presence of sucrose or HBsAg on storage at 4°C.

Formulation	Storage Time	DNA Retention (%)	Volume Mean or Z-Average Diameter (μm)	Zeta potential (mV)
PC/DOPE DRV entrapping CaPi-DNA complexes	1 week	84.62 ± 3.01	8.15± 2.78	-7.7 ± 0.3
	2 weeks	77.11 ± 2.12	5.67 ± 1.56	-7.2 ± 0.4
	4 weeks	69.34 ± 3.29	5.54 ± 1.66	-6.3 ± 0.5
	6 weeks	50.77± 8.38	5.67 ± 1.44	-6.2 ± 1.4
	8 weeks	47.45 ± 8.70	5.23 ± 1.34	-6.6 ± 0.5
PC/DOPE DRV entrapping CaPi-DNA complexes and HBsAg	1 week	87.92 ± 4.52	9.89 ± 0.35	-16.0 ± 1.0
	2 weeks	76.13 ± 0.62	9.28 ± 0.91	-17.6 ± 1.5
	4 weeks	70.23 ± 4.18	9.74 ± 0.80	-19.2 ± 0.6
	6 weeks	67.29 ± 1.94	9.77 ± 0.82	–17.6 ± 2.1
	8 weeks	58.15 ± 5.29	9.97 ± 0.96	-18.2 ± 1.5
PC/DOPE DRV entrapping CaPi-DNA complexes prepared in the presence of sucrose	1 week	50.79 ± 6.38	0.087 ± 0.004	-2.3 ± 0.6
	2 weeks	24.40 ± 4.66	0.099 ± 0.003	-5.2 ± 0.8
	4 weeks	16.59 ± 2.59	0.175 ± 0.002	-18.1 ± 6.1
	6 weeks	15.26 ± 4.68	0.171 ± 0.046	-21.7 ± 8.4
	8 weeks	12.40 ± 3.39	0.178 ± 0.052	-24.1 ± 3.6
PC/DOPE DRV entrapping CaPi-DNA complexes and HBsAg prepared in the presence of sucrose	1 week	49.90 ± 2.39	0.206± 0.036	-3.0 ± 3.9
	2 weeks	22.87 ± 5.88	0.283 ± 0.048	-5.9 ± 1.8
	4 weeks	20.27 ± 1.38	0.281 ± 0.058	-8.0 ± 6.9
	6 weeks	16.94 ± 3.08	0.370 ± 0.058	-14.9 ± 7.6
	8 weeks	11.91 ± 2.89	0.460 ± 0.046	-16.2 ± 3.5

(Data shown are mean ± SD of three experiments)

7.3.5.2 Release profiles of DNA from DNA-containing DRVs at 37°C

The release profiles of DNA from these formulations depended on the yield of liposomal entrapment, lipid composition and the existence of sucrose or HBsAg in formulation(Figure 7.8). Firstly, the released amount of DNA from cationic liposomes was more than that from CaPi-DNA complexes when they were incubated in the muscle tissue extracts. The ratio of DNA released from the calcium phosphate-DNA complexes in the muscle extract was around 10%, but its release faction from PC/DOPE/DC-Chol DRVs is 18% (Figure 7.8 (a)). Furthermore, liposomal entrapping CaPi-DNA complexes made the DNA be easy to release from the formulations in the presence of muscle extract. The ratio of DNA released from capisomes is 60% for PC/Chol DRVs, 30% for PC/DOPE DRVs and 20% for PC/DOPE/DC-Chol DRVs (Figure 7.8 (b)). Moreover, sucrose co-entrapment into capisomes increased the amount of DNA released form 30% to 90%. HBsAg co-entrapment into capisomes also increased the amount of DNA released from 30% to 80% in capisomes composed of PC/DOPE (Figure 7.8 (c) and (d)). Results indicated that the liposomes increased the release of DNA from CaPi formulations incubated in muscular extract. However, genetic immunization is based on the transfection *in vivo*. Thus, delivery of DNA into the targeted cells to express the plasmid is important to produce antigens *in vivo*. This is different from protein vaccination. In the immunisation with protein, the antigen presentation cells, such as macrophages, can move around and engulf the antigens *in vivo*. Consequently, the released protein can still be processed with these cells to induce the immune responses. However, the released DNA may result in different processing *in vivo*. The data of DNA release from these formulation incubated in muscular extract may be used to estimate the ratio of DNA processed in muscular tissue or local lymph node.

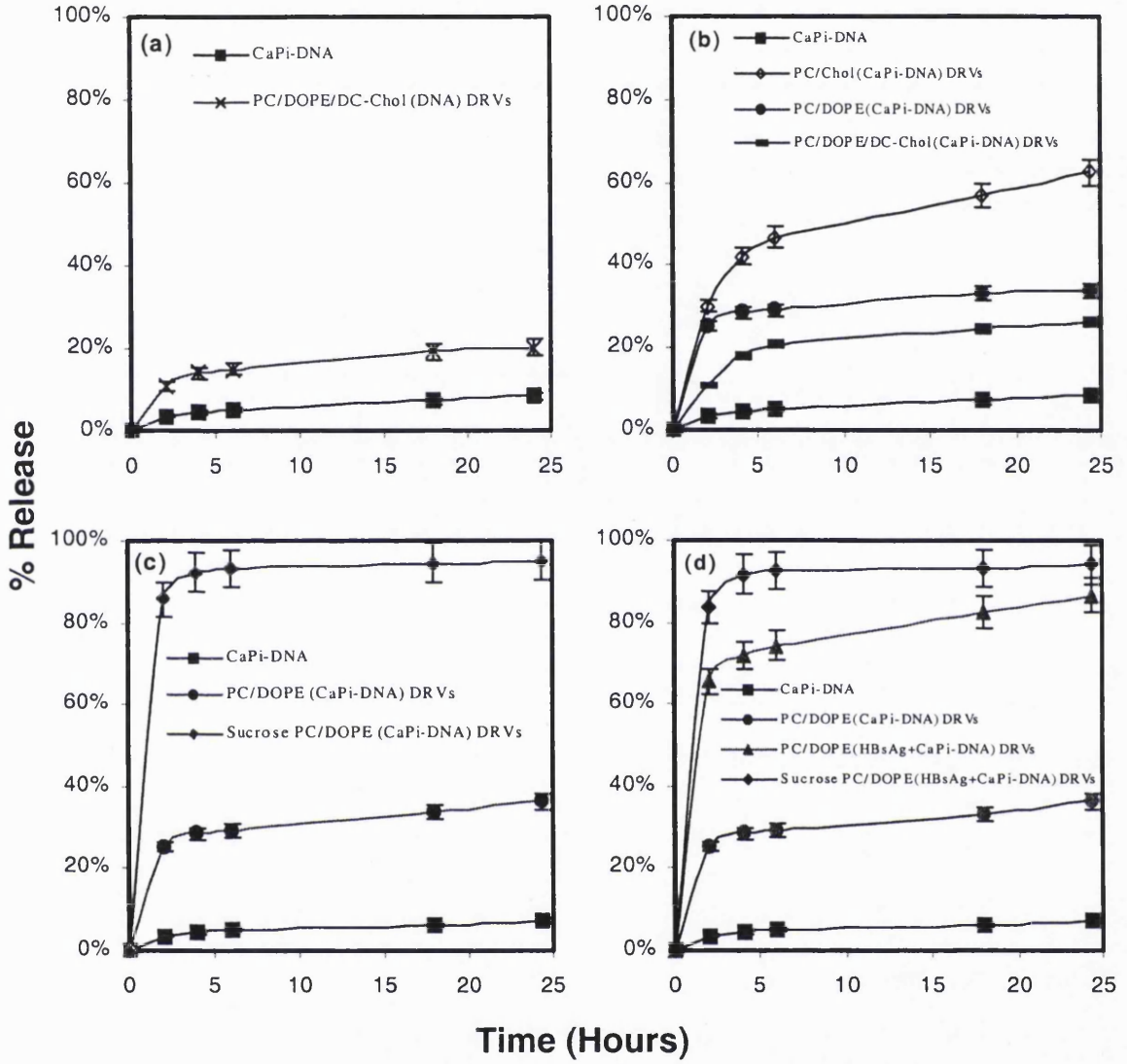

Figure 7.8: Release profiles of DNA from DRVs or complexes *in vitro*. (a) CaPi-DNA complexes and DNA-containing PC/DOPE/DC-Chol DRVs, (b) PC/Chol, PC/DOPE, or PC/DOPE/DC-Chol DRVs entrapping CaPi-DNA complexes, (c) PC/DOPE DRVs entrapping CaPi-DNA complexes prepared in the presence or absence of sucrose, (d) PC/DOPE DRVs entrapping CaPi-DNA complexes prepared in the presence or absence of HBsAg were resuspended in 10% muscle extract in PBS at 37°C. Aliquots were taken at different time intervals (2, 4, 6, 18 and 24 hours) and centrifuged on an the ultra-centrifuge to evaluate the release in the supernatant by measuring the ^{35}S radioactivity (Data shown are mean ± SD of three experiments).

7.4 IMMUNE RESPONSES OF DNA-CONTAINING DRVs

Liposomes entrapping plasmid DNA encoding HBsAg were intramuscularly injected into Balb/c mice using different schedules. The sera were taken at different time points up to 5 months to investigate the effect of liposomes on DNA-mediated vaccination. Firstly, 10μg of naked DNA and DNA entrapped in cationic liposomes composed of PC, DOPE and DC-Chol or capisomes were intramuscularly injected into mice weekly at 0, 7,14, 21, and 28 days. The antibody titres in mice treated with these formulations were monitored at 35, 42, 56, 84,112, and 140 days. Then, 10 or 100μg of naked DNA and DNA entrapped in capisomes prepared in the presence or absence of sucrose were intramuscularly injected into mice at 0 day. The antibody titres in the mice immunised with single-dose DNA vaccines were assayed at 28, 56, 84,112, and 140 days. Finally, capisomes entrapping HBsAg and DNA were intramuscularly injected into mice at 0 day. The antibody titres in mice vaccinated with these combined vaccines were monitored at 28, 56, 84,112, and 140 days. These results could be a step toward caposomes-formulated DNA vaccines against hepatitis B infection.

7.4.1 Immune responses after multiple-doses immunisation

The efficacy of intramuscular immunisation with plasmid encoding the HBsAg and its formulations was evaluated by antibody titres on serum with respect to the protocol of vaccination (Gregoriadis *et al.*,1997). Figure 7.9 and legend showed that the titres of total IgG responses against HBsAg were up to 10-fold greater than titres in mice immunized with naked DNA at 63, 112 and 140 days in mice injected weekly during a 28 day period with 10 μg of DNA entrapped in capisomes.

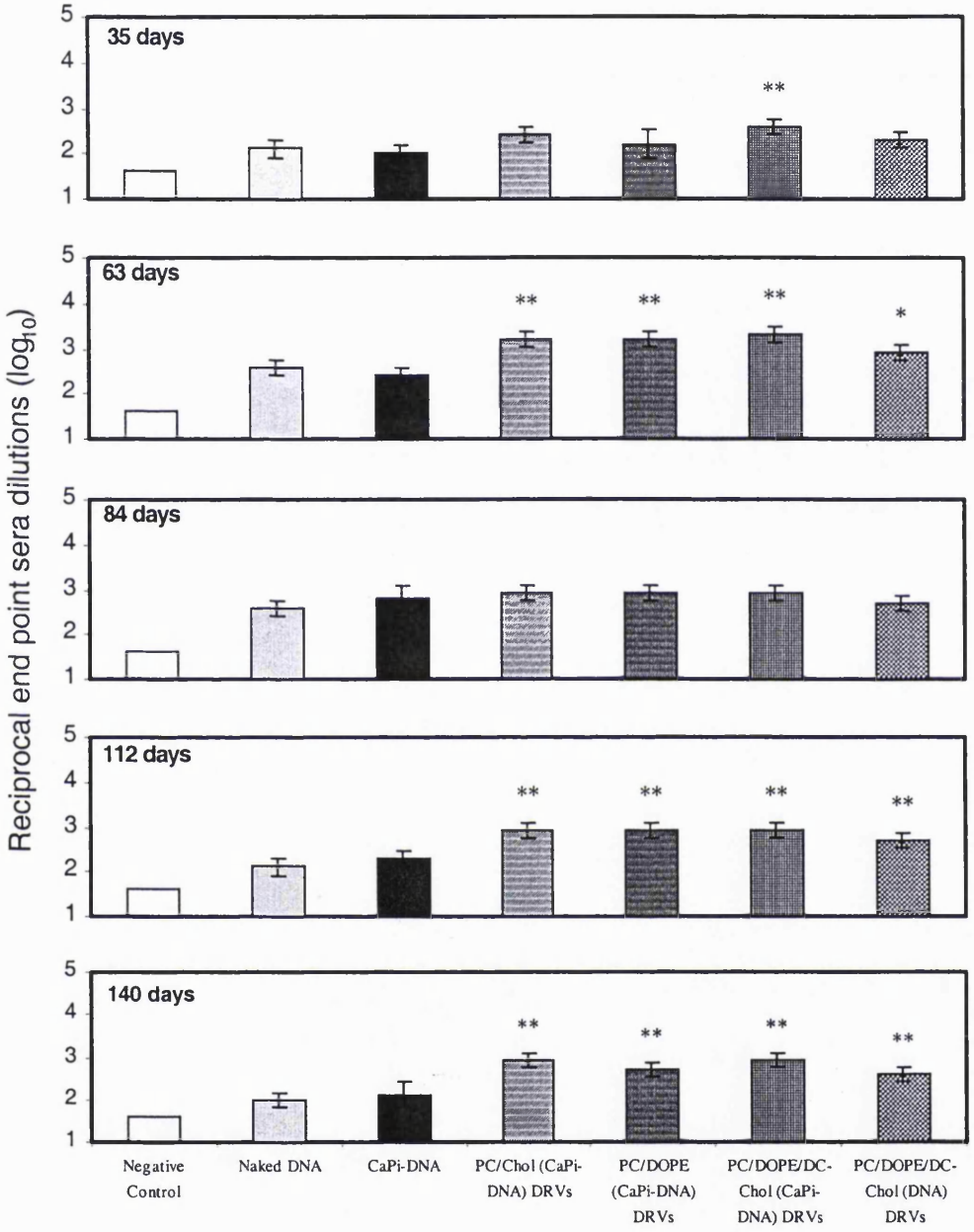

Figure 7.9: Comparison of total IgG responses in mice immunized with five doses of naked or liposome-entrapped pRc/CMV HBS DNA. Balb/c mice were injected intramuscularly on days 0, 7, 14, 21, and 28 with 10 μg of naked DNA or DNA entrapped in liposomes or capisomes. Animals were bled at 35, 63, 84, 112 and 140 days after the first injection and sera were tested by ELISA for IgG responses against the encoded HBsAg. The values in IgG responses are log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2. Bars are mean ± SD (n= 5).*$p<0.05$, **$p<0.005$ compared to the mice immunised with naked DNA (Student's unpaired *t*-test).

The total IgG responses induced by multiple DNA immunisation with these formulations at different time courses indicated that DNA entrapped in capisomes can produce similar IgG titres as cationic liposomes (DC-Chol) entrapping DNA (Figure 7.9). The means of log values of serum IgG titres in the mice immunised with liposomes entrapping DNA reached $\log_{10}$ values of 3.0 after 5 weeks of boosting. However, the means of $\log_{10}$ values of serum IgG titres in mice immunised by CaPi-DNA complexes or naked DNA were under 2.8 during 140 days. After immunisation with cationic liposomes entrapping CaPi-DNA complexes, the mice died six months later. The mice in this group exhibited hair loss and bleeding from the skin before they were killed according to Home Office regulations. In addition, there were no significantly different IgG responses between the mice treated by DNA-containing cationic liposomes and those vaccinated with capisomes. Capisomes prepared in the absence of cationic lipids may be another choice as DNA vaccine adjuvants. Moreover, in transfection experiments *in vitro*, the calcium phosphate can produce stable transfection cell colonies with calcium stimulating the gene expression in the process (Watanabe *et al.*, 1997 and Pine *et al.*, 1988). However, liposome-mediated gene transfection can form even more transfected colonies (Miller and Vile, 1995). By combining these two systems for gene transfection, capisomes may transfect cells *in vivo* to produce antigens for inducing the immune responses. Calcium phosphate can be entrapped into liposomes and applied in bone implants (Huang *et al.*, 1997), so the biocompatibility of capisomes would be better than that of other cationic substances for gene delivery. Consequently, capisomes are a promising system as a DNA vaccine adjuvant. It was, however, observed that multiple immunisation with capisomes by the intramuscular route induced fibrosis at the injection site. Therefore, to avoid this side effect, single-dose vaccines with high dose of DNA

in capisomes were evaluated.

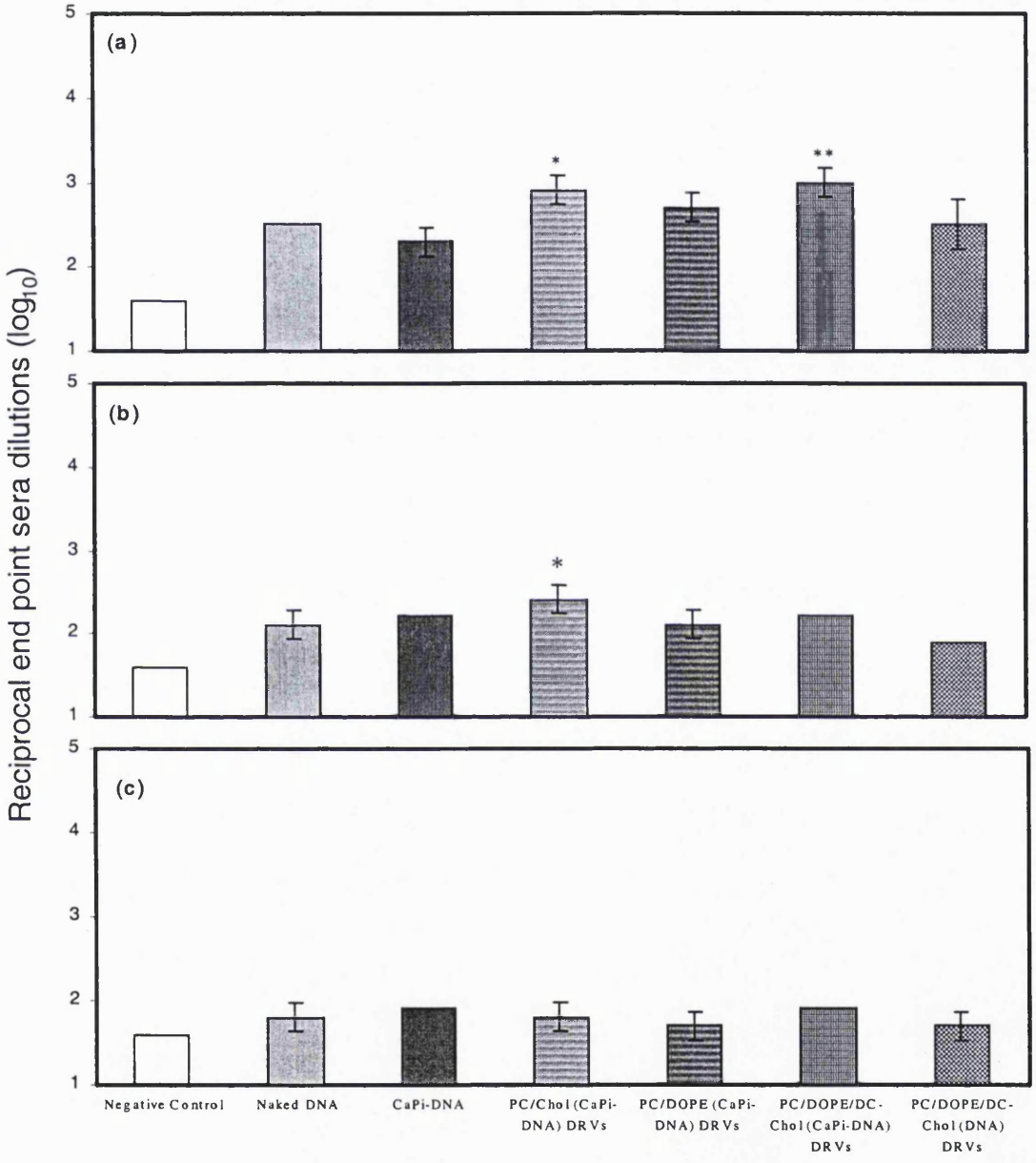

Figure 7.10: Subclass IgG responses in mice immunized with five doses of naked or liposome-entrapped pRc/CMV HBS DNA at 63 days after the first injection. Animals were treated as described in Figure 7.9. The sera tested by ELISA for (a) IgG_1, (b) IgG_{2a} or (c) IgG_{2b} responses against the encoded HBsAg. The values presented are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2 (n=5). Significantly different from the mice immunised with naked DNA: *$p<0.05$; **$p<0.005$ (Student's unpaired *t*-test).

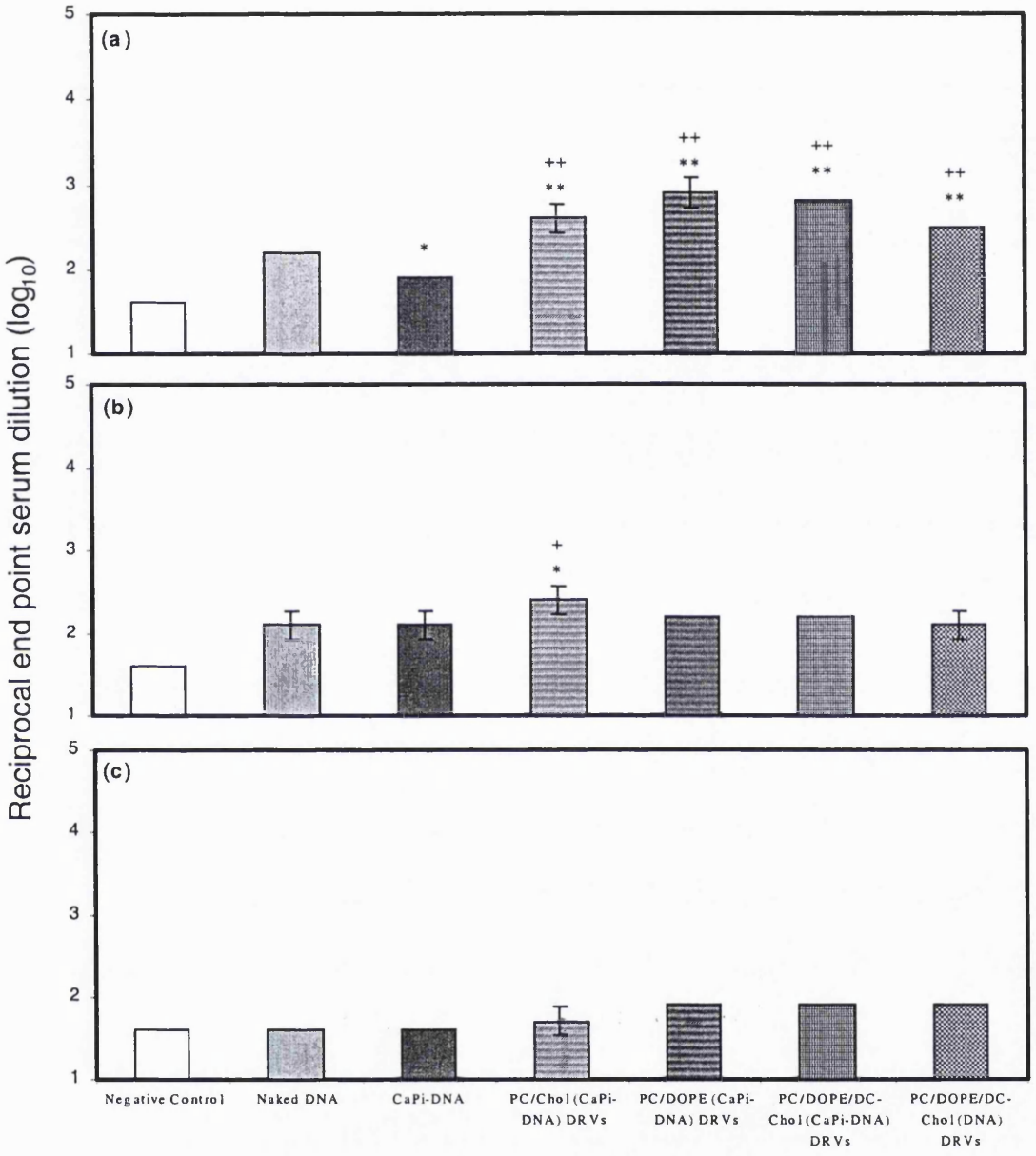

Figure 7.11: Subclass IgG responses in mice immunized with five doses of naked or liposome-entrapped pRc/CMV HBS DNA at 112 days after the first injection. Animals were treated as described in Figure 7.9. The sera tested by ELISA for (a) IgG_1, (b) IgG_{2a} or (c) IgG_{2b} responses against the encoded HBsAg. The values shown are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2 (n=5).*$p<0.05$, **$p<0.005$ compared to the mice immunised with naked DNA. Significantly different from the groups vaccinated with CaPi-DNA complexes: +$p<0.05$; ++$p<0.005$ (Student's unpaired *t*-test).

The subclass IgG responses in the mice immunised with multiple dose of DNA vaccines showed that the mice treated with liposome-entrapped DNA produced greater (over 10-fold) IgG_1 titres than those inoculated with naked DNA(10μg dose; 63 and 112days). IgG_{2a} titres for the liposomal DNA vaccination were also greater in the immunisation with PC/Chol capisomes entrapping DNA. However, IgG_{2b} titres for the liposoml DNA vaccination did not show any argument (Figure 7.10 and 7.11). The mice immunized with CaPi-DNA complexes exhibited different profiles in subclass IgG responses at various time points by comparison with those vaccinated with liposomes-entrapped DNA.

The mice treated with CaPi-DNA complexes were unable to enhance the subclass IgG titres at 63 days and even inhibited IgG_1 responses at 112 days after the first immunisation. The subclass IgG responses in the mice treated with CaPi-DNA complexes were almost as same as those induced by naked DNA vaccination. Generally, CaPi did not increase any subclass IgG titres in genetic immunisation in this study and might be unsuitable to develop as DNA vaccine adjuvants for hepatitis B genetic vaccination.

The mean of log values of serum IgG_1 titres in the mice treated with the liposomes entrapping CaPi-DNA complexes reached up to 3.0 (log_{10}). Only the mice treated with DNA entrapped in capisomes composed of PC/Chol DRVs induced higher IgG_{2a} titres than those in other groups. However, the mice inoculated with all these formulations did not enhance IgG_{2b} titres. By comparison with the mice immunized with naked DNA, the IgG_1 responses in the mice treated by PC/Chol or PC/DOPE/DC-Chol DRVs entrapping CaPi-DNA complex showed higher

titres at 63 days after the first immunisation. The IgG_{2a} titres in immunized mice were enhanced only in the groups treated with PC/Chol entrapping CaPi-DNA complexes. Furthermore, the antibody responses induced by cationic liposome entrapping CaPi-DNA complexes are similar to those by cationic liposome entrapping DNA. However, the antibody responses induced by capisomes with various lipid compositions did not show the significant difference between each other in IgG subclass titres. The results indicated that liposomes entrapping DNA can enhance the IgG_1 responses at different time courses and only PC/Chol DRVs entrapping CaPi-DNA complexes can induce higher IgG_{2a} response in immunized mice.

7.4.2 Immune responses after single-dose immunisation

Most reports on naked DNA vaccination have employed protocols of multiple injections, but a single-dose also produces a humoural response to the encoded antigen (Davis *et al.*, 1993a). In this study, 10 μg and 100 μg of naked or formulated DNA were injected intramuscularly into male Balb/c mice to induce the immune responses. The mean of $\log_{10}$ values (Figure 7.12) of serum IgG titres in the mice immunized with naked DNA or CaPi-DNA complexes (10 or 100 μg) were maintained as 2.2 during 140 days. However, liposome-entrapped DNA complexes (capisomes) enhanced in IgG titres between 56 and 112 days after immunisation. The mean of $\log_{10}$ values of serum IgG titres in mice immunised with capisomes prepared in the presence or absence of sucrose increased from 2.2 to 3.0 in the period of 56 and 112 days after immunisation.

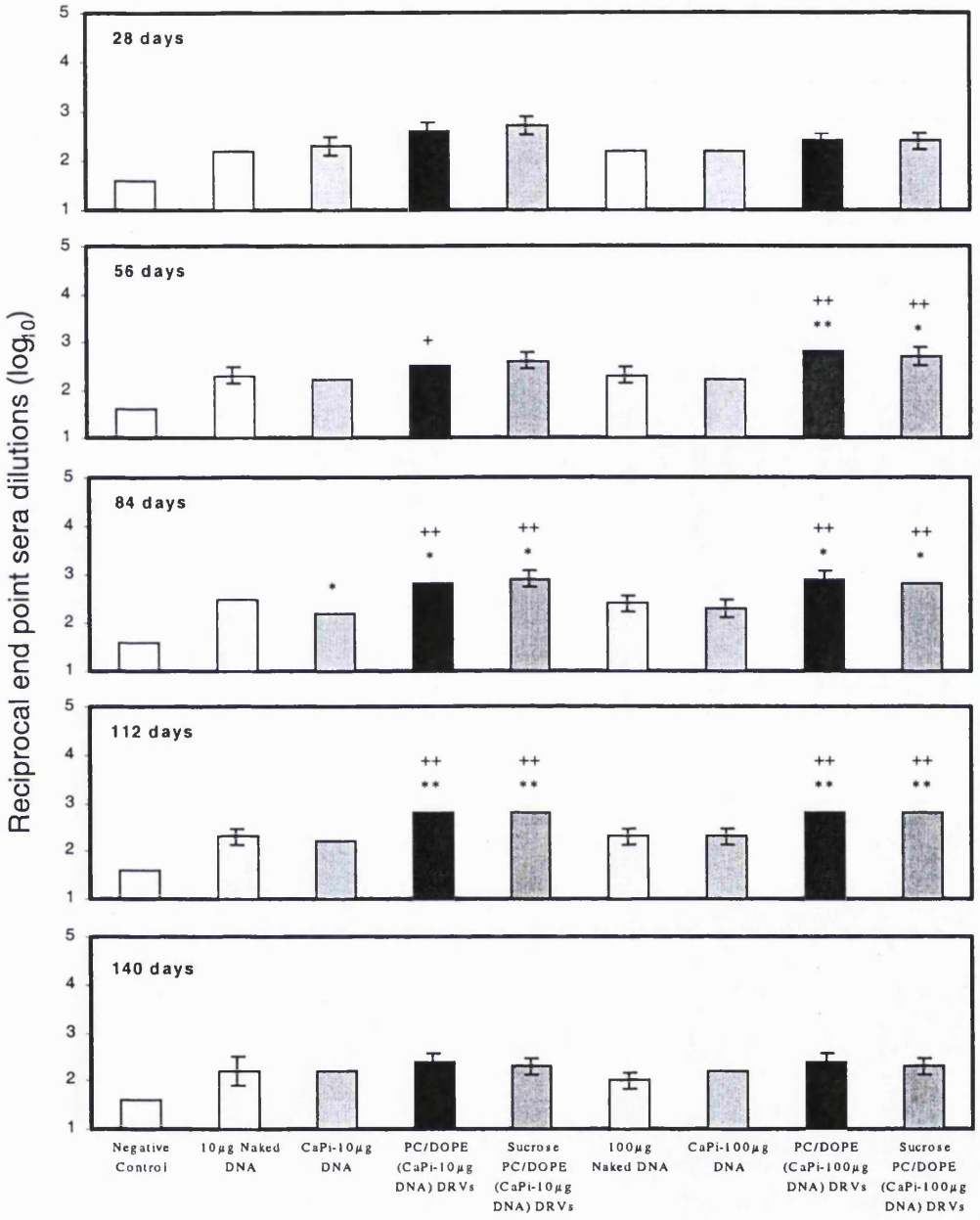

Figure 7.12: Total IgG responses in mice immunized with a single-dose of naked or liposome-entrapped pRc/CMV HBS DNA. Balb/c mice were injected intramuscularly on days 0 with 10 or 100 μg of naked DNA or DNA entrapped in liposomes. Animals were bled at 28, 56, 84, 116, and 140 days after the first injection and sera were tested by ELISA for IgG responses against the encoded HBsAg. The values in IgG responses are log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2. Each bar represents mean ± SD (n=5). *p<0.05, **p<0.005 compared to the mice immunised with naked DNA. Significantly different from the groups treated with CaPi-DNA: +p<0.05; ++p<0.005 (Student's unpaired *t*-test).

The subclass IgG responses in mice immunised with a single-dose of DNA showed that immunisation with liposome-entrapped CaPi-DNA complex can enhance serum IgG_1 titres from 2.2 to 3.0 at 56 days after injection (Figure 7.13). However, there was no significant enhancement in antibody responses at 112 days (Figure 7.14). In the IgG_{2a} responses, the mice treated with liposome-entrapped CaPi-DNA complexes were also higher (100μg DNA) at 56 days. However, the IgG_{2b} responses remained low in all groups at both 56 and 112 days (Figures 7.13 and 7.14 respectively).

Compared to the subclass IgG responses induced by the vaccination with multiple-doses (Figure 7.10 and 11), vaccination with multiple-doses of DNA could obtain higher titres at 112 days and still keep IgG_1 dominance. The subclass IgG responses at 56 and 112 days after a single-dose of DNA vaccination showed that the IgG_1 dominance in the mice treated with liposome-entrapped CaPi-DNA complexes just happen at 56 days. Thus, the boosting in DNA vaccination maintained IgG responses in immunised mice by maintaining the IgG_1 titres, but it had no significant effect on IgG_{2a} and IgG_{2b} subclass. However, there is no dose-dependent relationship between 10μg and 100μg of DNA vaccination. The mice immunised with liposome-entrapped CaPi-DNA complexes induced higher IgG_{2a} titres than those treated with CaPi-DNA complexes in the high dose (100μg) at 56 days. These subclass IgG responses in single-dose genetic immunisation showed that the mice treated with DNA-entrapping capisomes elicited stronger IgG_1 responses than those immunized with naked DNA vaccination.

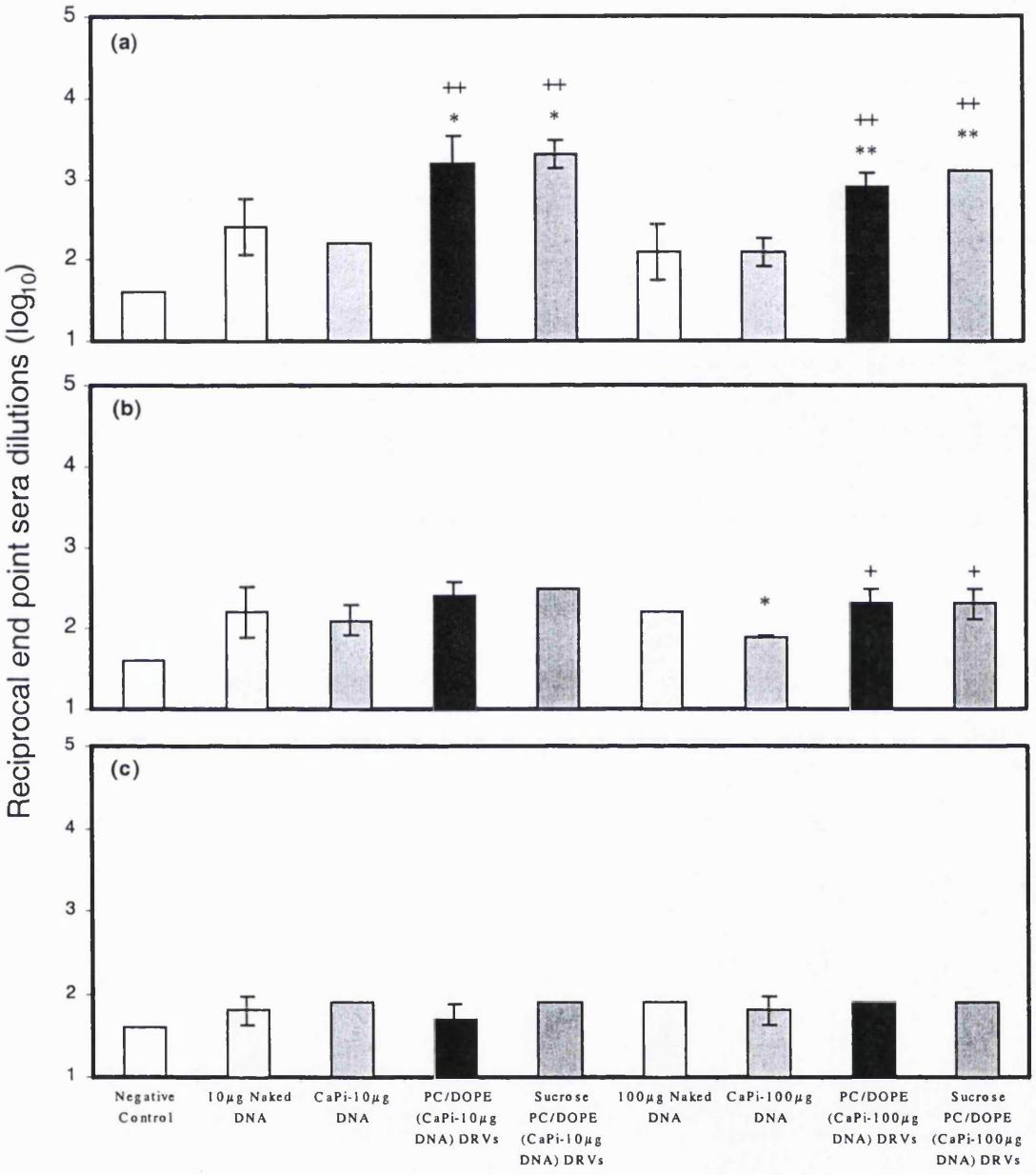

Figure 7.13: Subclass IgG responses in mice immunized with a single-dose of naked or liposome-entrapped pRc/CMV HBS DNA at 56 days after the injection. Animals were treated as described in Figure 7.12. The sera were tested by ELISA for (a)IgG_1, (b)IgG_{2a} or (c)IgG_{2b} responses against the encoded HBsAg. The values are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2 (n= 5). *p<0.05, **p<0.005 compared to the mice immunised with naked DNA. Significantly different from the groups treated with CaPi-DNA complexes:+p<0.05; ++p<0.005 (Student's unpaired *t*-test).

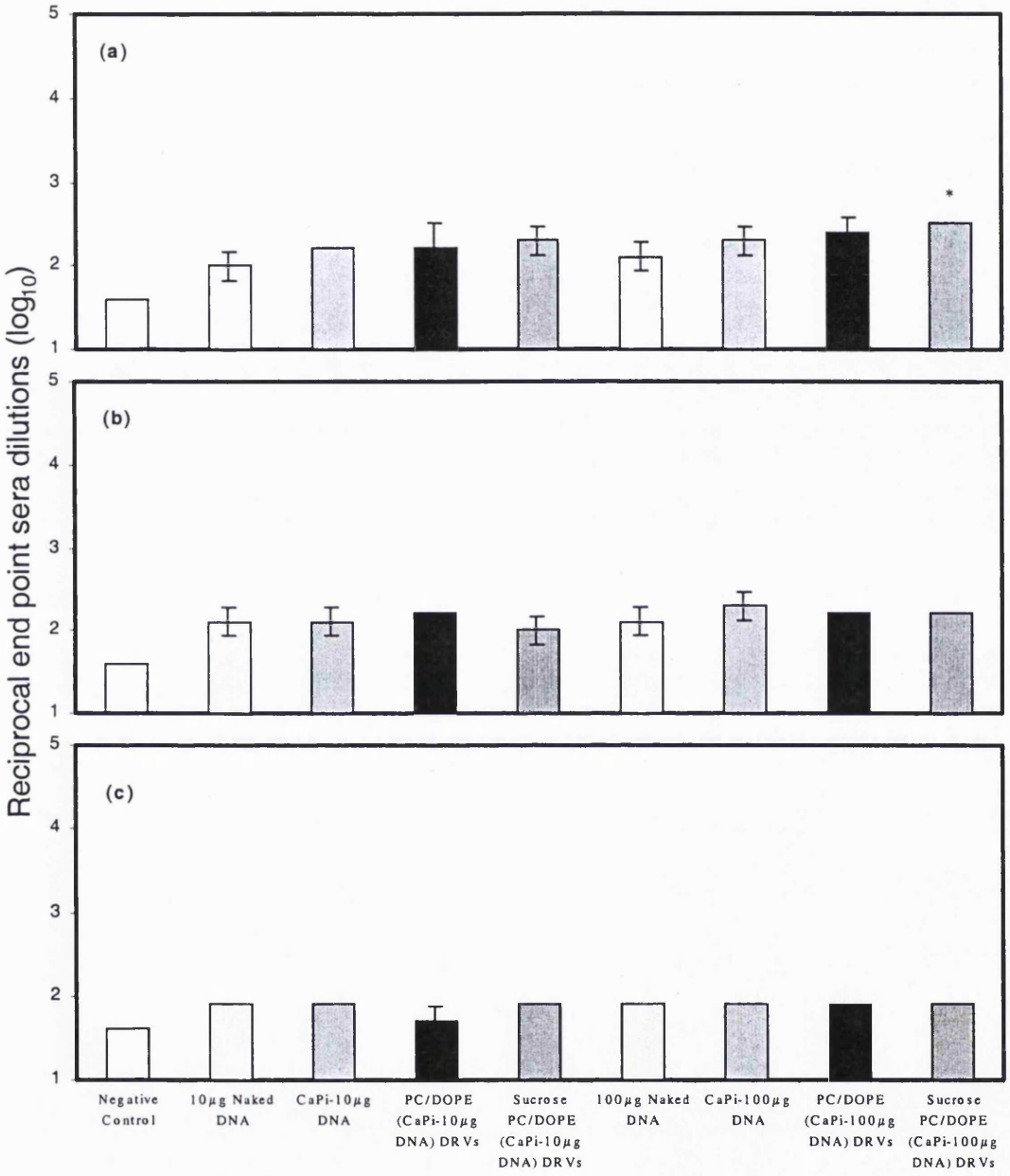

Figure 7.14: Subclass IgG responses in mice immunized with a single-dose of naked or liposome-entrapped pRc/CMV HBS DNA at 112days after the injection. Animals were treated as described in Figure 7.12. The sera were tested by ELISA for (a)IgG_1, (b)IgG_{2a} or (c)IgG_{2b} responses against the encoded HBsAg. The values shown are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2 (n= 5). Significantly different from the mice immunised with naked DNA: *$p<0.05$ (Student's unpaired *t*-test).

7.4.3 Immune responses to the combination of protein and DNA immunisation

Immunity in DNA vaccination follows DNA uptake by muscle fibres, leading to the expression and extracellular of the antigen. Combining protein and its encoding plasmid DNA in vaccination, the protein could induce the primary antibody responses and the following antigens expressed from DNA transfected cell could work as boosting dose. This would have the advantage to develop as single-dose vaccines. Consequently, the pRc/CMV HBS DNA-containing capisomes prepared in the presence of HBsAg to conduct a combined vaccine. The mice were immunised with these formulations and their sera were monitored to confirm the possibility of single-dose vaccination (Figure 7.15).

The total IgG responses of Balb/c mice treated with these formulations showed that CaPi maintained IgG titres induced by HBsAg but not by plasmid-DNA encoding HBsAg. At early stage (28 days after the injection), IgG titres in the mice treated with HBsAg-containing formulations were lower than in those immunized with HBsAg-free ones. In contrast, IgG titres induced by HBsAg-containing formulations were higher than those elicited by HBsAg-free preparations after 56 days after the first immunisation. It indicated that IgG responses in mice immunized with HBsAg are delayed by co-immunization with CaPi-DNA complexes. Furthermore, CaPi maintained IgG responses in mice immunized with HBsAg until 140 days after the first immunisation. It increased the mean of log values of serum IgG titres induced by HBsAg from 2.2 to 3.5 (log_{10}). Results showed that the mice immunised with formulations entrapping HBsAg and pRc/CMV HBS DNA induced stronger responses than those treated with DNA vaccines.

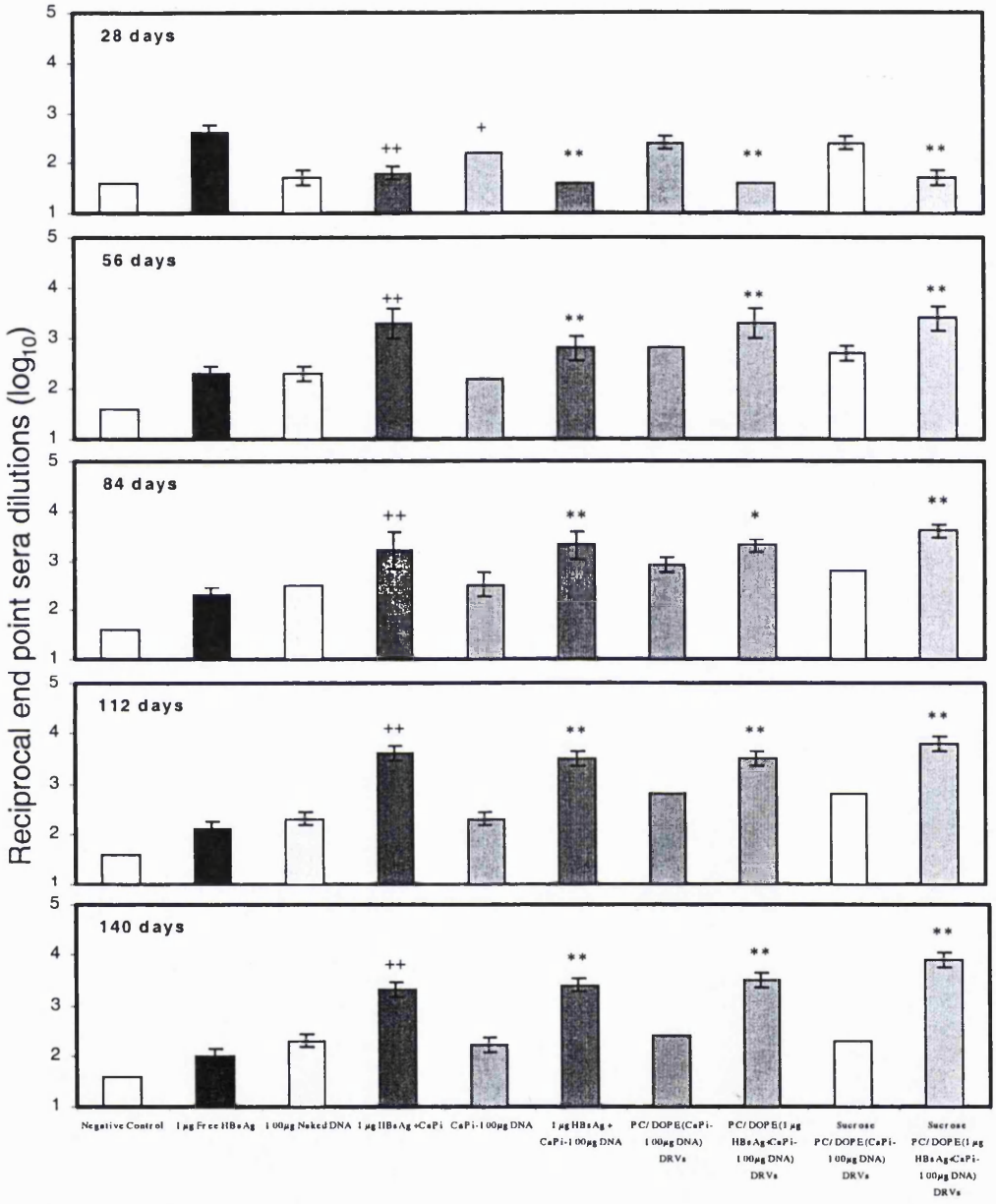

Figure 7.15: Total IgG responses in mice immunized with a single-dose of naked or formulated pRc/CMV HBS DNA prepared in the presence or absence of HBsAg. Balb/c mice were injected intramuscularly on days 0 with naked DNA (100 µg) or DNA (100 µg) entrapped in liposomes prepared in the presence or absence of HBsAg (1µg). Animals were bled at 28, 56, 84, 116, and 140 days after the injection and sera were tested by ELISA for IgG responses against HBsAg. The values are log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2. Each bar represents mean ± SD (n= 5). +p<0.05, ++p<0.005 compared to the mice immunised with free protein or naked DNA. Significantly different from the groups treated with the formulation prepared in the presence of HBsAg: *p<0.05; **p<0.005 (Student's unpaired *t*-test).

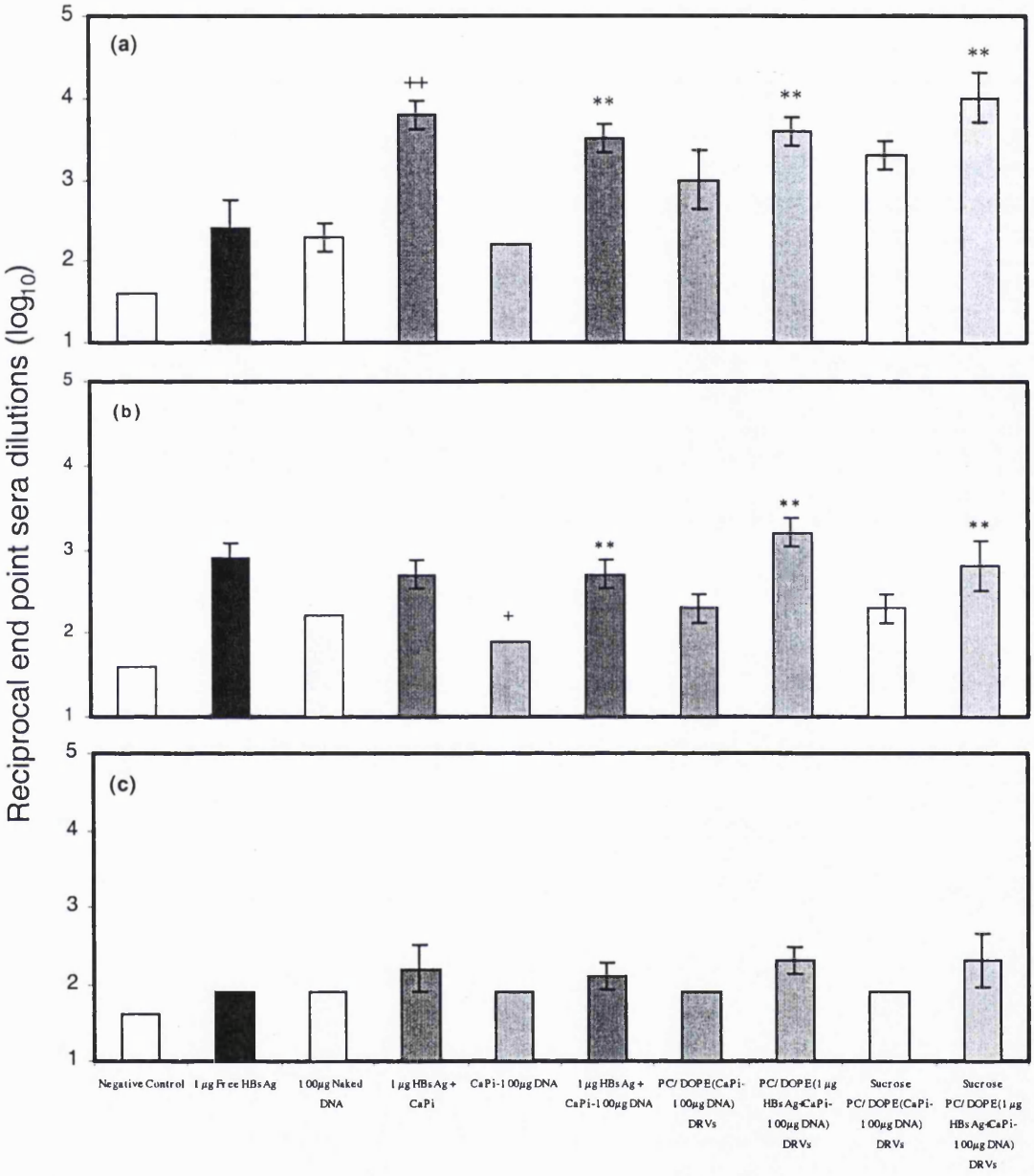

Figure 7.16: Subclass IgG responses in mice injected with a single-dose of naked or formulated pRc/CMV HBS DNA prepared in the presence or absence of HBsAg at 56 days after the injection. Animals were treated as described in Figure 7.15. The sera were tested by ELISA for (a)IgG_1, (b)IgG_{2a} or (c)IgG_{2b} responses against the encoding HBsAg. Values presented are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2 (n=5). +$p<0.05$, ++$p<0.005$ compared to the mice immunised with free protein and DNA. Significantly different from the groups treated with the formulations prepared in the absence of HBsAg: **$p<0.005$ (Student's unpaired *t*-test).

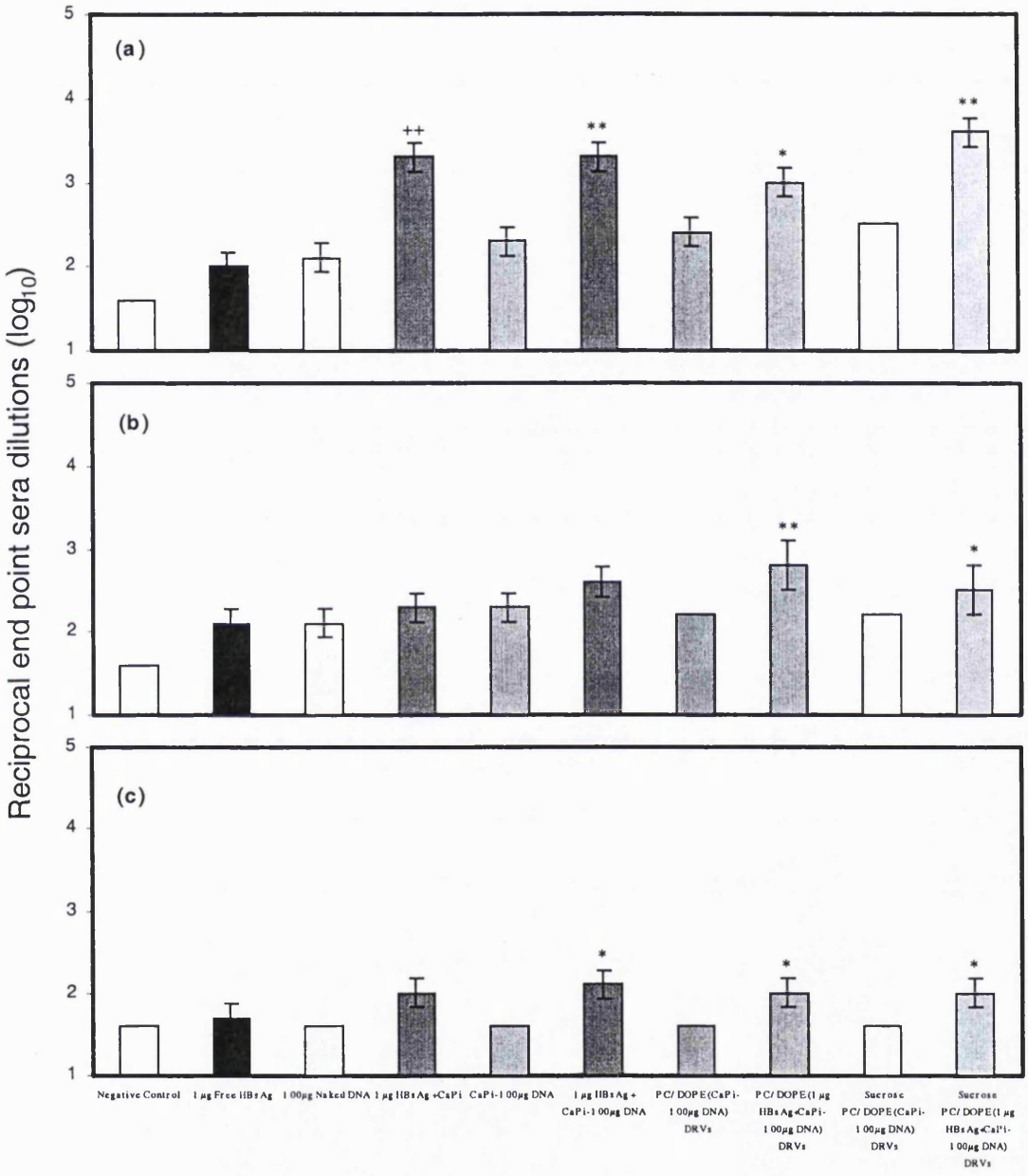

Figure 7.17: Subclass IgG responses in mice injected with a single-dose of naked or formulated pRc/CMV HBS DNA prepared in the presence or absence of HBsAg at 112 days after the injection. Animals were treated as described in Figure 7.15. The sera were tested by ELISA for (a)IgG_1, (b)IgG_{2a} or (c) IgG_{2b} responses against the encoding HBsAg. Bars shown are mean ± SD of log_{10} of reciprocal end point serum dilutions required for OD_{492} to reach readings of about 0.2 (n=5). ++$p<0.005$ compared to the mice immunised with free protein and DNA. Significantly different from the groups treated with the formulations prepared in the absence of HBsAg: *$p<0.05$; **$p<0.005$ (Student's unpaired *t*-test).

Single-dose immunisation by using CaPi as an adjuvant enhanced the IgG_1 responses induced by HBsAg vaccination, but inhibited IgG_{2a} titres for DNA vaccination at 56 days (Figure 7.16). Furthermore, the mean of log values of serum IgG_1 titres for HBsAg vaccination increased from 2.5 to 3.7 and maintained this level until 112 days (Figure 7.17). However, the mice immunized by the HBsAg mixed with CaPi or CaPi-DNA complexes did not show the difference in IgG subclasses. Thus, the subclass IgG responses in the vaccination with combination of HBsAg (1μg)and plasmid DNA encoding HBsAg (100μg) exhibited the protein dominance in switching the regulation of Th pathways in this dose level.

Immunised by liposomes entrapping HBsAg and CaPi-DNA complexes, the mean of log values of serum IgG_1 titres increased from 3.0 to 3.5 (log_{10}) and IgG_{2a} titres increased from 2.0 to 3.0 (log_{10}) at 56 days after first immunisation. However, the mean of log values of serum IgG_1 titres induced by the formulations prepared in the absence of HBsAg decreased to 2.5 (log_{10}). In addition, those induced by the formulations with HBsAg still maintained at 3.5 (log_{10}) at 112 days after the immunisation. Moreover, IgG_{2a} titres induced by these formulations exhibited high titres in the immunisation with HBsAg-containing formulations at 56 days after first immunisation. However, these IgG_{2a} levels decreased at 112 days after first immunisation. In IgG_{2b} responses, the titres induced by all formulations did not show any significant difference between each other at 56 days after first immunisation. However, the titres induced by the formulations with HBsAg were higher than control at 112 days after first immunisation. These results indicated that HBsAg switched subclass IgG titres induction to IgG_1 dominance when mice were immunised with the combination of HBsAg and plasmid DNA encoding HBsAg.

7.5 DISCUSSION AND CONCLUSIONS

In this study, DNA entrapment into capisomes has been developed successfully. The preparation, characterisation, stability and immune responses in these studies was conducted. The results indicated that capisomes may be an alternative method for DNA vaccine formulations as they appear to act as immunological adjuvants augmenting immune responses against the encoded antigen. Furthermore, the immune responses induced by the HBsAg protein antigen or protein encoded by pRc/CMV HBS DNA were compared. Combination of protein and DNA into liposomes provided a new approach to develop a single-dose DNA vaccine. These findings are discussed in the following section.

DNA delivery is an important issue in the development of gene therapy and genetic immunisation (Davies, 1993; Gregoriadis, 1998). *In vitro*, a variety of methods, such as CaPi precipitation (Song and Lahiri,1995), cationic lipids (Felgner *et al.*,1987; Gao and Huang, 1995) and polymers (Lopata *et al.*, 1984), have been used for gene transfection in studying the function of genes in cell cultures. Several types of viruses, including retrovirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus, have been modified in the laboratory for use in gene therapy (Robbins and Ghivizzani, 1998). Retroviral vectors can permanently integrate into the genome of the infected cell, but require mitotic cell division for transduction (Naldini *et al.*, 1996). Adenoviral vectors can efficiently deliver genes to a wide variety of dividing and nondividing cell types. However, elimination of infected cells by CTLs often limits gene expression *in vivo* (Kovesdi *et al.*, 1997). Herpes simplex virus can deliver large amounts of exogenous DNA; however, cytotoxicity and maintenance of transgene expression remain as

obstacles (Spaete and Frenkel, 1982). AAV also infects many nondividing cell types,but has a limited DNA capacity (Grimm *et al.*, 1998). Because these vector systems have specific advantages and limitations, each one has applications for which it is best suited. Although virus-mediated gene delivery has proved to be one of the most efficient means of gene transfer, nonviral means are also under development (Ledley, 1994). However, these systems are still far from ideal for DNA delivery *in vivo*. Clinical application of gene therapy depends on the development of suitable gene transfer vehicles (vectors). Problems include the targeted delivery into specific cells, expression of gene and the delivery of gene expression products *in vivo* (Ledley,1995). Although generally not as efficient as viral vectors, nonviral systems such as lipidic vectors have the potential advantages of being less toxic and nonrestrictive in the size of DNA. They can be accomodated, targetable, and easy to produce in relatively large amounts (Lee and Huang, 1997).

Considering the gene expression *in vivo*, the gene therapy still has many problems, including the expression vector, used dose, expression site and dose-response relationship (Anderson,1998). By comparison to the gene therapy, DNA vaccination may be more easy to achieve and have the advantage of preventing virus infection. However, DNA vaccination is still in the early stage and the immune responses induced by genetic immunisation is under study. In addition, the carriers to mediate DNA vaccination, such as microbes (Sizemore *et al.*, 1995), gene gun (Fynan *et al.*, 1993), cochleates (Gould-Fogerite and Mannino, 1996), vesicle-DNA complexes (Szoka *et al.*, 1996) and liposomes (Gregoriadis *et al.*, 1996), had been used to enhance the immune responses induced by DNA vaccination. Cationic liposomes had been found

to promote immunity to the encoding antigen (Gregoriadis *et al.*, 1997). Cationic lipids can interact with DNA molecules leading to DNA condensation (Reimer *et al.*, 1995). Thus, cationic liposomes can entrap DNA and act as adjuvant for DNA vaccination. Moreover, CaPi can precipitate DNA for gene transfection and have an adjuvant effect for some antigen molecules (Relyveld and Chermann, 1994). In a previous study, CaPi was used as a delivery system for gene therapy of β-thalassaemia (Anderson, 1992). The clinical trials failed, but reasons for this unsuccessful attempt are still not clear. However, the CaPi had been used as vaccine adjuvant for several years (Relyveld, 1986). It showed a haemolytic effect but no other toxic effects (Goto *et al.*,1993). Consequently, CaPi was selected to condense the DNA in the present research and used to entrap the complex formed into liposomes in order to increase both the biocompatibility (avoidance of cationic lipid) and transfection efficiency.

CaPi had been entrapped into liposomes for the regeneration of bone transplant for several years (Huang *et al.*, 1997). The interaction between CaPi and lipid bilayers during bone formation has been studied (Plate *et al.*, 1996). This study suggested that entrapment of calcium phosphate-DNA complexes into liposomes may be possible. In the present study, CaPi-DNA complexes were found to have very low zeta potential. Consequently, the aggregation of CaPi-DNA complexes increased the particle size up to 20μm. This size rendered the formulation unsuitable for injection. Phospholipids can interact with the surface of CaPi-DNA complex particles and thus prevent the aggregation of these complexes. Furthermore, CaPi will dissociate under pH 5, which is the pH value within late endosomes. Thus, CaPi can protect DNA before it was delivered into the cellular nucleus (Owens *et al.*, 1986). Calcium ions are membranolytic

in vitro (Wallingford and McCarty, 1971) and, associated with plasmid DNA, they could allow DNA molecules to leave endosomes. In fact, it is generally accepted that the CaPi-DNA complexes fuse with organelle membrane leading to the escape of DNA into the cytosol. Another possibility is that CaPi causes a swelling and rupture of organelles by sequestering protons and its counter ions creating an osmotic disequilibrium (Wattiaux *et al.*, 2000). Consequently, CaPi can get the DNA out of endosome and deliver it into the cytosol.

The immune responses for DNA vaccination in mice immunised with these formulations showed that the antibody titres are affected by the immunisation doses, schedules as well as the compositions of formulations. Eventually, the immune responses induced by DNA vaccination follows the gene transfection, its expression and genetic product processing or presentation in the antigen presentation cells (APCs). Some expressed proteins will be exported out of the cells to be processed to other APCs, which may induce antibody responses. Moreover, if the transfected cell can produce antigen continually, the expressed antigens can be processed by APC to produce immunological memory at the initiation stage. The following expressed product will induce high titres as secondary boosting in protein vaccination. Unfortunately, the genetic immunisation with a single-dose in this study cannot confirm this idea. The death of the transfected cells or the neutralization of antibody with expressed antigen *in vivo* may explain the low titres induced by this single-dose genetic immunisation. Furthermore, the combination of protein and DNA vaccination induced high titres at 56 days after the immunisation. Previous study (Donnelly *et al.*, 1997) had shown that DNA has adjuvant effect for protein vaccine. Results in this study suggested that this approach could develop a single-dose vaccination against

hepatitis B. However, the interaction between protein and DNA immunisation is still not clear. More studies are required in further research.

It is clear that the dose-dependent immune stimulatory effects primarily result from the presence of an unmethylated CpG dinucleotide in particular base contexts (CpG motif). CpG DNA causes the rapid activation of innate immune defenses through direct stimulation of monocytes, macrophages and dendritic cells (APCs) which produce Th1-like cytokines such as IL-12 promoting the activation of natural killer (NK) cells by CpG (Krieg, 1999). The current generation of DNA vaccines are quite effective in mice, but much less effective in primates (Prince *et al.*, 1997). The recent results suggested that attaining the full clinical potential of DNA vaccines may require using engineered vectors in which CpG-N (CpG-neutralizing) motifs have been deleted and CpG-S (CpG-suppression) motifs have been added. When they were used as a DNA vaccine, the anti-HBs response at 4 and 6 weeks was substantially stronger with DNA vaccines from which CpG-N motifs had been deleted and even more so when 16 CpG-S motifs had been inserted. Removal of CpG-N motifs and addition of CpG-S motifs resulted in a more than 3-fold increase in the proportion of IgG_{2a} relative to IgG_1 anti-HBs antibodies, indicating an enhanced Th-1 response. This accentuated Th1 response also was demonstrated by the striking progressive increases in CTL responses induced by vectors from which CpG-N motifs were deleted and/or CpG-S motifs added (Krieg *et al.*, 1998). The immune effect of these immune stimulatory sequence (CpG motifs) can be mitigated by certain modifications of the cytokines and inhibitors of endosomal acidification or perhaps by some delivery systems. This DNA vaccine with CpG motifs can be applied in using capisomes to study the co-adjuvant effect.

The DNA vaccination with multiple doses can maintain the antibody titres longer than the immunisation with a single dose. The compositions of capisomes are not important for the adjuvant effect on the genetic immunisation. This is different from the results obtained from protein immunisation which was founded to depend on the lipid compositions (Chapter 3). Liposomes act as carrier to deliver DNA into the transfected cell for genetic immunisation. However, they enhance the immune responses to proteins by delivering these antigens to local lymph nodes. Moreover, the genetic immunisation with multiple doses did not show high titres as multiple protein vaccination. The indirect way to induce immune responses by protein expressed from injected DNA may make liposomes lose their depot effect to enhance the immune response. Another reason responsible of low titres obtained by DNA vaccination may be the endogenous protein expressed from injected DNA passing through MHC I pathway dominantly and inhibit the humoral responses. This may be similar to the antibody responses induced by mannosylated HBsAg in Chapter 5. More studies are required for the investigation of the cellular or humoral immunity at the same time in the future.

In conclusion, capisomes had been successfully used in genetic vaccination. The investigations of the immune responses induced by these formulations were conducted. The protein and DNA combined vaccination offered a new opportunity to develop the vaccine with a single dose through the biological sustained release mechanism. Moreover, the capisomes provide an alternative formulation to deliver DNA *in vivo*. The surface charge can be controlled by varying these lipid composition. This may be a promising way to deliver DNA for the gene therapy and DNA vaccination in further studies.

Chapter 8

General Discussion and Conclusions

8.1 LIPOSOMES AS HEPATITIS B VACCINE ADJUVANTS

In order to optimize the liposomes as vaccine carriers for hepatitis B immunisation, the effects of liposomal characteristics, the delivery pathways and the approaches to supply the antigen to antigen presentation cells have been studied in this thesis. Firstly, HBsAg was entrapped into DRVs and applied in immunisation to investigate the effect of lipids on immune responses (Chapter 3). DSPC/Chol DRVs were selected to study the adjuvant effects of liposomes of various sizes for HBsAg vaccination (Chapter 4). Microfluidised HBsAg-containing DRVs (small vesicle size) were not able to enhance antibody responses. However, HBsAg-containing DRVs prepared in the presence of sucrose, which have a size similar to that of microfluidised DRVs, were found to enhance the IgG responses to HBsAg. In addition, liposomes showed varying adjuvanticity for mannosylated HBsAg with only DSPC/DOPE DRVs eliciting high antibody titres after immunisation with mannosylated HBsAg (Chapter 5). In other experiments, CTB was conjugated to the liposomal surface. CTB-conjugated DRVs were shown to induce systemic and mucosal immunity by oral administration (Chapter 6). Finally, the plasmid pRc/CMV HBS DNA encoding HBsAg was used in the form of capisomes to induce antibody titres to HBsAg by indirect immunisation (Chapter 7). Capisomes exhibited a similar adjuvant effect as the cationic liposomes which, however, can be potentially toxic, and provided an opportunity to combine protein and DNA vaccines to produce a single-dose vaccine. These preparations were evaluated in terms of manufacture, characterisation, stability on storage and antigen release profiles *in vitro*. In addition, mice were immunised with these formulations and the antibody responses elicited were measured. Results have been illustrated and discussed in previous chapters. Pharmaceutical and immunological considerations are discussed as follows.

In the pharmaceutical considerations, the substances to induce antibody responses, such as HBsAg and plasmid DNA encoding HBsAg, should be analysed quantitatively in the formulations before they are used for the immunisation studies. This work confirmed that the equivalent dose was applied in immunisation to study the effect of adjuvants. Furthermore, the characteristics of these vesicles played an important role to affect their fates and immune responses *in vivo*. In order to check the reproducibility of these formulations prepared in different batches, the characteristics of each preparation were examined. These data had been used to control the immunisation dose at the same level in different experiments. In practical applications, the retention of active substances in the liposomes and the aggregation of these vesicles are quite important for the stability of these formulations. Moreover, the release profiles of protein or DNA molecules from DRVs incubated with tissue extracts could be used to estimate the ratio of entrapped to free molecules *in vivo*. These evaluations in pharmaceutical characterisation could improve the applications of liposomes in vaccine formulations.

The immune responses induced by these formulations in mice were assayed to evaluate liposomal adjuvanticity in vaccination. In order to evaluate the new formulations for hepatitis B vaccines, the antibody responses induced by each formulation in mice had been measured. In addition, the subclass IgG responses were assayed to understand the effect of liposomes on the immunological modulation for antibody induction. These results indicated that liposomal adjuvanticity for hepatitis B vaccines depended on many factors, such as liposomal characteristics, antigen properties, routes of administration and immunisation methods. These results provided the concepts to design liposomal formulations for hepatitis B vaccines.

8.2 PHARMACEUTICAL CONSIDERATIONS

Since 1974, liposomes had been applied as vaccine adjuvants. Until now, many antigens had been incorporated into the liposomes and shown the adjuvant effect (Table 1.3). However, most of the studies were focussed on the biological effect not on the evaluation of pharmaceutical applications. The studies in this thesis had carried out these characteristics to confirm the formulations applied in immunisation in the same quality. The preparation procedures had significant effect on the entrapment of substances into DRVs (Figure 3.3 and 5.5). These results can improve the quality control and scale-up techniques in liposomal production. Moreover, the monitoring of these vesicles upon storage exhibited the problems associated with the stability. Lyophilized liposomal powder may be the alternative solution for these problems. However, the stability of freeze-dried powder is still unknown and requires further studies. Furthermore, the fate of liposomes *in vivo* will affect the effect of liposomes as the carriers for antigen molecules. In order to evaluate the possibility of liposomes as antigen carriers, the release kinetics *in vitro* had been tested (Figure 3.7, 4.4, 4.9, 5.9, 6.7 and 7.8). These results estimated the antigen forms processed *in vivo*. Further research in antigen pharmacokinetics were required to investigate the relationship between the antigen forms and immune responses induced by these antigens. In addition, the direct and convenient method to analyse the amount of protein or DNA in liposomal formulations is required to conduct. These studies improve the availability of liposomes in vaccine pharmaceutics.

8.2.1 Preparation of liposomes for pharmaceutical application

The preparation procedures of DRVs determined their quality. In this thesis, the results

in HBsAg incorporation into DRVs displayed the preparation procedures should be controlled to make these formulations be reproducible (Figure 3.3). DRVs entrapped mannosylated HBsAg had the similar phenomena as HBsAg. Generally, the sonication disperse the lipid molecules in the aqueous phase to supply more surface area of liposomes contacted with entrapment substances. The rehydration of freeze-dried lipid powders led the formation of vesicles. The phase transition of lipid bilayers in the aqueous solution may be the major factor to affect the interaction between proteins and lipid bilayers (Epand, 1998). This interaction will decide the entrapment efficiency and amount of proteins in DRVs. However, more advanced experiments are required to explore the mechanism of liposomal entrapment. These studies improve the liposomal quality and make liposomes be easy to use in pharmaceutical application.

Moreover, this study (Chapter 7) had developed a new technique to entrap DNA molecules into the liposomes without cationic lipids. In the experiments of DNA vaccines, the DRVs were applied to entrap the calcium phosphate-DNA complexes. The photographs under transmission electron microscopy showed that capisomes had changed the multilayer structures, but the cationic DRVs preserved the structural integrity. Calcium ions could interact with the phosphate groups of DNA and phospholipid to form the spiral structures in the previous studies (Mozafari, 1998; Bailey and Sullivan, 2000). In this study, the calcium phosphate and DNA constructed the complexes. Then, these complexes were entrapped into liposomes by dehydration-rehydration procedure. The photos (Figure 7.7)of capisomes under transmission electron microscopy indicated that these CaPi-DNA complexes may be blent with phospholipids but not be coated with lipid bilayers in capisomes. This made the particles be different from the

cochelate structures of calcium-DNA-lipid complexes (Papahadjopoulos *et al.*,1975). However, the detail structure of capisomes is still unknown and requires more studies. This technique provides a new option to entrap DNA into liposomes. Furthermore, calcium phosphate-DNA complexes can dissociate in low pH conditions and may be able to deliver DNA into the cellular nucleus as virus uncoating in the host cells (Marsh and Helenius, 1989). Viruses have excellent efficiency to deliver DNA into the host cells. The virus-like structures may have the potentials to be developed as the delivery system for DNA molecules.

Finally, liposomes also exhibited some advantages to apply in the vaccine adjuvants for HBsAg. They included incorporation of this membrane protein into lipid bilayer, reconstruction of the viral envelope to entrap DNA complexes and the surface modification for targeting delivery of antigens. These properties make liposomes be ideal systems to entrap HBsAg and construct the structure as the envelope of hepatitis B viruses. The traditional vaccine, plasma vaccine, for hepatitis B is purified from HBs positive and HBe negative plasma. It is constituted of well defined morphological particles, containing two major polypeptides P22 and P27, and without any trace of viral DNA. When HBsAg was incorporated into liposomes, the structures of these HBsAg-containing liposomes can be achieved to small size as viral envelops. These are similar to the plasma vaccines. However, plasma vaccine has a risk of contamination in the purification processes. The recombinant HBsAg from yeast contains some lipids which assemble into aggregates as a natural liposome had been reported (Diminsky *et al.*, 1996). The phospholipid composition can alter liposomal adjuvanticity for incorporated antigens (Gómez-Gutiérrez *et al.*, 1995). Consequently, applications of liposomal formulations in HBsAg are still

useful to develop the new formulations for hepatitis B vaccines. In addition, these virosomes, HBsAg-containing liposomes, can entrap DNA complexes encoded the same antigen to construct virus-like particles. These formulations may work as the biological sustained release dosage form to generate antigens continuously and maintain the immune responses in a long period after immunisation.

Generally, the new immunisation methods produce the new generation of vaccines. They needed more efficient adjuvant systems. Liposomes showed their potential in the development of vaccine formulations. They could be applied in the vast field of development ranging from protein to DNA vaccination. This study were aimed at discussing the effect of liposomes on hepatitis B subunit and DNA vaccines. HBsAg and the plasmid DNA encoding this antigen had been incorporated into the liposomes successfully by the new preparation techniques. Liposomes exhibited their versatility in allowing various formulations of hepatitis B vaccines. However, many problems, such as stability and mass production, remained unsolved in the development of these formulations. These studies concerned the preparation of these DRVs and showed some solutions to prepare liposomal vaccines. These results provide the useful information that can be used to develop and scale-up these formulations.

8.2.2 Stability of liposomal formulations on storage

In order to design a successful vaccine product, considerations must be given for the formulation and regulatory issues. Most important, a vaccine must be both efficacious and safe, but it also is stable and relatively easy to use. An ideal vaccine formulation designed for

parenteral application should consist of a liquid formulation that does not require cold or frozen storage and can be used directly from a single vial. The ideal characteristics are not achieved by most vaccines available on the market. Hence, the opportunity for marketing new vaccines is great even if competitive products exist (Newman and Powell, 1995).

Vaccine stability can be defined in numerous ways and under many storage conditions. A reasonable standard of stability is a shelf life of two years or more, where the shelf life is defined as the time point for which the product remains at least 90% active or efficacious (Newman and Powell, 1995). In this study, the retention of HBsAg in liposomes was monitored to evaluate the stability of these formulations. The results showed that these liposomal formulations were still far from ideal in the aqueous dispersion. The shelf-life of these formulations are around six weeks or less. Some of the formulations had serious aggregation phenomena. The results in this study only focussed on physical stability of these formulations. More studies, such as chemical and microbiological stability, are demanded to investigate in near future.

Furthermore, the aggregation of liposomal vaccine formulations depended on the vesicle coalescence and the lipid interaction. The interaction between vesicle and vesicle may be explained by the mathematical model in colloid stability (DLVO theory). The contribution to the equilibrium of the colloidal dispersion included the attraction of the vander Waal force and the repulsion resulting from the electrostatic force. The electrostatic interaction can be measured through the zeta potential. In pharmaceutics, zeta potential of a colloidal dispersion is applied to

evaluate the stability of this system, such as aggregation, coagulation and flocculation (Kayes, 1977). In this work, the zeta potentials of these vesicles were measured to evaluate the stability of these formulations on storage. The zeta potentials of these vesicles became more negative upon storage except microfluidised DRVs (Table 4.1). The charge on liposomal surfaces made these vesicles be stable by electrostatic repulsion in the dispersion. The DSPC/DOPE DRVs entrapping mannosylated HBsAg and sucrose were the formulation had been found in aggregation. Their zeta potentials were around -20mV. The reduction of electrostatic repulsion is one of the reasons which are responsible of this increased aggregation. However, the fusion of lipid bilayers still plays a crucial role in inducing the vesicle aggregation. Generally, this aggregation can be evaluated by measuring the zeta potential of the liposomal formulation. The flexible liposomal surface and the occurring fusion of lipids in lipid bilayers resulted in disagreement with the prediction from the DLVO theory.

In summary, the physical stability of liposomal vaccine formulations in this work had been characterized by the retention of the active substances, the vesicle sizes and the zeta potentials. The zeta potentials can be related to the vesicle sizes in aggregation. However, the results required more mathematical analysis to compare with the ideal model. The reasons for instability of these vesicles are still unclear. They may include physical, chemical or microbial factors. The further studies in the aspect are recommended. Furthermore, lyophilized liposomal powder had been reported as the ideal form to store for a long period (Zou *et al.*, 1996). This may be solution of the instability of liposomal formulations in dispersion. However, the stability of these lyophilized powder would be next considerations in further research.

8.2.3 Fate of liposomal formulations *in vivo*

The fate of liposomal formulations *in vivo* depended on the vesicle properties and the route of administration. The vesicle properties included the lipid composition, vesicle size and the surface charge. The routes of administration included intravenous injection, intramuscular injection, transdermal, pulmonary or oral pathways. The immunisation route used to deliver antigens can dramatically influence both the type and magnitude of the resulting immune responses (Webster, 1968; Pierce, 1984; Fadda, *et al.*, 1987). Generally, the immunisation was employed by the intramuscular injection. However, the oral vaccination showed the advantage over parenteral administration to induce the mucosal immunity, including secretory IgA. This study started from the intramuscular immunisation to test the effect of liposomes as vaccine adjuvants (Chapter 3, 4 and 5). In addition, the formulations were targeted to the mucosa through oral delivery (Chapter 6). Moreover, the release amounts of immunological substances, HBsAg and its encoded plasmid DNA, from these formulations in the media (PBS or tissue extracts) were monitored at different time courses. These results can be used to estimate the ratio of entrapped to free molecules in the process of delivery *in vivo*. The data may be able to relate with the fate of liposomal formulations after intramuscular injection or oral administration.

Liposomes will accumulate on the local lymph node after injecting these formulations intramuscularly for several hours. This is the deposit effect of liposomes to work as immunological adjuvants. However, the macrophages can engulf the liposomes *in vivo* and they exist everywhere including the local lymph nodes. This causes that liposomes can not be so concentrate on the local lymph node. The antigen forms *in vivo* is not so easy to be analysed.

Hence, this limitation brings the difficulty to evaluate the bioavailability of these antigen molecules and the relationship between antigens and immune responses. Furthermore, the release kinetics of HBsAg from the DRVs demonstrated that the protection effect of liposomes for antigens in the delivery processes. However, pharmacokinetics of the liposomal antigens still have many gaps required to be investigated. More experiments are required in the further research.

Oral administration of liposomal formulations follows different pathways from the intramuscular injection. Cholera toxin or its B subunit (CTB) had been used successfully to induce mucosal immunity. IgA production and secretion were significantly enhanced when administered in vaccine formulations (Elson and Ealding, 1984a; McKenzie and Halsey, 1984; McGhee *et al.*, 1993). The mechanisms of CTB as an adjuvant were poorly defined but known to bind to GM_1 ganglioside receptors in the gut-associated lymphoid tissue, possibly targeting the immunogen for increasing uptake amounts (McCann *et al.*, 1997). The CTB-conjugated DRVs increased the uptake of HBsAg in GI tract may confirm this mechanism. However, the mechanism of CTB escaping from the gastric digestion is another interesting issue in further studies. Furthermore, the immune responses obtained from the oral administration can achieve the antibody level of free HBsAg induced by intramuscular injection. It indicated that liposomes can protect antigens in GI tracts and deliver them to the antigen-presentation cells. This is also proved by the release kinetics of HBsAg in gastrointestinal exacts. Consequently, the stability of these vesicles in GI tracts would be the main consideration to improve their adjuvanticity by oral administration.

In conclusion, liposomes are useful carriers to deliver the HBsAg through intramuscular injection and oral administration. Liposomes have different fate *in vivo* by various routes of administration. After injected intramuscularly, liposomes will accumulate on the local lymph node. However, CTB-bearing DRVs may deliver directly to the mucosal surface of the intestine by oral administration. Due to the limitation of analysis methods *in vivo*, the antigen forms processed *in vivo* are still unclear. It bring in the difficulty in evaluating antigen delivery by these vesicles. This analytical techniques are required to build up in the further research.

8.3 IMMUNOLOGICAL CONSIDERATIONS

Liposomes had been considered as a substitute for aluminium-based adjuvants, the one that only presently incorporated in licensed products for human use, in the vaccine development (Aprile and Wardlaw, 1966). The flexibility and versatility of liposomal preparation methods made liposomes be an ideal system to probe the immunological responses (Gregoriadis, 1990). Phospholipid-based liposomes are not associated with serious local or systemic reactions and have not been linked with significant antigen accumulation in the blood or internal organs of animals or humans (Kramp *et al.*, 1982; Eichler *et al.*, 1988). In addition, practical large-scale methods are now available for preparing liposomes for use in clinical studies (Amselem *et al.*, 1993; Barenholz *et al.*, 1993). Several years ago, clinical studies investigating liposomes as vaccine adjuvants had also been conducted (Fries *et al.*, 1992; Glück *et al.*, 1992). In all cases, liposomes were found to be safe with varying degrees of effectiveness. In this thesis, the liposomes had been applied in the hepatitis B subunit and DNA vaccines to investigate the effect of liposomes on the humoral immune responses. The impact of these formulations on the

humoral immunity is discussed as follows.

8.3.1 Evaluation of liposomes as vaccine adjuvants

The vaccine adjuvants are materials that help the antigens to increase the humoral or cellular immune responses to them (Gupta and Siber, 1995). To evaluate liposomes as vaccine adjuvants for hepatitis B vaccines, HBsAg, the major components to induce the antibody against hepatitis B viruses, was chosen as antigen. However, the recombinant HBsAg from yeast has lipid components in a bilayer form in the protein assembly particles. Indeed, it has been described as a kind of natural liposome already (Diminsky *et al.*, 1996). The recombinant HBsAg from yeast is an entity of 20nm but the HBsAg-containing DRVs can be prepared with size ranging from 100nm up to 5μm. Thus, by incorporating the HBsAg into liposomes, the vesicles will have a similar structure to that of the recombinant antigens. Moreover, the lipid composition and vesicle size of liposomes can be controlled by choosing the lipid components and preparation methods. Furthermore, SDS was applied to unfold the HBsAg molecules in this study (Chapter 5). Then, the mannose moiety was conjugated with the amino groups on the antigen polypeptide chains. The mannosylated HBsAg has the same molecular weight as the HBsAg monomer. However, mannosylation may have changed the characteristic of HBsAg from membrane protein to water-soluble one. This modification is expected to change the protein property but not the amino acid sequence. The antibody responses induced by this mannosylated HBsAg (Figure 5.10) indicated that the modified HBsAg reduced the ability to elicit antibody responses. The loss of such ability may be due to the partial denaturation of protein or the masking of HBsAg antigenicity as a resulting conformation change.

The determination of antigen doses is another important issue to study the effect of liposomes as vaccine adjuvants. Dose-response relationships are very important in therapeutic decisions and in experimental pharmacology. Measured effects are frequently recorded as maxima at time of peak effect or under steady-state conditions (e.g., during continuous IV infusion). Drug effects may be quantified at the level of molecule, cell, tissue, organ, organ system, or organism. Graphing dose-response curves of drugs studied under identical conditions can help the pharmacologic profiles of the drugs be compared. However, the antibody responses induced by antigen looks not so simple as the pharmacological response of drugs. Eventually, the antibody response induced by antigen includes three steps. Firstly, antigens are processing and presentation. Then, B-cell is activated and differentiated. Finally, antibody is produced and secreted out (Tonegawa, 1983). The complexity in antibody induction results in the difficulty to evaluate the vaccine formulations. So far, the dose determination is still by experience. The antibody response is the popular method to evaluate the effect for vaccine formulations. In this study, the dose-response curve between antigen and antibody had been tested before the evaluation of liposomal adjuvant effect and the dose in the linear range had been chosen for the advance studies.

The preparation of liposomal antigen is an important factor to affect the adjuvant effect of liposomes. The liposomal antigen preparation can be modified by the ratio of protein to lipid, the composition and the preparation methods, such as DRVs, microfluidisation, or sucrose co-entrapment. In order to investigate the effect of liposomes as vaccine adjuvants, the ratio of protein to lipid was kept as constant and the effect of compositions and preparation had been

carried out. The cholesterol-containing liposomes composed of saturated phospholipid (DSPC) induced stronger antibody responses for HBsAg than those composed of unsaturated lipids (PC). However, the responses induced by the mannosylated HBsAg are reverse. The results in this thesis (Chapter 3 and 5) confirmed the previous study that the liposomes composed of high melting phospholipid will increase the antibody responses for membrane protein but inhibit the responses induced by water-soluble protein (Gregoriadis, 1988). Furthermore, the methods to reduce vesicle size alter liposomal adjuvanticity for HBsAg. These results indicated that HBsAg-containing DRVs prepared in the presence of sucrose is the most promising candidate as liposomal formulations for HBsAg vaccination. The optimisation of HBsAg-containing DRVs had been done by controlling the various factors, such as lipid compositions, vesicle size and protein characteristics. However, the further improvements need more molecular mechanism in immunology to design the formulations.

The routes of administration affected greatly on the biological activity for vaccine formulations. In order to evaluate the effect of liposomes as vaccine adjuvants, the route of administration should be considered in advance. Generally, the vaccination is done by intramuscular injection to get the sustained release of antigens and induce protection effect for a long period. However, the intramuscular immunisation cannot induce the mucosal immunity, which can protect the infection of pathogens from mucosa. For some cases, mucosal immunity is very important for protection of pathogen invasions. In addition, oral vaccination is still more safe than injection even if the mucosal immunity was not so important. Morover, the targeting delivery of antigen molecules *in vivo* is another advance in the administration. Cholera toxin has

been used to target to the mucosal surface in the intestine and its B subunit (CTB) can bind glycosides on the intestinal mucosa (Kuziemko *et al.*, 1996). Not only the specific ligands for binding but also the barrier in the delivery routes should be considered in the targeting delivery. In Chapter 6, the targeting delivery of HBsAg by CTB-conjugated DRVs entrapping HBsAg through oral administration had shown the possibility to deliver the antigen to specific sites. Generally, the routes of administration will affect antigen delivery and should be considered in the evaluation of liposomes as vaccines adjuvants.

In summary, there are many factors to be considered and well-defined to evaluate the effect of liposomes as vaccine adjuvants. The property of antigens, the dose for immunisation, the preparation of vaccine formulations and the route of administration had been evaluated in this study. However, the optimisation of liposomes as vaccine adjuvants may consider from the various views but not just from one side. These results indicated that the effect of antigen characteristics is important to induce the antibody responses. HBsAg-containing DRVs prepared in the presence of sucrose is a promising candidate to develop as the formulation for hepatitis B vaccination. Furthermore, the oral vaccination by CTB-conjugated DRVs encapsulating HBsAg showed the possibility to develop a targeting vaccine. Results suggested that liposomes conjugated with specific ligands were able to deliver the entrapped antigens to the target organs, if there were no other barriers existing in the delivery pathways.

8.3.2 Effect of liposomes on antigen processing and presentation

In the immune system, specialized cells known as antigen presenting cells (APCs) provided mechanisms both for initial intracellular processing of protein antigens and for causing interactions with T lymphocytes that ultimately lead to specific humoral or cellular immune responses (Figure 8.1). An APC can express either class I or class II MHC molecules on its surface. Although all nucleated cells can express class I MHC, only a few cells, such as macrophages, B lymphocytes and dendritic cells of skin and lymphoid organs, have the capacity to express class II MHC (Austyn, 1989).

Current evidence suggested that two distinctive categories of antigens may be recognized by APCs and presented to T lymphocytes: extracellular antigens that are phagocytosed or endocytosed or otherwise taken up by APCs and intracellular cytoplasmic antigens (Hanahan, 1990). Either category of antigens may lead to presentation with class II MHC as part of an initial immune response resulting in the induction of antibodies or cytotoxic T lymphocytes (CTLs). The second type of antigens mentioned above may also lead to presentation with class I MHC as part of an effector mechanism process that leads to recognition and killing of the affected cells by CTLs (Garcon and Six, 1991).

The interaction between liposomes and macrophages has been referred and the presumed role of macrophages as APCs for liposomes has served as the major rationale for using liposomes as carriers of vaccines (Alving, 1991). The importance of macrophages in the immune response to liposomal antigens was suggested by *in vivo* studies in which macrophages were deleted in

animals by either injection of carrageenan (Shek and Lukovich, 1982) or liposomes containing cytotoxic agents (Su and Van Rooijen, 1989). These treatments resulted in severe suppression of immune responses to liposomal antigens. Recovery of the immune response occurred in parallel with reappearance of the macrophages. It appears that macrophages are probably the most important element in the processing of liposomal antigens. However, presentation by B cells and dendritic cells is not necessarily excluded.

From an immunological standpoint, an initial immune response leading to generation of $CD8^+$ CTLs can involve interactions between antigens and macrophages. Also, it can involve the participation of $CD4^+$ Th in the process of induction of CTLs (Fayolle *et al.*, 1991). After phagocytosis of liposomes by macrophages, liposomes containing encapsulated antigens accumulated in large intracytoplasmic vacuoles and within the vacuoles. Then, liposomes were often closely associated with the vacuolar membrane (Verma *et al.*, 1991). However, the unique ability of liposomes to deliver liposomal antigens both to intracellular acidified vacuoles and to cytoplasm. It is possible that the ability of liposomes to serve as carriers both for induction of humoral immune responses and for generation of CTLs may occur (Verma *et al.*, 1992). Thus, the antigen may be processed in the cytosol and the epitope fragments are transported into the ER to be complexed with MHC I molecules to induce CTL response. Otherwise, the antigens and liposomal lipids are delivered to and degraded in the lysosome. Then, the antigen fragments are retransported to a late endosomal compartment to be complexed with MHC II molecules. These complexes are exported and displayed on the surface membrane for recognition and antibody induction.

Until now, most of these works had done *in vitro*, which lacks of the regulation of cytokines and the interaction between immunological cells. This is different from the phenomena *in vivo*. Therefore, understanding the function of liposomes as vaccine adjuvants *in vivo* is the next issue to improve liposomal formulations for immunisation.

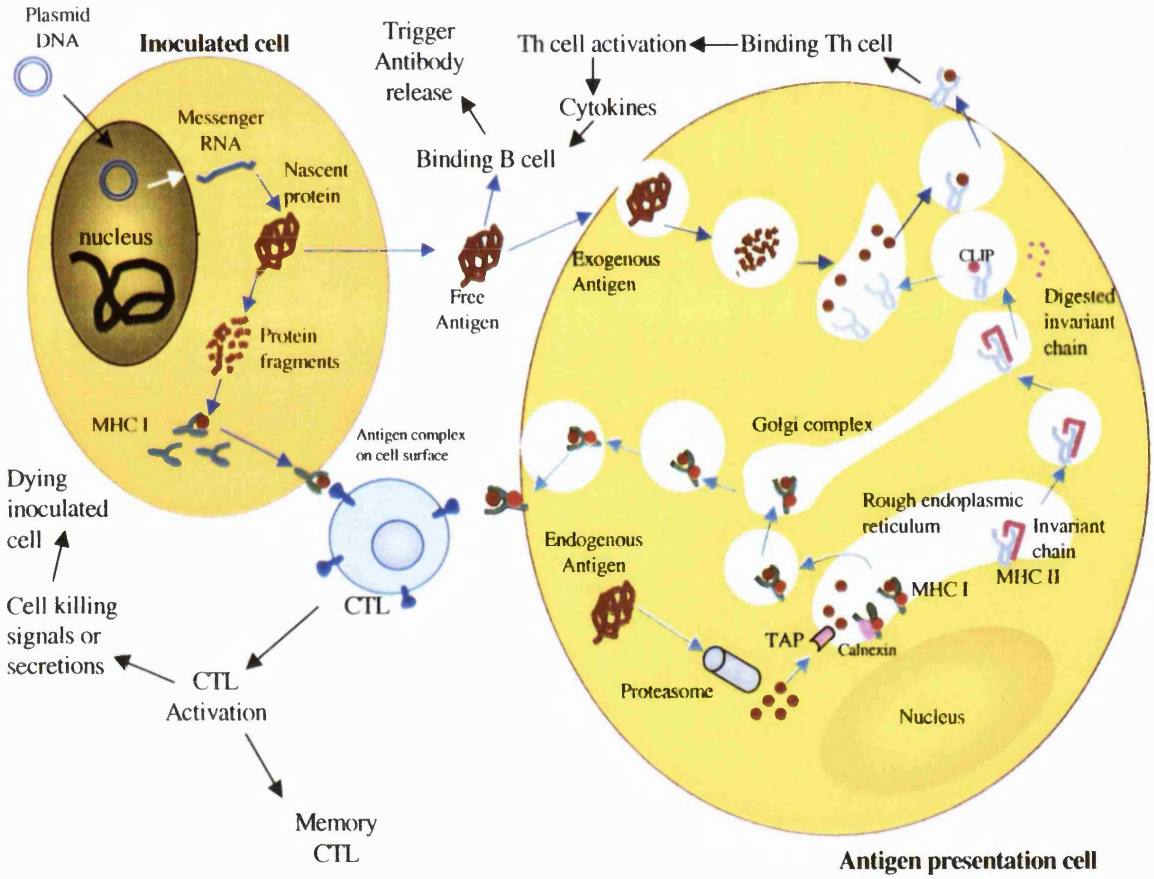

Figure 8.1: Antigen presentation and the immune response in vaccination with DNA or protein (Adapted from Weiner and Kennedy, 1999).

8.3.3 Liposome mediated DNA immunisation: problems and opportunities

Recently, a novel and exciting concept developed, namely de novo production of required vaccine antigens by the host's cells *in vivo*, promises to revolutionize vaccination, especially where vaccines are either ineffective or unavailable. The concept entails the direct injection of antigen-encoding plasmid DNA which, following its uptake by cells, finds its way to the nucleus where it transfects the cells episomally. Produced antigens are then subjected to pathways similar to those undergone by the antigens of internalized viruses (but without their disadvantages) leading to protective humoral and cell mediated immunity (Manickan *et al.*, 1997). However, vaccination with naked DNA by the intramuscular route relies on the ability of myocytes to engulf the plasmid. The extent of DNA degradation by extracellular deoxyribonucleases is unknown but, depending on the time of its residence interstitially, degradation could be considerable (Manickan *et al.*, 1997). It follows those approaches to protect DNA from the extracellular milieu, introduce it into cells more efficiently or target it to immunologically relevant cells should contribute to optimal DNA vaccine design.

By comparison with the immunisation with protein vaccines, the DNA vaccination needed one more step, the gene expression *in vivo*, to achieve the initial state of protein vaccination. It meant that the DNA vaccination is a kind of indirect vaccination. However, the gene expression of foreign DNA *in vivo* is still unclear and has difficulty to evaluate it. This made the genetic immunisation be more difficult to apply in the future, but this method still has its advantage in vaccine development. The continuous expression and secretion of antigens will provide the possibility to prepare the single-dose vaccination. In addition, previous study had shown the

ability to induce CTL responses by DNA vaccine (Spier, 1996). These may be the opportunities to develop the single-dose or therapeutic vaccines.

Moreover, the vaccine adjuvants for genetic immunisation will be completely different from the traditional vaccine adjuvant for protein or other vaccines. Due to the procedure of gene expression, the adjuvants for DNA vaccines should consider the effect to improve the transfection efficiency of DNA *in vivo*. In order to improve the efficiency of gene transfection, many materials had been tried in the DNA transfection in cell culture, such as calcium phosphate, cationic polymers and liposomes. However, the low efficiency and high toxicity in these systems made them still be far from ideal *in vivo*. Therefore, the way to improve the transfection efficiency *in vivo* is quite important for the development of non-viral gene delivery vectors. Calcium phosphate-mediated gene transfection can produce the stable transfected colony in cell culture and liposomes can get more colonies in gene transfection. Combining these two systems, liposomes entrapping calcium phosphate-DNA complexes showed the possibility to be developed as new adjuvants for DNA vaccines. The works in the Chapter 7 were the initial studies about this system. The concept that liposomes entrapping condensed DNA complexes may improve the development of DNA delivery in gene therapy and genetic immunisation.

Finally, the protein and the DNA encoding this protein had been co-entrapped into DRVs to prepare the virus-like particles by liposome technology. Compared these particles with the hepatitis B viruses, they keep the components to induce the antibody responses to HBsAg and replace the structural components with calcium phosphate and liposomes. Immunisation with

these preparations keep the antibody titres in mice be longer than those in animals vaccinated with protein or DNA vaccines alone (Figure 7.15). This may result from the continuous DNA expression to keep the titres induced by proteins. Protein antigens might induce the primary responses and memory cell to produce antibodies. Then, the proteins secreted form transfected cells induce the secondary or following responses to keep the antibody titres. This can be considered as the biological sustained release of antigens. Consequently, it may have the possibility to develop as single-dose immunisation for hepatitis B vaccines.

Generally, liposomes mediated-DNA vaccination is possible, but the effects depended on their preparation and the immunisation procedures. Combination of protein and DNA vaccines showed the possibility to develop the single-dose vaccines, which maintained the antibody protection effect for a long period. The further works may improve these works to develop a promising vaccine formulation against the infection of hepatitis B viruses. More immunological works are required to investigate about the regulation of cytokines and the induction of CTL responses in the immunisation by these formulations.

8.4 CONCLUDING REMARKS

In this study, the liposomes as vaccine adjuvants for hepatitis B vaccination had been investigated from the pharmaceutical points and the results indicated that liposomes have the advantages to develop as the vaccine adjuvants in traditional vaccines, oral vaccines, DNA vaccines and even single-dose combined vaccines. From the first study for liposomes as vaccine adjuvants (Allison and Gregoriagis, 1974), the liposomes had been used in the various antigens,

including organisms, proteins, peptides and the plasmid DNA encoding antigens in the previous studies (Table 1.3). However, there is no study had investigated the effect of liposomes as vaccine adjuvants from the pharmaceutical views until now. This thesis followed the pharmaceutical approaches to study the application of liposomes in vaccine pharmaceutics. Results and further research recommended are summarized as follows.

8.4.1 Conclusion

From these studies, the effect of liposomes as vaccine adjuvant for HBsAg vaccination can be concluded as the following points. Firstly, the compositions of liposomes could affect on their adjuvanticity for HBsAg. DRVs composed of saturated phospholipids (DSPC) can induce stronger antibody titres than those constituted of unsaturated phospholipids(PC). The strains of mice showed different tolerance to these formulations (Figure 3.9). Antibody responses in the Balb/C mice were stronger than in the T.O. mice. The protein to lipid ratio in liposomal formulations is important for inducing the antibody titres in the animals immunised with these formulations. The dose for vaccination could be reduced by using the liposomes as adjuvants. Then, the method of vesicle size reduction resulted in different effect of liposomes as vaccine adjuvants. HBsAg-containing DRVs prepared in the presence of sucrose induced the strongest antibody responses in these formulations. It may be a promising candidate in the formulations for hepatitis B vaccines. However, the mannosylated HBsAg changed the protein property. Liposomes have varying adjuvanticity for mannosylated HBsAg by comparison with native HBsAg. Results (Figure 5.10) agreed with the previous study using other water-soluble antigen (Gregoriadis *et al.*, 1987). However, the DSPC/DOPE DRVs enhanced the antibody responses

in the mice immunised with mannosylated HBsAg. In addition, CTB was conjugated to the surface of HBsAg-containing DRVs. This formulation was successfully used to immunize mice by oral administration. Finally, plasmid DNA encoding HBsAg was entrapped into capisomes. Results from mice immunised with capisomes entrapping DNA showed the adjuvanticity of capisomes for DNA vaccination. Moreover, HBsAg was co-entrapped with the plasmid DNA encoding this antigen to develop a single-dose vaccine for hepatitis B prevention. Liposomes were shown to be versatile on their application as vaccine adjuvants by this study. These results provide information as how to prepare new liposomal formulation for subunit and DNA vaccines.

8.4.2. Further research

In this thesis, studies have been focussed on liposomes as vaccine adjuvants for hepatitis B vaccines, including subunit and DNA vaccines. Some promising formulations are recommended for further development as new vaccines against hepatitis B. However, there are still many problems to be overcome, especially with regard to mechanisms of liposomes as vaccine adjuvants and scale-up preparation and technology for clinical use. Improvement of the stability and safety of these formulations is also important for applying these formulations in the clinic.

In terms of immunology, the present studies compared the ability of liposomal formulations to induce antibody titres to HBsAg in mice. However, the details of cytokine profiles and cellular immunity induced by these formulations in animal models are still unknown and need more investigation. Furthermore, co-adjuvants, such as cytokines, lipid-A, can be

applied in liposomal systems to induce various immune responses and provide the opportunity to prevent or cure infection by the hepatitis B virus. Although chemotherapy for hepatitis B had been developed, vaccination is still the most economic and convenient method to prevent the disease. After all, there are still 5% population in the world suffering with this disease. The development of therapeutic vaccines may be required urgently to solve the problem of chronic hepatitis B carriers.

Capisomes provide a novel method to entrap DNA into liposomes, allowing them to deliver DNA into cells. Capisomes could also be developed as delivery systems for gene therapy and as adjuvants for genetic vaccines. The mechanism of DNA delivery by capisomes requires more studies for its elucidation. Furthermore, immune stimulatory sequence (CpG motifs) can be inserted into plasmid DNA encoding antigens or co-entrapped with this plasmid DNA into liposomes. Such approaches may improve the immune responses induced by these formulations and provide the opportunity to develop new DNA vaccine products. Moreover, viral vectors used for gene therapy can be condensed and entrapped into liposomes. Surface modification of these vesicles and additional investigations of their pharmacokinetics can improve the targeted delivery of DNA *in vivo*. Such studies may contribute to the academic background for the development of delivery systems for gene therapy. In this post-genomic era, molecular medicine, such as gene therapy and DNA vaccination, needs more research leading to the application of these concepts in clinical therapy.

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Appendix

Results had been published:

Abstracts

Liposome-entrapped plasmid DNA: Vesicle Characteristics and Immunisation Studies (1999) *Jyh-Chyang Yang, Yvonne Perrie, Milka Obrenovic and Gregory Gregoriadis* in NATO Advanced Studies Institute, Targeting of Drugs: Strategies for Gene Constructs and Delivery.

Liposomes as immunological adjuvants for hepatitis B surface antigen (1999) *Jyh-Chyang Yang and Gregory Gregoriadis* in 136th British Pharmaceutical Conference.

A novel liposomal DNA vaccine formulation with improved efficiency (1999) *Jyh-Chyang Yang and Gregory Gregoriadis* in 4th International Conference Liposome Advances: Progress in Drug and Vaccine Delivery.

Chapter

Genetic Vaccines: A Role for Liposomes (2000) *Gregory Gregoriadis, Brenda McCormark, Milka Obrenovic , Yvonne Perrie and Jyh-Chyang Yang* in Targeting of Drugs: Strategies for Gene Constructs and Delivery. IOS Press.